UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE CIÊNCIAS MÉDICAS

JOSÉ BRITTO JÚNIOR

DOPAMINA ENDOTELIAL: MAIOR MODULADOR DO SISTEMA CIRCULATÓRIO

ENDOTHELIAL DOPAMINE: MAJOR MODULATOR OF THE CIRCULATORY SYSTEM

Campinas
2022

JOSÉ BRITTO JÚNIOR

DOPAMINA ENDOTELIAL: MAIOR MODULADOR DO SISTEMA CIRCULATÓRIO

Tese apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de doutor em Farmacologia.

ORIENTADOR: PROF. DR. GILBERTO DE NUCCI
COORIENTADORA: DRA. NATALÍCIA DE JESUS ANTUNES

ESTE TRABALHO CORRESPONDE A VERSÃO
FINAL DA TES DEFENDIDA PELO
ALUNO JOSÉ BRITTO JÚNIOR E ORIENTADA
PELO PROF. DR. GILBERTO DE NUCCI.

Campinas

2022

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Ciências Médicas
Ana Paula de Morais e Oliveira - CRB 8/8985

B778d

Britto Júnior, José, 1991-
Dopamina endotelial : maior modulador do sistema circulatório / José Britto Júnior. – Campinas, SP : [s.n.], 2022.

Orientador: Gilberto De Nucci.
Coorientador: Natalícia de Jesus Antunes.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Ciências Médicas.

1. Catecolaminas. 2. Sistema vascular. 3. Receptores dopaminérgicos. 4. Cordão umbilical. I. De Nucci, Gilberto, 1958-. II. Antunes, Natalicia de Jesus. III. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. IV. Título.

Informações Complementares

Título em outro idioma: Endothelial dopamine : major modulator of the circulatory sistem
Palavras-chave em inglês:
Catecholamines
Vascular system
Receptors, Dopamine
Umbilical cord
Área de concentração: Farmacologia
Titulação: Doutor em Farmacologia
Banca examinadora:
Gilberto De Nucci [Orientador]
Emer Suavinho Ferro
Fabio Husemann Menezes
Lindemberg da Mota Silveira Filho
Marcelo Nicolás Muscará
Data de defesa: 18-11-2022
Programa de Pós-Graduação: Farmacologia

Identificação e informações acadêmicas do(a) aluno(a)
- ORCID do autor: https://orcid.org/0000-0003-0250-8468
- Currículo Lattes do autor: http://lattes.cnpq.br/5498384555654298

COMISSÃO EXAMINADORA DA DEFESA DE DOUTORADO

JOSÉ BRITTO JÚNIOR

ORIENTADOR: PROF. DR. GILBERTO DE NUCCI

MEMBROS TITULARES:

1. PROF. DR. GILBERTO DE NUCCI

2. PROF. DR. EMER SUAVINHO FERRO

3. PROF. DR. FABIO HUSEMANN MENEZES

4. PROF. DR. LINDEMBERG DA MOTA SILVEIRA FILHO

5. PROF. DR. MARCELO NICOLÁS MUSCARÁ

Programa de Pós-Graduação em FARMACOLOGIA da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

A ata de defesa com as respectivas assinaturas dos membros encontra-se no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da FCM.

Data de Defesa: 18/11/2022

Dedico este trabalho em memoria de meu Pai José Brito Filho, que sempre esteve ao meu lado em cada conquista.

AGRADECIMENTOS

Agradeço primeiramente a Deus pelo conhecimento, amor, paz, sustento, por ter abençoado e tornado este trabalho realidade.

Agradeço ao Prof. Dr. Gilberto De Nucci, por ter confiado a mim a realização deste trabalho. Obrigada pela orientação e amizade que ficará para sempre em toda a minha vida.

Agradeço a minha mãe Maria Ivani da Silva, por ter me sustentado com palavras de vitória, com amor, carinho, por me acompanhar a cada fase da minha vida

Agradeço ao meu pai, José Brito Filho, por ser presente mesmo estando longe. Por sempre me incentivar a estudar e crescer cada vez mais.

Aos professores do departamento de farmacologia da UNICAMP, pelo carinho e exemplo.

Agradeço a minha namorada, amiga Marina Buzatto Gilioli por sempre está do meu lado acompanhando cada conquista do meu doutorado.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001

Muito obrigado

"Fé é acreditar no que você ainda não vê; a recompensa por essa fé é ver em que você acredita."

Santo Agostinho

Dopamina endotelial: maior modulador do sistema circulatório

Britto-Júnior J.[1] **e De Nucci G.** [1]

[1]Faculty of Medical Sciences, Department of Pharmacology, University of Campinas (UNICAMP), Campinas, Brazil.

RESUMO

O endotélio é um órgão capaz de regular tônus vascular através da liberação de agentes relaxantes (óxido nítrico e prostaciclina) ou contráteis como a endotelina e o EDCF (*Endothelium-Derived Contracting Factor*). A estimulação de campo elétrico (EFS, *Electrical Field Stimulation*) induz contrações da aorta da cobra e dos vasos do cordão umbilical humano (HUCV, *Human Umbilical Cord Vessels*), que são dependentes da presença do endotélio. Este estudo teve como objetivo estabelecer a natureza do(s) mediador(es) responsável(is) pelas contrações induzidas por EFS no HUCV. Anéis com ou sem endotélio da artéria umbilical humana (HUA, *Human Umbilical Artery*) ou veia (HUV, *Human Umbilical Vein*) foram montados em câmaras de banho de órgãos contendo solução de Krebs-Henseleit oxigenada e aquecida. A liberação basal de dopamina (DA), noradrenalina e adrenalina foi medida por LC-MS-MS (*Liquid Chromatography coupled to tandem Mass Spectrometry*). As curvas cumulativas de concentração-resposta foram realizadas com dopamina na ausência e na presença de L-NAME ou de antagonistas da dopamina. Os estudos de EFS foram realizados na presença e ausência de L-NAME, tetrodotoxina (TTX), atropina, indometacina, glibenclamida, phentolamina, prazosin e idazoxano, e os antagonistas da dopaminergicos SCH-23390 e haloperidol. A identificação da presensa da enzima tirosina hidroxilase (TH, *Tyrosine Hydroxylase*) e a dopa-descarboxilase (Dopa-DesCarboxylase) foram avaliadas por imunohistoquímica a presença do RNA mensageiro da enzima TH foi avaliado por hibridizações fluorescentes *in situ* (FISH, *Fluorescence In Situ Hybridization*). A liberação basal de dopamina requer um endotélio intacto tanto no HUA quanto no HUV. TH e DDC estão presentes apenas no endotélio de HUA e HUV, conforme determinado por imuno-histoquímica. A dopamina induziu contrações em HUA apenas na presença de L-NAME. As contrações induzidas por dopamina em HUV foram fortemente potencializadas por L-NAME. As contrações induzidas por EFS em ambos HUA e HUV foram potencializadas por L-NAME e inibidas pelo antagonista do receptor D2-like haloperidol. TTX, prazosin e idazoxan, atropina, indometacina, inibidor dos canais K-ATP glibenclamida e o antagonista do receptor tipo D1-like SCH-23390 não tiveram efeito nas contrações induzidas por EFS de HUA e HUV. Nossos resultados demonstram que dopamina é liberada pelo endotélio e modula a reatividade vascular no cordão umbilical humano.

Palavras-Chave: Catecolamina, Sistema vascular, Receptor dopaminérgico, Cordão umbilical.

Endothelial dopamine: major modulator of the circulatory system

Britto-Júnior J.[1] and De Nucci G. [1]

[1]Faculty of Medical Sciences, Department of Pharmacology, University of Campinas (UNICAMP), Campinas, Brazil.

ABSTRACT

The endothelium is an organ capable of regulating vascular tone through the release of relaxing agents (nitric oxide and prostacyclin) or contractile agents such as endothelin and endothelium-derived contracting factor. Electric field stimulation (EFS) induces contractions of snake aorta and human umbilical cord vessels (HUCV), which are dependent on the presence of endothelium. This study aimed to establish the nature of the mediator(s) responsible for EFS-induced contractions in the HUCV. Human umbilical artery (HUA) and vein (HUV) rings with or without human endothelium were mounted in organ bath chambers containing heated (37C°), oxygenated (95%O_2/5%CO_2) Krebs-Henseleit's solution. Basal release of dopamine (DA), noradrenaline and adrenaline were measured by liquid chromatography coupled to mass spectrometry (LC-MS-MS). Cumulative concentration-response curves were performed with dopamine in the absence and presence of L-NAME and dopamine receptor antagonists. EFS studies were performed in the presence and absence of L-NAME, tetrodotoxin (TTX), atropine, indomethacin, glybenclamide, phentolamine, prazosin and idazoxan, and the dopaminergic antagonists SCH-23390 and haloperidol. The identification of the presence of the enzyme tyrosine hydroxylase (TH) and dopa-decarboxylase (DDC) was evaluated by immunohistochemistry and the presence of TH enzyme messenger RNA (mRNA) was evaluated by FISH (Fluorescence in Situ Hybridization). Basal dopamine release requires an intact endothelium in both the HUA and the HUV. TH and DDC are present only on the endothelium of HUA and HUV, as determined by immunohistochemistry, and TH mRNA identified by FISH in the endothelium of both HUA and HUV. Dopamine induced contractions in HUA only in the presence of L-NAME. Dopamine-induced contractions in HUV were strongly potentiated by L-NAME. EFS-induced contractions in both HUA and HUV were potentiated by L-NAME and inhibited by the D_{2like}-receptor antagonist haloperidol. TTX, prazosin and idazoxan, atropine, indomethacin, glybenclamide and the $D_{1\text{-}like}$ receptor antagonist SCH-23390 had no effect on contractions. induced by EFS of HUA and HUV. Our results demonstrate that dopamine is released by the endothelium and modulates vascular reactivity in the human umbilical cord and that dopamine is the major EDCF.

Keywords: Catecholamine, Vascular system, Dopaminergic receptor, Umbilical cord.

SUMÁRIO

1 INTRODUÇÃO

1.1. Catecolaminas

As catecolaminas são compostos orgânicos que possuem em sua estrutura o grupo catecol (3,4-diidroxibenzeno) conectado a um grupo amina por uma ponte etil. As principais catecolaminas são dopamina, noradrenalina e adrenalina as quais possuem papel importante na neurotransmissão e hormonal em vários processos fisiológicos, tais como controle cardiovascular (1), regulação do humor e estresse (2), controle de funções motoras, (3), funções renais entre outros (4,5).

As catecolaminas são sintetizadas através do aminoácido tirosina, podendo ser derivado diretamente da dieta (fonte exógena) ou ser sintetizado no fígado (fonte endógena) a partir do aminoácido fenilalanina. A primeira etapa (Figura: 1) para sua formação é realizada pela enzima tirosina hidroxilase, a qual converte a tirosina em L-DOPA (l -3,4- diidroxifenilalanina) por oxidação da posição 3 no anel benzênico (5,6). A conversão da L-DOPA em dopamina é feita pela enzima aminoácido aromático descarboxilase (AADC) que está presente no cérebro, e é expressa por neurônios dopaminérgicos. A AADC cliva o grupo carboxila do carbono alfa da cadeia lateral de etilamina, liberando dióxido de carbono no qual requer o co-fator fosfato de piridoxal (5,6)em seguida ocorre a hidroxilação pela atividade enzimática da dopamina-β-hidroxilase, a qual converte a dopamina em noradrenalina. A noradrenalina é o transmissor liberado pelas terminações nervosas simpáticas pós-ganglionares do sistema nervoso autônomo. Em outras células, a noradrenalina pode ser convertida subsequentemente em adrenalina pela feniletanolamina N-metiltransferase. A adrenalina é o hormônio secretado pela medula da glândula supra-renal. A dopamina é um transmissor (ou neuromodulador) no sistema nervoso central (6).

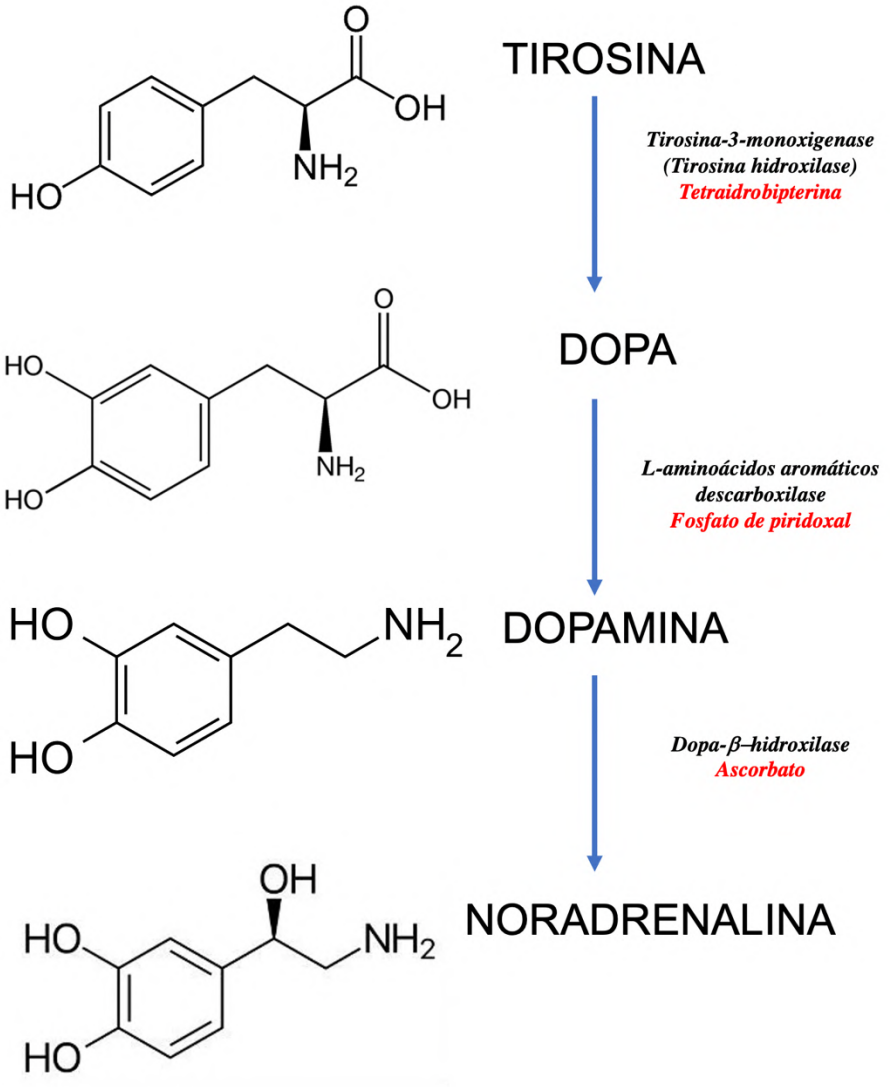


Figura 1: Síntese das catecolaminas (7)

Grande parte da noradrenalina liberada pelo neurônio é capturado e recondicionado dentro de vesículas para nova utilização, enquanto uma pequena porção é capturada pelas células não neurais. A captura neural é feita pela família das proteínas transportadoras de neurotransmissores específicas - NET (transportador de noradrenalina, do inglês norepinephrine transporter). Tais transportadores também atuam como contransportadores de sódio, cloreto e amina. Já o transporte até as vesículas dá-se pelo transportador vesicular de monoaminas (VMAT) e a captura extraneural (no tecido alvo) ocorre pelo transportador extraneural de monoaminas (EMT).

A metabolização das catecolaminas ocorre pela ação das enzimas monoamino-oxidase (MAO-A e MAO-B) e dcatecol-o-metiltransferase (COMT). A MAO, abundante nas terminações nervosas adrenérgicas, está ligada à membrana externa das mitocôndrias, convertendo catecolaminas em seus aldeídos correspondentes e metabolizados pelo aldeído desidrogenase ao ácido carboxílico correspondente. A COMT envolve a metilação de um dos grupos hidroxila do catecol, que produzir um derivado metoxi. Após a ação de MAO + COMT, o produto final é o 3-metoxi-4-hidroxifenilglicol (MHPG), eliminado pela urina em forma de glicuronídeo ou sulfato, e em grande parte convertido em ácido vanililmandélico - VMA. (8–10).

Atuando as catecolaminas possui uma ação endógena nos receptores adrenérgicos, sobre receptores alfa 1 e 2, e beta 1, 2 e 3 (11,12), tendo respostas diferentes para cada receptor e tecido(13,14). Os efeitos fisiológicos das catecolaminas são mediados por receptores acoplados à proteína G (GPCR), que controlam a sinalização do efetor, ou seja, o segundo mensageiro(15)

1.2. DOPAMINA

A dopamina, é uma catecolamina endógena e é o precursor da produção de noradrenalina, e foi sintetizado pela primeira vez por George Barger, James Ewens, e Henry Dale em 1910(16,17). Um dos mais importantes mediadores cardiovasculares, a dopamina é caracterizada como um importante modulador/mediador da pressão arterial por meio de um sistema dopaminérgico periférico independente (18). No estudo realizado por Holtz e Credner (1944; (19) os autores descrevem que a dopamina, em contraste com noradrenalina e adrenalina, produzia um efeito vasopressor em cobaias e coelhos. Os efeitos vasopressores foram produzidos injeções intravenosas de dopamina (>1 mg i.v.). Em humanos, a primeira evidencia da dopamina foi relatado nos estudos de McDonald et al., 1963 (20) em voluntários saldáveis ocasionou-se uma redução acentuada da resistência vascular renal. McDonald e Goldberg (1963; (21) também relataram que em cães anestesiados a dopamina causa uma vasodilatação em doses baixas, porém em does mais altas ocorre uma vasoconstrição nas artérias renais. Atualmente foram caracterizados 5

receptores dopaminérgicos classificado em duas famílias principais famílias: $D_{1\text{-like}}$ e $D_{2\text{-like}}$.

Os receptores de dopamina estão em diversos régios e órgão do corpo sendo identificados em vasos sanguíneos, néfron, sistema nervoso. Sua ação/efeito biológico são mediados através de cinco principais receptores dopamina geneticamente distintos: D_1, D_2, D_3, D_4 e D_5 tendo sua classificação em duas principais famílias $D_{1\text{-like}}$ (D_1 e D_1) e $D_{2\text{-like}}$ (D_2, D_3 e D_4) – que estão acoplados à proteína G, com base na estimulação (Gs) e inibição (Gi) da adenilato ciclase, respectivamente (22). A dopamina desempenha um papel importante na patogênese da hipertensão. A primeira demonstração do efeito vasodilatador da dopamina foi relatado por Toda e Goldberg em 1973 (23) em artérias mesentéricas e renais isoladas de caninos contraídas com cloreto de potássio (KCl; 10-30mM) no qual a dopamina causou relaxamento em concentrações 1-30 μM no qual produziu relaxamento na maioria dos testes, porém não em todas as artérias.

Os receptores que estimulam a adenilato ciclase, os receptores dopaminérgicos $D_{1\text{-like}}$ estão acoplados à proteína Gs, a qual estimula a adenilato ciclase. A adenilato ciclase catalisa a conversão de adenosina trifosfato (ATP) em adenosina monofosfato (AMP) cíclico, o qual se liga à proteína quinase A (PKA) para desinibir/ativar as subunidades catalíticas. As primeiras evidências dos receptores de dopamina da família D_1 foram identificadas através da estimulação da adenilato ciclase pela dopamina, levando ao acúmulo de AMP cíclico na retina (24) e no neostriatum de rato (25) . Os receptores que inibem a adenilato ciclase, são os receptores dopaminérgicos $D_{2\text{-like}}$ os quais são mediados pela ativação das proteínas G heterotriméricas G i/o, inativando/reduzindo a adenilato ciclase. Os receptores da família $D_{2\text{-like}}$ são responsáveis pela inibição da adenilato ciclase no qual age em oposição aos receptores da família $D_{1\text{-like}}$, diminuindo a fosforilação de substratos de PKA. A primeira via de sinalização identificada para receptores do tipo D_2 foi a inibição de acumulação de AMP cíclico em adenomas hipofisários secretores de prolactina humana (26,27)

A dopamina, um neurotransmissor bem conhecidos no sistema nervoso central, também é um modulador importante da pressão arterial, do equilíbrio de sódio, das funções renal e adrenal e é relevante para a patogênese e/ou manutenção de

hipertensão (28). Curiosamente, derivados de dopamina, como o araquidonoil-dopamina, foram encontrados no sistema nervoso central (29,30), onde atua como um agonista endógeno dos receptores canabinóides. Outro derivado endógeno da dopamina, a 6-nitrodopamina, também apresentou liberação basal em vasos de cordão umbilical humano(31), anéis aórticos de *Chelonoidis carbonarius* (32), anéis aórticos de *Pantherophis guttatus* (33), vas deferens de *Rattus norvegicus* (34) e *Homo sapiens* (35) e átrio direito de *Rattus norvegicus* (36)

1.3 Morfologia do cordão

O cordão umbilical é uma estrutura vital para o crescimento e bem-estar do feto sendo o único órgão que "morre no início da vida". É uma estrutura fundamental que liga o embrião à placenta (37). Seu comprimento pode variar entre 50 a 60 centímetros de comprimento no final da gravidez possuindo em sua morfologia três vasos sanguíneos que atravessam em espiral a geléia de Wharton, completando em média 10 a 11 voltas entre os fetos (38). O cordão umbilical possui duas artérias umbilicais (Figura 02) responsáveis por transportar o sangue venoso do embrião para a placenta e uma única veia, a qual e responsável de transportar o sangue rico de oxigênio e nutriente da placenta para o embrião (39), Juntas, essas estruturas fornecem nutrientes e respiração ao embrião.

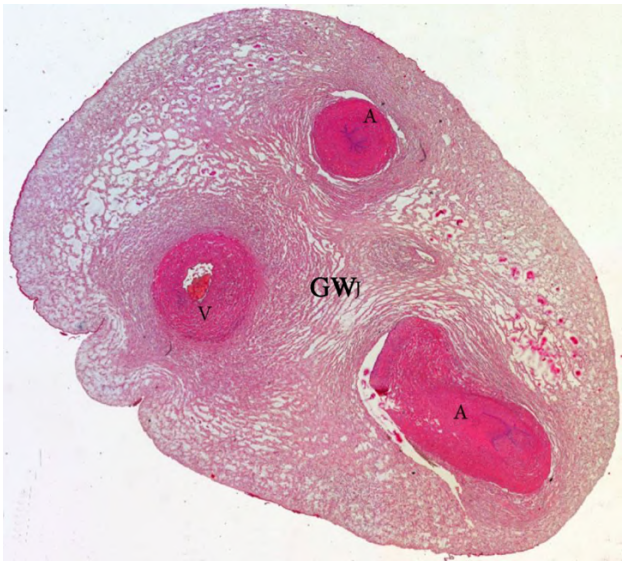


Figura 2: Corte transversal do cordão umbilical humano: A: Artérias; V: veia; GW: Geleia de Wharton (40)

A geleia de Wharton consiste em grande número de fibras colágenas, um pequeno número de fibroblastos, uma matriz contendo ácido hialurônico e glicosaminoglicanos sulfatados (41). As artérias umbilicais são circundadas apenas por uma camada plana ou cúbica de células epiteliais e o endotélio, não contendo nenhuma camada subendotelial ou endoelástica subjacente, a qual é adjacente à camada muscular. A camada muscular consiste em uma camada longitudinal interna (com lúmens irregulares) sem nervos ou "ductos alimentadores".

Nos vasos de cordão umbilical humano foi caracterizado farmacologicamente por ensaios de reatividade vascular que a acetilcolina induz a contração na veia umbilical humana (HUV), através da ativação dos subtipos de receptores colinérgicos M_1 e M_3 (42). A acetilcolina, mas não a nicotina, causa a contração das artérias umbilicais humana (HUA), a qual é sensível à atropina (43). O tromboxano A2 causa contração no vasos do cordão umbilical humano semelhante ao seu mimético U46619 contrai HUA (44) e HUV (45). A endotelina (ET) é um potente vasoconstritor tanto *in vitro* quanto *in vivo*. Ambas ET-1 e ET-2 são potentes agentes contráteis de HUA e HUV (46), e HUV é mais sensível à ET-1 em comparação com HUA(42) . No HUA, noradrenalina, oximetazolina e fenilefrina, provocam uma contração dependente da concentração com uma ordem de potência agonística: noradrenalina = oximetazolina ≫ fenilefrina, a qual é consistente com uma interação com α_2-adrenoceptores (47). O antagonista dos adrenoceptores α_1, prazosin, causou uma redução dependente da concentração da resposta contrátil à noradrenalina e antagonizou a contração induzida pela fenilefrina no HUA, indicando a presença de α_1-adrenoceptores (48).

1.4 Endotélio Vascular, fator relaxante derivado do endotélio

A identificação da importância do endotélio na regulação do tônus vascular foi observada no estudo de Furchgott e Zawadzki (1980; (49), analisando a relação entre a acetilcolina (ACh) e a vasodilatação em aorta isolada de coelho. Em algumas preparações notou-se que a acetilcolina produzia a vasodilatação. Isso ocorria devido a presença do endotélio durante a preparação do vaso, originalmente denominado “fator relaxante derivado do endotélio” (do inglês, EDRF ou endothelium derived relaxing factor), identificado mais tarde que esse efeito era devido a liberação de óxido nítrico (NO).

O endotélio consiste numa monocamada de células que constitui a estrutura mais interna de todo sistema circulatório, separando o sangue da parede vascular e do interstício (Figura 3). As células endoteliais comunicam-se com células do músculo liso vascular por meio de junções “gap” mioendoteliais, as quais permitem a transferência de íons ou pequenas moléculas (50).

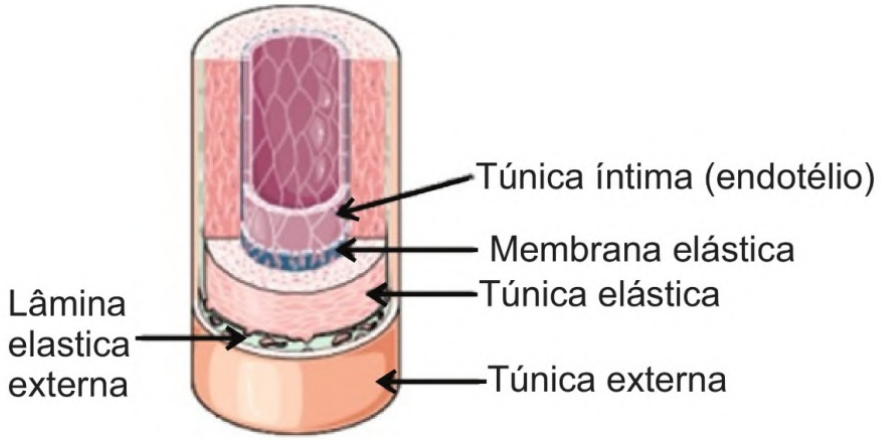


Figura 03: Fonte: Adaptada de Servier Medical Art – Powerpoint Image Bank (acessado em 25/08/2022).

As respostas vasodilatadoras dependentes do endotélio envolvem predominantemente fatores como NO (51), hiperpolarização derivada do endotélio (EDH; (52) e endotelina (53). Entre os fatores derivados do endotélio, o NO é gerado pela NO sintase endotelial (eNOS) a partir de L -arginina e é considerado como a mais importante substância moduladora do tônus vascular, devido à sua capacidade de regular uma grande variedade de respostas vasodilatadoras que contribuem para a homeostase vascular (51).

As células endoteliais produzem e liberam outras substâncias vasodilatadoras. As funções do endotélio através do NO e de outros mediadores vasodilatadores, é modular processos como a angiogênese, respostas inflamatórias, imunológicas, homeostase, tônus e permeabilidade vascular, fatores antioxidantes (enzima superóxido dismutase), além de atuarem na prevenção de trombose e promoverem a manutenção da integridade dos vasos (54).

A reação da L-Arginina e oxigênio molecular leva à produção de NO e citrulina, esta reação é catalisada por uma família de enzimas denominada de óxido nítrico sintase (NOS; (55). Até o momento foram descritas três isoformas de NOS no qual são caracterizadas de acordo com sua expressão em cada tecido especifico: NOS neuronal (nNOS ou NOS I; (56) que está presente nos sistemas nervosos central e periférico, no qual produz NO que atua como neurotransmissor; NOS induzível (iNOS ou NOS II), cuja indução ocorre por ação de citocinas inflamatórias e a NOS endotelial (eNOS ou NOS III; (57), expressa de maneira constitutiva predominantemente nas células endoteliais.

Na célula endotelial o NO formado difunde-se rapidamente até as fibras musculares lisas onde se combina com o ferro da porção ativa da enzima guanilato ciclase solúvel (GCs), que catalisa a saída da sitio de guanosina trifosfato (GTP) em guanosina monofosfato cíclica (GMPc), no qual cicla a GMPc e subsequente ativação da proteína quinase dependente de GMPc (PKG) nas células do musculo liso (58). Essa ativação resulta no relaxamento celular reduzindo os níveis intracelular de ìon de cálcio (Ca^{2+}), fosforilando a cadeia leve de miosina e inibindo a contração dependente de cálcio. A fosforilação da PKG promove o aumento do sequestro de Ca^{2+} para o retículo endoplasmático, no qual também impulsiona a abertura de canais de potássio (K^{+}) e fechamento de canais de cálcio do tipo-L, proporcionando o relaxamento vascular (59).

1.5 Endotélio Vascular, fator contraturante derivado do endotélio

A primeira evidência que apoia a hipótese de que o endotélio pode liberar um vasoconstritor veio de experimentos em artérias coronárias caninas isoladas, nos quais um experimento em hipóxia ou anoxia produziu contrações dependentes do endotélio (60). Foi demostrado que as contrações dependentes do endotélio também através da modulação da pressão arterial e estimulação elétrica (61) em artérias cerebrais de gatos. A liberação basal de fator contraturante derivado do endotélio (EDCF), no inglês “endothelium derived contracting fator” foi relatada em cultura de células endoteliais bovinas, demostrando assim sua origem biológica (62).

As contrações dependentes do endotélio induzidas por ácido araquidônico (63,64) são prevenidas pelos inibidores de ciclooxigenase (bloqueadores da síntese de prostaglandinas) tais como indometacina, meclofenamato e ácido acetilsalicílico, inibidor da tromboxano sintetase dazoxibeno (63) indicando que o EDCF possivelmente seja um prostanóide. Porém as respostas contráteis dependentes do endotélio à anóxia ou hipóxia não são inibidas por inibidores da ciclooxigenase, propondo que a resposta contrátil por 'hipóxia' não é afetada por inibidores de lipoxigenase, fosfolipase A2, e antagonistas adrenérgicos, receptores serotoninérgicos e histaminérgicos (65). Os efeitos vasoconstritores produzido por incubação de células endoteliais aorta bovinos não foram afetadas pelos inibidores acima mencionados, porém foram inibidas por tripsina e por hidrólise alcalina ou ácida, sugerindo que o EDCF possa ter uma estrutura peptídica (62).

A endotelina (ET) é um peptídeo liberado do endotélio, possuindo na sua estrutura 21 aminoácidos, e apresentando um potente efeito vasoconstritor (66). No estudo de De Nucci et al., (1988(53) foi demostrado que é removida pela circulação pulmonar do rato *in vitro* e *in vivo* e pelo pulmão da cobaia *in vitro*. A endotelina também causa de prostaciclina (PGI_2) e tromboxano (TXA_2) e de óxido nítrico. Existem três genes de endotelina no genoma humano sendo ET-1, ET-2 e ET-3 (67). ET-1 foi a primeira a ser identificado/detectado por Yanagisawa et al. (1988) (66) em cultura de células endoteliais aórticas porcinas. O ET-2 é a sequência Leuh-Met^7 de ET-1 é substituída por Trph-Leu^7. ET-3 é a forma originalmente encontrado no genoma do rato, no qual os resíduos ET-1 Ser^2-Ser^4-Ser^5-Leuh-Met^7-$Phei^4$ foram substituídos por Thr^2-Phe^4-Thr^5-Tyrh-Lys^7-$Tyrl_4$ (68).

Um novo mediador liberado do endotélio temos as catecolaminas que curiosamente, foi identificada por imuno-histoquímicas presença da enzima tirosina hidroxilase, responsável por catalisar a conversão de L-tirosina em L-DOPA, em vasos de cobras "*Crotalus durissus terrificus* e *Bothrops jararaca"* (69)e jabuti *"Chelonoidis carbonarius*" (70) estando presente apenas nas células endoteliais. Como a imunohistoquímica não conseguiu identificar terminais nervosos em vasos de *Chelonoidis carbonarius* (70), os resultados indicam uma fonte não neuronal de síntese de catecolaminas. Em anéis aórticos de *Chelonoidis carbonaria* por cromatografia líquida acoplada ao espectrômetro de massas em tandem (LC-MS/MS) revelou a liberação basal de dopamina, noradrenalina e adrenaline (71). Está bem

estabelecido que as células endoteliais modulam a reatividade vascular através da liberação de mediadores como prostaciclina (72), óxido nítrico (49) e endotelina (66). As catecolaminas modulam o tônus, no entretanto, a produção e a liberação de catecolaminas estão associadas à existência de terminais nervosos nos vasos (73,74). Neste estudo, foi investigada a natureza do mediador liberado pelo endotélio dos vasos do cordão umbilical humano (HUCV) por cromatografia líquida acoplada a espectrometria de massa (LC-MS-MS) e caracterizado farmacologicamente o mecanismo das contrações induzidas por EFS em artérias umbilicais humanas (HUA) e veias umbilicais humanas (HUV).

2 OBJETIVOS

2.1. Objetivo Geral

Investigar a ação *in vitro* da dopamina liberada pelo endotélio do cordão umbilical humano.

2.2. Objetivos Específicos

- Avaliar o papel do endotélio como fonte de catecolaminas na contração induzida por EFS em HUCV, na presença e ausência de endotélio íntegro;
- Caracterizar o mecanismo farmacológico das contrações induzidas por EFS em músculo liso vascular de cordão umbilical humano;
- Quantificar a liberação de catecolaminas por LC-MS/MS;
- Identificar e localizar enzimas envolvidas na síntese de catecolaminas (tirosina hidroxilase, dopamina beta-hidroxilase) em vasos isolados de cordão umbilical humano através de ensaios de imuno-histoquímica;
- Realizar uma investigação morfológica pelo ensaio de hibridização *in situ* para comprovação da imuno-histoquímica.

3 MATERIAIS e MÉTODOS

3.1 População e amostra do estudo

Parturientes maiores de 18 anos, em parto natural ou cesárea realizados na Santa Casa de Vinhedo (Vinhedo - SP) e na Maternidade de Campinas (Campinas - SP), foram convidadas a participar do estudo. Foram convidados a participar desse estudo mulheres normotensas, que não apresentavam pré-eclâmpsia, diabetes mellitus pré-gestacional ou gestacional e que não estavam fazendo uso de medicação regular. O consentimento livre e esclarecido foi assinado pelas parturientes que concordaram em participar desse estudo. Foi utilizados cordões umbilicais de 99 voluntários com idades entre 18 e 47 anos. A investigação seguiu os princípios contidos na Declaração de Helsinque, esse estudo foi aprovado pelo Comitê de Ética e Pesquisa (CEP) do Instituto de Ciências Biomédicas da Universidade de São Paulo - ICB/USP (protocolo n º 3.165.417).

3.2 Protocolo de reatividade vascular em anéis de artéria e veia de cordão umbilical humano

Para a avaliação da reatividade vascular os tecidos de HUA e HUV foram colocados em banho de órgãos com 10 mL em solução de Krebs-Henseleit, gaseificada com mistura carbogênica (O_2:CO_2, 95:5%) a uma temperatura mantida de 37 °C.

Após um período de estabilização de 90 minutos, os anéis foram pré-contraídos com serotonina (5-HT; 1 μM) e a integridade do endotélio foi avaliado por incubação com ATP (10 μM) na presença e ausência de inibidores da síntese de óxido nítrico, L -NAME (100 μM). A integridade do músculo liso foi avaliada pelo relaxamento induzido por nitroprussiato de sódio (SNP; 10 μM).

Posteriormente, os anéis HUA e HUV foi submetido a estimulação de campo elétrico (EFS) a 60 V por 30 s usando um estimulador da Grass S88 com pulsos de onda quadrada de 8-16 Hz, largura de pulso de 0,3 ms e atraso de 0,1 ms. (Astro-Medical, Industrial Park, Rhode Island, EUA). Para avaliar e caracterizar o mecanismo de contração do EFS foi utilizado tetrodotoxina (TTX; 1 μM), atropina (10 μM e 100 μM), indometacina (10 μM), glibenclamida (10 μM) e fentolamina (10 μM e 100 μM).

Em um segundo experimento, a EFS foi realizada em vasos sem endotélio. Já em um terceiro experimento, o efeito dos antagonistas foi avaliado na presença de L-NAME (100 μM) para os antagonistas do receptor $D_{1\text{-like}}$ da dopamina SCH-23390 (10 μM), receptor $D_{2\text{-like}}$ da dopamina haloperidol (10 μM), antagonista do receptor α_1-adrenérgico prazosin (100 μM), e do antagonista do receptor α_2-adrenérgico idazoxan (100 μM).

Para o ensaio "sanduíche", três tecidos (três artérias ou três veias) foi colocado em um banho de órgãos contendo 10 mL de solução de Krebs-Henseleit borbulhando por uma mistura produtora de carbono (O_2:CO_2, 95:5%) a uma temperatura 37°C. Os dois anéis com endotélio foram chamados de "tecido doador" (6 mm) e o anel sem endotélio foi chamado de "tecido receptor" (3 mm). Os anéis foram mantidos no mesmo banho de órgãos pelo mesmo tempo e nas mesmas condições para investigar se fatores liberados pelo tecido doador poderiam afetar o tecido receptor durante a EFS.

Foi também realizado curvas concentração resposta à dopamina (10 nM a 3 mM) realizadas em anéis de HUA e HUV com o endotélio preservado na ausência e na presença de oxido nítrico L-NAME (100 μM). Para investigar os efeitos dos receptores dopaminérgicos foi utilizados os antagonistas do receptor $D_{1\text{-like}}$ SCH-23390 (10 μM) e do antagonista do receptor $D_{2\text{-like}}$ haloperidol (10 μM) nas contrações induzidas por dopamina em vasos intactos com endotélio tratados com L-NAME.

3.3 Análise por LC-MS/MS

As concentrações de dopamina, noradrenalina e adrenalina na solução de Krebs-Henseleit foram determinadas por LC-MS/MS. Resumidamente, 100 μL dos padrões internos (dopamina-d3, noradrenalina-d6 e adrenalina-d6 a 100 ng/mL) foram adicionados à solução de Krebs (2 mL) seguido por 1.5 mL de água deionizada. Após agitação em vórtex por 10 seg, 100 mg de Al_2O_3 foram adicionados e as amostras foram incubadas por 20 min em um agitador orbital (Centrífuga 5810/5810 R). Os tubos foram então centrifugados a 2,000 g por 4 min a 4 °C e o sobrenadante descartado. O resíduo foi lavado 4 vezes com 2 mL de água deionizada. Após a lavagem final, foram adicionados 200 μL de uma solução contendo ácido trifluoroacético 0,1% em ACN/H_2O (60/40; v/v). Após agitação em vórtex por 40 seg, os tubos Eppendorf foram centrifugados a 2,000 g por 5 min e os sobrenadantes transferidos para *vials* e submetidos à análise por LC-MS/MS (Shimadzu; (75).

3.4 Imuno-histoquímica

Amostras de cordão umbilical humano (n= 5) foram fixadas em formalina tamponada 10%, incluídas em parafina, e posteriormente cada bloco foi cortado em seções seriadas de 4-5 µm de espessura. Foram realizadas coloração H&E e, com relação às amostras encaminhadas para imuno-histoquímica ou hibridização *in situ* de fluorescência (FISH) foram utilizadas lâminas silanizadas e carregadas positivamente.

As amostras de tecido foram primeiramente desparafinizadas e reidratadas em álcoois graduados em água destilada e, em seguida, incubadas em peróxido de hidrogênio a 3% por 10 min para bloquear a peroxidase endógena. A recuperação antigênica foi feita com tampão citrato (10 mM, pH 6,0) a 95 °C por 20 min (em panela de vapor). Posteriormente, as lâminas contendo as amostras foram lavadas em PBS. Foi feita a incubação das secções com os respectivos anticorpos primários descritos abaixo por 2 horas, a temperatura ambiente. Os anticorpos primários usados foram os seguintes: (1) anti-calretinina humana (*mouse* monoclonal IgG1, clone: DAK-calret 1, 1:200 in PBS; DAKO/Agilent, USA) (2) anti-tirosina hidroxilase (*chicken* polyclonal IgY, ab76442, 1:1500, Abcam, Cambridge, UK) e (3) anti-dopa descarboxilase (*mouse* monoclonal IgG1; clone: CL 2962; ab211535, Abcam, Cambridge, UK). Para detecção de tirosina hidroxilase foram usados anticorpos secundários (*goat anti-chicken* IgY, ab150169, 1:500, Abcam, Cambridge, UK) e terciário (*rabbit anti-goat* IgG, AP106P, 1:250, Sigma/ Merck, Germany) (Britto-Júnior *et al*., 2020). As lâminas foram incubadas com o anticorpo secundário por 1 hora a temperatura ambiente, lavadas com PBS. E, em seguida, as lâminas foram incubadas com anticorpo terciário por 1 hora a temperatura ambiente.

O sistema de detecção utilizado neste estudo foi o NovoLink™ Max Polymer Detection System (catalog code RE7280-k, Leica Biosystems, UK), seguindo o protocolo do fabricante. Foi usado o cromógeno 3,3′ diaminobenzidine (DAB, DAKO). No final, as laminas foram desidratadas e contracoradas com hematoxilina de Harris e montadas com Entellan (Sigma/Merck). Os controles negativos consistiram na omissão do anticorpo primário e incubadas com o diluente (bem como com os anticorpos secundários/terciários, quando aplicável, e o sistema de detecção). No ensaio de imuno-histoquímica foi feito um controle negativo por seção, para identificar qualquer reação de fundo.

Todas as laminas imunomarcadas foram analisadas, e as imagens capturadas utilizando um microscópio trinocular Eclipse 50i microscope (Nikon) coupled to a 10MP CMOS digital camera (AmScope, EUA). A positividade foi avaliada por um médico

patologista (AAS), que desconhecia a presença ou ausência de anticorpo primário na amostra (o observador não sabia se uma amostra teste ou um controle de omissão estava sendo avaliada). As etiquetas das lâminas foram cobertas com um adesivo de oclusão removível.

Com relação à hibridização *in situ* (FISH), foram selecionados aleatoriamente 5 amostras dentre 8 cordões umbilicais humanos usados para a realização de imuno-histoquímica. As amostras foram desparafinizadas com xilol e reidratadas em álcoois graduados por 5 min cada. Em seguida, foram incubados em solução de HCl 0,2 N por 20 min e, posteriormente, tratados com tampão citrato (ZytoVision kit, catalog code Z-2028-20, Germany), pH 6,0 a 80 ºC por 1 h. Em seguida, os cortes foram incubados com pepsina por 8 min em temperatura ambiente. As lâminas foram lavadas com 2X SSC (ZytoVision kit, catalog code Z-2028-20, Germany), desidratadas em uma sequência de etanóis (75%, 80% e 100% de etanol por 2 min cada) e, em seguida, secas ao ar. As lâminas foram incubadas com 100 µL da sonda de mRNA de TH (na concentração de 100 µM, em água livre de RNAse) por 10 min a 75 ºC e *overnight* em um Hybridizer Dako a 37 ºC. A sequência da sonda de mRNA de TH foi a seguinte: 5′- AACCGCGGGGGACATGATGGCCT - 3 ′ (RNA Tm = 77,8 °C) (RNA Tm = 77.8°C) (catalog code: VC00021, Sigma/Merck, Germany). As sondas foram marcadas com fluoresceína 6-FAM na região 5’.

No dia seguinte, os cortes foram colocados em solução de UREA/0,1Xssc, a 45°C por 30 minutos e, em seguida, lavadas com solução 2xSSC por 2 minutos. Depois, os cortes foram desidratados em etanol 75%, 85% e 100% por 2 minutos cada, e secados ao ar. Por fim, as lâminas foram montadas com 15 µL de DAPI contendo meio de montagem (kit ZytoVision) e lamínula (sendo a lamínula selada com Fixogum Rubber Cement, de Marabu, Alemanha). Os controles negativos consistiram na omissão da sonda e foram realizados em todos os ensaios FISH (um controle negativo por seção) para controlar qualquer autofluorescência significativa. Todas as lâminas de FISH foram examinadas e fotomicrografadas usando um microscópio trinocular DM4000 B LED (Leica Microsystems, Wetzlar, Alemanha) acoplado a uma câmera de 1,4 MP DFC 310 FX (Leica, Suíça).

4 RESULTADOS

4.1 Artigo 1 – Electrical field stimulation induces endothelium-dependent contraction of human umbilical cord vessels.

Autores: José Britto-Júnior, Felipe Fernandes Jacintho, Guilherme M Figueiredo Murari, Rafael Campos, Ronilson Agnaldo Moreno, Edson Antunes, Fabiola Z Mónica, Gilberto De Nucci.

Revista: Life Sciences

Situação: Aceito a publicação em 31 de dezembro de 2019. Publicado on-line em 07 de janeiro de 2020

Life Sciences 243 (2020) 117257

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Electrical field stimulation induces endothelium-dependent contraction of human umbilical cord vessels

José Britto-Júnior[a,*], Felipe Fernandes Jacintho[a], Guilherme M. Figueiredo Murari[a], Rafael Campos[b], Ronilson Agnaldo Moreno[a], Edson Antunes[a], Fabiola Z. Mónica[a], Gilberto De Nucci[a,c,d]

[a] Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (Unicamp), Campinas, Brazil
[b] Superior Institute of Biomedical Sciences, Ceará State University (UECE), Fortaleza, Brazil
[c] Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo (USP), São Paulo, Brazil
[d] Metropolitan University of Santos (UNIMES), Santos, Brazil

ARTICLE INFO

Keywords:
Tetrodotoxin
Adenosine triphosphate (ATP)
N_{ω}-Nitro-L-arginine methyl ester (L-NAME)
Catecholamine
Vascular smooth muscle

ABSTRACT

Electrical field stimulation (EFS) has been used for decades in classical pharmacological preparations in order to characterize the mediators released by neural endings involved in smooth muscle contraction or relaxation. Since most of the human umbilical cord has no innervation, EFS has never been used in this preparation. This study aimed to investigate the effect of EFS on vascular responsiveness from human umbilical cord. Segments of the human umbilical cord were obtained from normotensive parturients and the human umbilical artery (HUA) and the human umbilical vein (HUV) were isolated and mounted in organ bath chambers. Electrical field stimulation-induced contractions in both HUA (2.35 ± 1.31 mN and 3.77 ± 2.31 mN for 8 Hz and 16 Hz respectively, n = 24) and HUV (3.81 ± 2.54 mN and 6.26 ± 4.51 mN for 8 Hz and 16 Hz respectively, n = 25). The addition of tetrodotoxin (1 μM) did not alter the EFS-induced contractions in both tissues (n = 5). Pre-incubation with atropine (10 and 100 μM), glibenclamide (10 μM) and indomethacin (10 μM) did not affect the EFS-induced contractions in both tissues. The contractions of both vessels were significantly reduced by pre-incubation of the tissues with phentolamine (10 and 100 μM). The endothelium removal almost abolished the EFS- induced contractions in both vessels (n = 5). In sandwich preparation, *donor* tissue (with endothelium) released a factor (s) that promoted contraction of the *recipient* tissue (endothelium removal) in both HUA and HUV (n = 5, respectively). Our findings indicate a potential role of endothelium-derived catecholamines in modulating HUA and HUV reactivities.

1. Introduction

Endothelial dysfunction plays an important role in the pathogenesis of preeclampsia [1]. Flow-mediated vasodilation is lower during pregnancy in preeclamptic and hypertensive women compared with the control group [2]. Electrical field stimulation (EFS) is a technique in which the stimulus is applied uniformly to an isolated tissue in short pulse width waves, often used as a method of selectively stimulating intramural nerves [3–5]. It may cause tissue contraction or relaxation depending on the mediators released [6,7].

The innervation of the umbilical cord is controversial. Apparently, it has no innervation since no cholinergic or adrenergic nerve fibers have been identified by fluorescence [8]. Immunohistochemistry assay performed with monoclonal and polyclonal antibodies against neuron-associated antigens has also proven negative [9]. However, a positive presence for acetylcholinesterase in the nerve endings in the proximal 20 cm of the umbilical cord was demonstrated with the thiocholine technique [10]. Furthermore, adrenergic innervation (S100 protein), glial fibrillary acidic protein, neurofilaments, γ-subunit of neuron-specific enolase, myelin-associated glycoprotein, protein gene product 9-5 (PGP9.5) and neuroblastoma associated antigen were identified at the proximal side of the cord close to the fetus [11,12]. Interestingly, EFS-induced contractions of snake aorta are dependent on the endothelium [13].

Since EFS has never been used in this preparation, this study aimed to investigate its effect on human isolated umbilical vessels *in vitro* and

* Corresponding author at: University of Campinas (Unicamp), Department of Pharmacology, Rua Tessália Vieira de Camargo, 126 - Cidade Universitária, 13083-887 Campinas, São Paulo, Brazil.
E-mail address: josebrittojr@gmail.com (J. Britto-Júnior).

https://doi.org/10.1016/j.lfs.2020.117257
Received 17 November 2019; Received in revised form 22 December 2019; Accepted 31 December 2019
Available online 07 January 2020
0024-3205/ © 2020 Published by Elsevier Inc.

whether the endothelium would modulate its effect.

2. Methods

2.1. Study participants

Parturients over the age of eighteen, undergoing cesarean or natural delivery from Santa Casa de Vinhedo and Maternity of Campinas Hospital (São Paulo, Brazil), were invited to take part in the study. The women included in this study were normotensive and did not have preeclampsia, pregestational or gestational diabetes mellitus and none were on regular medication. Written consent was obtained from those who agreed to participate. The umbilical cord from 32 volunteers aged 20–35 years was used.

The investigation conformed to the principles outlined in the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Institute of Biomedical Sciences of the University of São Paulo – ICB/USP (protocol number 3.165.417).

2.2. Reagents

Serotonin (5-HT), adenosine 5′-triphosphate (ATP), sodium nitroprusside (SNP), N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), atropine, indomethacin, glibenclamide, phentolamine and tetrodotoxin (TTX) were purchased from Sigma-Aldrich Chemicals Co. (Missouri, USA). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$), potassium phosphate monobasic (KH_2PO_4) and glucose were purchased from Merck KGaA (Darmstadt, Germany).

2.3. Tissue preparation

A segment of the umbilical cord – 10–20 cm from the insertion point in the placenta and 5 cm from the umbilicus was removed by the obstetrician and placed in a container with Krebs-Henseleit solution. The Warton's jelly was removed and the HUA and the HUV were dissected. Vessel rings (3 mm) were suspended vertically between two metal hooks in 10 mL organ baths containing Krebs-Henseleit solution: (mM) NaCl (118), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (1.2), $NaHCO_3$ (25), KH_2PO_4 (1.2) glucose (5.6) gassed with a mixture of 95%O_2: 5% CO_2 (pH 7.4) at 37 °C and coupled to isometric transducer. The initial smooth muscle tension was set at 10 mN. The tensioning force was recorded using a PowerLab 400 data acquisition system (Software Chart, version 7.0; ADInstruments, Colorado Springs, CO, USA).

2.4. Vascular reactivity protocol in human umbilical cord artery and vein rings

Following a 90-min stabilization period, rings were pre-contracted with 5-HT (1 μM), and the integrity of the endothelium was evaluated by incubating ATP (10 μM) in the presence and in the absence of the nitric synthase inhibitor L-NAME (100 μM). Seroronin was chosen as the contracturant agent since induces contractile responses at sufficiently low (physiologic) concentrations to be involved in the control of vascular tone in umbilicoplacental circulation [14]. In another set of experiments, the endothelium was removed with the aid of a thin stick, and the integrity of the smooth muscle was assessed by a relaxation induced by SNP (10 μM).

Human umbilical cord arteries and veins rings were subsequently submitted to EFS at 60 V for 30 s, at 8–16 Hz in square-wave pulses, 0.3 ms pulse width, and 0.1 ms delay, using a Grass S88 stimulator (Astro-Medical, Industrial Park, RI, USA). Electrical-field stimulations were performed in the presence and absence of the sodium channel blocker tetrodotoxin (TTX; 1 μM), the peripheral muscarinic acetylcholine receptor antagonist atropine (10 μM and 100 μM), the prostaglandin synthesis inhibitor indomethacin (10 μM), the inhibitor of KATP channels glibenclamide (10 μM) and the nonselective alpha-adrenergic antagonist phentolamine (10 μM and 100 μM). In another set of experiments, EFS was performed in vessels without endothelium.

For the "sandwich" assay, a protocol previously described (Furchgott and Zawadzki, 1980; Plane et al., 1995 and Dong et al., 1997) was used. Three tissues (either three arteries or three veins) were placed in an organ bath in 10 mL in Krebs-Henseleit solution, gassed with carbogenic mixture (O_2:CO_2, 95:5%) at a maintained temperature of 37 °C. The two rings with endothelium were referred as the "donor tissue" (6 mm) and the ring without endothelium as the "recipient tissue" (3 mm). Each ring was maintained in the same organ bath for the same period of time and under the same conditions to investigate whether factors released by the donor tissue could affect the recipient tissue during EFS [15–17].

2.5. Data analysis

Data are expressed as mean ± standard deviation (SD) and mean ± standard error of mean (SEM) of the number of experiments. Paired or unpaired Student's *t*-test was performed according to the protocol and a p-value < 0.05 was considered significant.

3. Results

3.1. Endothelium evaluation

Serotonin (5-HT, 1 μM) induced contractions in both HUA and HUV (9.7 ± 2.6 mN and 11.4 ± 1.5 mN, n = 5 for each group). ATP (10 μM) induced relaxation in both HUA and HUV (37.9 ± 1.8% and 40.3 ± 1.9%; Fig. 1A and B). The relaxation-induced by ATP was abolished by pre-incubation with L-NAME (100 μM; Fig. 1C and D).

3.2. Electrical field stimulation

The HUA contraction magnitudes were 2.35 ± 1.31 mN and 3.77 ± 2.31 mN for 8 Hz and 16 Hz, respectively (n = 24; Fig. 2A and C). The HUV contraction magnitudes were 3.81 ± 2.54 mN and 6.26 ± 4.51 mN for 8 Hz and 16 Hz, respectively (n = 25; Fig. 3A and C). Mechanical removal of the endothelium practically abolished EFS-induced contractions in both HUA (Fig. 2B and C) and HUV (Fig. 3B and C).

3.3. Tetrodotoxin

Incubation with tetrodotoxin (1 μM), a neurotoxin commonly used as a pharmacological tool to block sodium channels on nerve cells, had no effect in the EFS-induced contractions of HUA (Fig. 4A and C) and HUV (Fig. 4B and D).

3.4. Atropine

Incubation with the muscarinic receptor antagonist atropine (10 μM and 100 μM), had no effect in the EFS-induced contraction of HUA (2.3 ± 0.6 and 2.7 ± 1.1 mN for 8 Hz and 3.6 ± 2.2 and 3.9 ± 2.5 mN for 16 Hz; n = 5, for control and atropine 10 μM pre-treated vessels, respectively and 2.6 ± 0.7 and 2.9 ± 1.1 mN for 8 Hz and 3.5 ± 2.1 and 3.8 ± 2.2 mN for 16 Hz; n = 5 for control and atropine 100 μM pre-treated vessels, respectively). Similar results were obtained in HUV (2.3 ± 1.2 and 2.4 ± 1.5 mN for 8 Hz and 3.6 ± 2.1 and 3.4 ± 1.6 mN for 16 Hz; n = 5, for control and atropine 10 μM pre-treated vessels, respectively and 3.4 ± 2.2 and 3.6 ± 2.1 mN for 8 Hz and 5.2 ± 3.4 and 5.1 ± 3.7 mN for 16 Hz; n = 5 for control and atropine 100 μM pre-treated vessels, respectively).

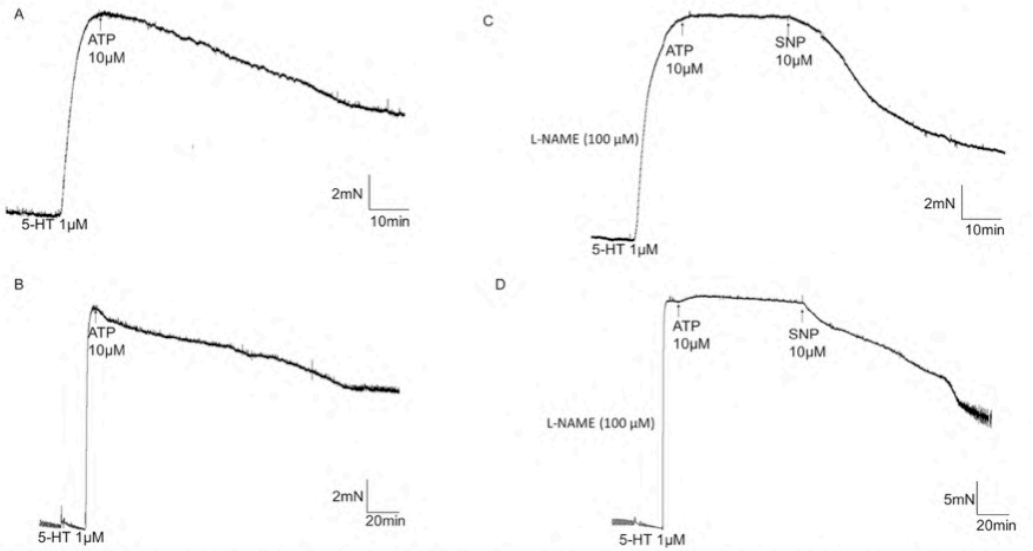


Fig. 1. Endothelium viability evaluation. ATP-induced (10 μM) relaxation of 5HT-precontracted HUA (A) and HUV (B) in the absence or presence of L-NAME (100 μM) in the HUA (C) and HUV (D; n = 5, for each group).

3.5. Indomethacin

Incubation with indomethacin (10 μM), a prostaglandin synthesis inhibitor, had no effect in the EFS-induced contraction of HUA (1.7 ± 0.9 and 1.7 ± 1.0 mN for 8 Hz and 2.2 ± 1.7 and 2.1 ± 1.9 mN for 16 Hz; n = 5, for control and indomethacin pre-treated vessels, respectively). Similar results were obtained in HUV (3.2 ± 2.4 and 3.1 ± 2.2 mN for 8 Hz and 3.7 ± 2.4 and 3.6 ± 2.1 mN for 16 Hz; n = 6 for control and indomethacin pre-treated vessels, respectively).

3.6. Glibenclamide

Incubation with glibenclamide (10 μM), an inhibitor of KATP channels, had no effect in the EFS-induced contraction of HUA (3.2 ± 2.1 and 3.6 ± 1.8 mN for 8 Hz and 3.7 ± 1.7 and 3.9 ± 1.8 mN for 16 Hz; n = 5, for control and glibenclamide pre-treated vessels, respectively). Similar results were obtained in HUV (4.1 ± 2.6 and 4.1 ± 2.5 mN for 8 Hz and 5.3 ± 1.4 and 5.3 ± 1.1 mN for 16 Hz; n = 4 for control and glibenclamide pre-treated vessels, respectively). In this set of experiments, both HUA and HUV were incubated with L-NAME.

3.7. Phentolamine

Pre-treatment with the non-selective α-adrenergic antagonist phentolamine (10 μM) caused a reduction of EFS-induced contraction of the HUA (2.9 ± 1.8 and 1.8 ± 1.0 mN for 8 Hz; p = 0.04 and 3.1 ± 1 and 1.5 ± 1.1 mN for 16 Hz; p = 0.01 n = 5, for control and phentolamine pre-treated vessels, respectively; the data obtained for phentolamine 100 μM is illustrated in Fig. 5A and C). Pre-treatment of HUV with phentolamine caused a reduction of EFS-induced contractions at 10 μM (3.5 ± 2.8 and 1.3 ± 0.9 mN for 8 Hz; p = 0.03 and 5.3 ± 5.1 and 3.2 ± 3.2 mN for 16 Hz; p = 0.01; n = 5, for control and phentolamine pre-treated veins, respectively; the reduction caused by phentolamine 100 μM was significant and it is illustrated in Fig. 5B and D).

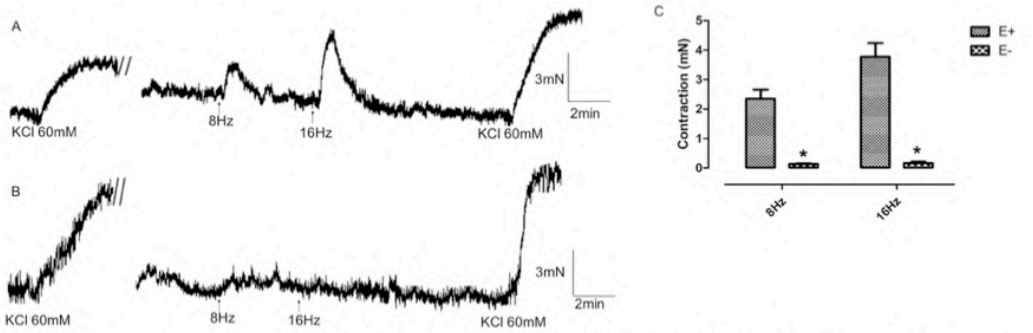


Fig. 2. EFS-induced contractions of HUA with preserved endothelium (A). The contraction was abolished by endothelium removal (B). Data expressed as mean ± SEM (C; n = 24, for each group).

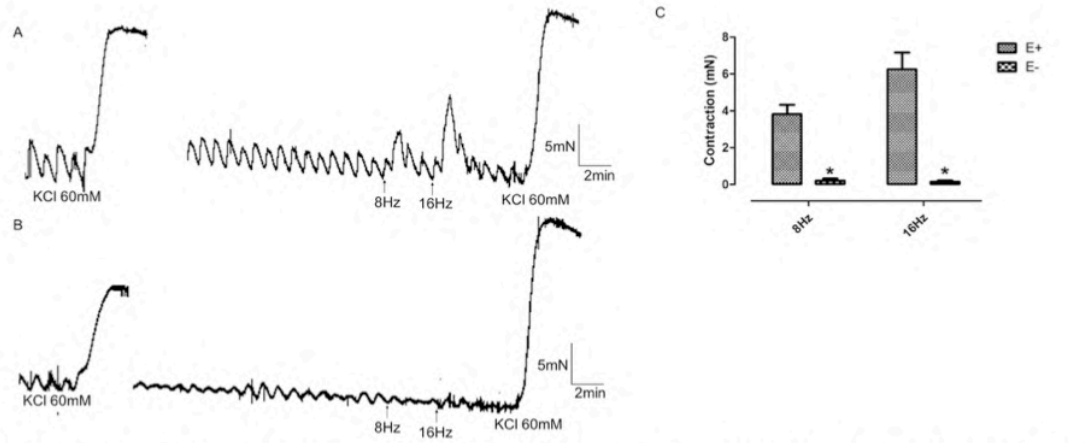


Fig. 3. EFS-induced contractions of HUV with preserved endothelium (A). The contraction was abolished by endothelium removal (B). Data expressed as mean ± SEM (C; n = 25, for each group).

3.8. Sandwich experiment

EFS of a sandwich preparation containing *donor* HUAs (with preserved endothelium; Fig. 6A and C) and a *recipient* HUA (endothelium denuded; Fig. 6B and D), contracted both tissues (Fig. 6). Similar results were observed in the sandwich preparation containing *donor* HUVs (with preserved endothelium; Fig. 7A and C) and a *recipient* HUV (endothelium-denuded; Fig. 7B and D).

4. Discussion

The classical mechanism proposed for EFS in isolated tissues is by stimulation of intramural nerve endings. The sodium channel blocker tetrodotoxin is classically used to block neural stimulation [18]; however, TTX did not affect the EFS-induced contractions in the umbilical cord vessels, excluding the idea that EFS is acting on nerve terminals. Indeed, the finding that the removal of the endothelium almost abolished EFS-induced contractions in both HUA and HUV, indicates that the endothelium is responsible for the contractile activity induced by EFS. Furthermore, the sandwich experiments demonstrate that there is release of mediator(s) responsible for these contractions, which remains to be identified.

The endothelial cell is known to release several substances that may cause vascular smooth muscle contraction. For instance, the endothelium contains the enzymes necessary to synthesize, store and breakdown ACh [19,20]. Acetylcholine induces contraction of HUV *via* activation of M_1 and M_3 receptor subtypes [21]. Acetylcholine but not nicotine causes HUA contraction which is sensitive to atropine [22]. Acetylcholine has been proposed to be released *in vivo* by endothelial cells [23], however the evidence was indirect since it is based on the finding that flow-evoked endothelial Ca^{2+} signaling was insensitive to tetrodotoxin, but abolished by the choline acetyl transferase (ChAT) inhibitor bromoacetylcholine. The present finding that atropine had no effect on the EFS-induced contractions of both HUA and HUV excludes the contribution of acetylcholine as a mediator for the contractions induced by EFS. Thromboxane A2 is produced by endothelial cells and

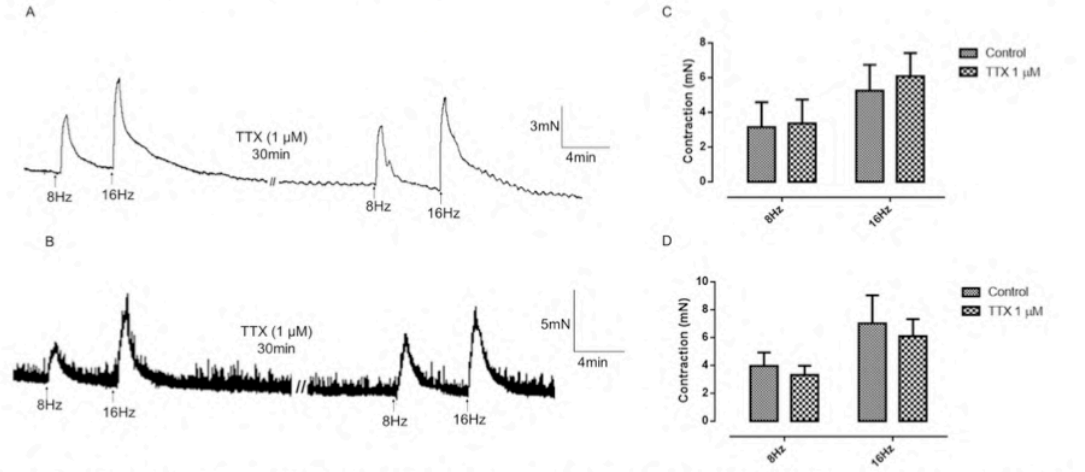


Fig. 4. Effect of tetrodotoxin (TTX; 1 µM) on EFS-induced contractions of HUA (A) and HUV (B). Data are expressed as mean ± SEM (C and D; n = 5, for each group).

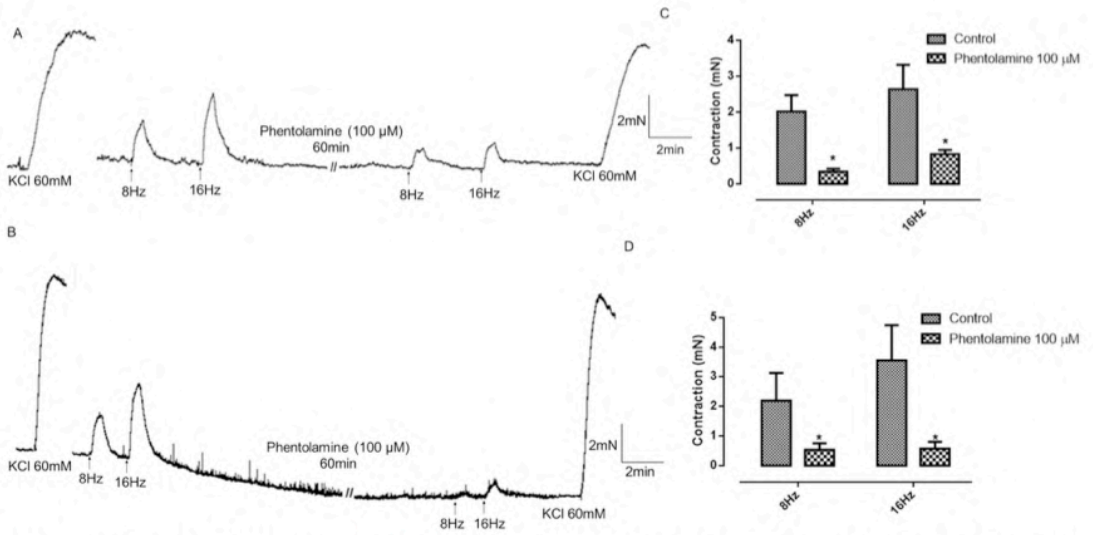


Fig. 5. Effect of phentolamine (100 μM) on EFS-induced contractions of HUA (A) and HUV (B). Data is expressed as mean ± S.E.M. $p < 0.05$ *vs* control (C and D; n = 5, paired Student's *t*-test).

the thromboxane mimetic U46619 contracts both HUA [24] and HUV [25]. Interestingly, an endothelium-derived contracting factor released by the aorta of spontaneous hypertensive rats requires activation of cyclo-oxygenase type 1 [26]. Pre-incubation with indomethacin had no effect in EFS-induced contractions of HUA and HUV, indicating that the mediator is not a cyclo-oxygenase metabolite.

Potassium channels are important regulators of vascular tone, because through their role in cell membrane potential regulation, they can determine the activity of voltage-operated calcium channels and hence the degree of vessel contraction [27]. However, KV channels are not involved in determining basal contractile tone in HUA, since the KV blocker 4-AP (5 mmol/L) had no effect on *in vitro* resting tone of these arterial rings [28]. Furthermore, the K+ channels blocker tetraethylammonium (TEA; 5 mmol/L) induced vascular contraction on nonstimulated HUA rings, whereas the BKCa channels activator phloretin (50 mmol/L) produced relaxation [29] indicating that activation of these K channels is unlikely to be responsible for the EFS-induced contractions. Glibenclamide is known to be one of the most selective blockers of KATP channels, although when used in high concentrations, it may block some other types of K+ channels [30]. Glibenclamide had no effect on the EFS-induced contractions of either HUA and HUV, excluding the role of KATP channel activation by EFS.

Endothelin (ET) is a potent vasoconstrictor both *in vitro* and *in vivo* [31]. Both ET-1 and ET-2 are potent contractile agents of HUA and HUV [32], and HUV is more sensitive to ET-1 as compared with HUA [24]. In our study, it is unlikely that the contraction induced by EFS is caused by ET release because ET-1-induced vascular smooth muscle contractions are long-lasting whereas those caused by EFS is short-lasting [33].

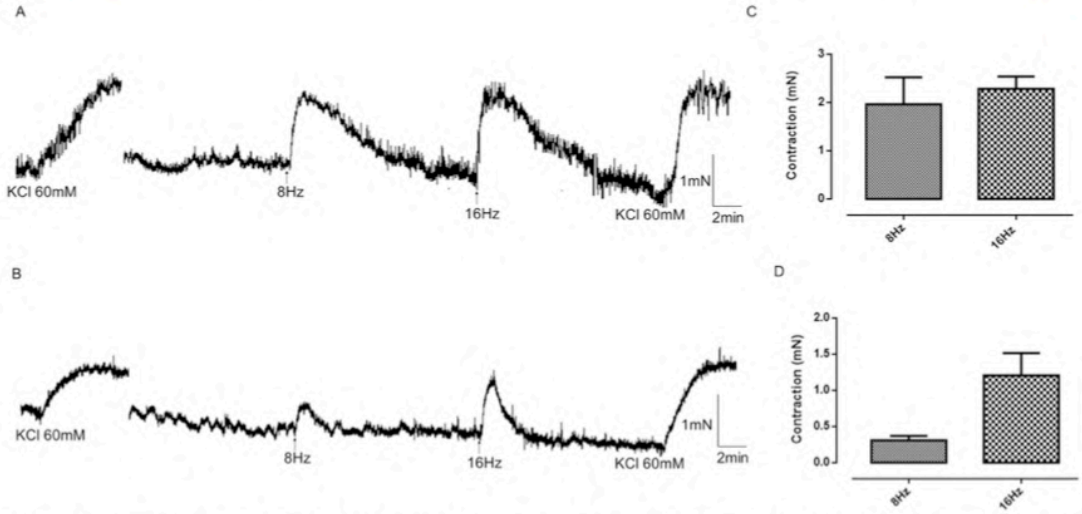


Fig. 6. EFS-induced contractions of HUA in Sandwich Experiment in *donor* tissue, with preserved endothelium (A), with endothelium denuded (B). Data expressed as mean ± SEM (C and D; n = 5, for each group).

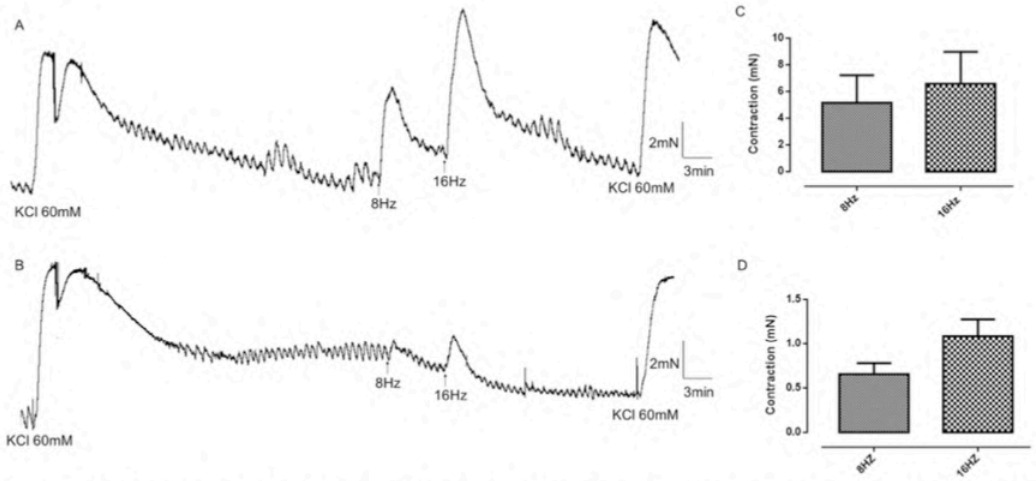


Fig. 7. EFS-induced contractions of HUV in Sandwich Experiment in *donor* tissue, with preserved endothelium (A), and in *recipient* tissue, with endothelium denuded (B). Data expressed as mean ± SEM (C and D; n = 5, for each group).

Phentolamine, a non-selective α-adrenergic antagonist [34], significantly reduced EFS-induced contractions of umbilical vessels suggesting that endothelium-derived catecholamines may be responsible for this effect. Indeed, contractions of vascular tissue induced by EFS, insensitive to tetrodotoxin and abolished by endothelium removal have been observed in aortic rings of the snakes *Crotalus durissus terrificus* and *Bothrops jararaca* [13] and *Panterophis guttatus* [35]. In all three cases, EFS-induced contractions were abolished by the use of adrenergic antagonists such as phentolamine and guanethidine, indicating a role for endothelium-derived catecholamine release. Interestingly, bovine aortic cells can synthesize and release catecholamines *in vitro* [36]. Our results indicating that phentolamine significantly reduced EFS-induced contractions of umbilical vessels show a potential role of endothelium-derived catecholamines for modulating vascular reactivity in humans. Human vessel endothelium does express tyrosine hydroxylase as detected by immunohistochemistry in paraganglioma vascular tissue [13].

In the HUA, noradrenaline, oxymetazoline and phenylephrine all elicited a concentration-dependent contraction with an agonist order of potency: noradrenaline = oxymetazoline ≫ phenylephrine [37], which is consistent with an interaction with α2-adrenoceptors [38]. The α1-adrenoceptor antagonist prazosin caused a concentration-dependent reduction of the contractile response to noradrenaline and antagonized the contraction induced by phenylephrine on HUA, indicating the presence of α1-adrenoceptors [37]. The HUV was more sensitive to EFS when compared to HUA; similar difference in sensitivity has been observed in ovine umbilical vessels where noradrenaline was more potent in UV than in UA [39].

Phentolamine only caused a significant inhiation of EFS-induced contractions at higher concentration, suggesting that it may be acting in a different population of receptors. Interestingly, dopamine D1 receptors are expressed in HUA [40] and phentolamine at high concentrations (25 μM) blocks dopamine binding in calf brain membranes [41].

Sympatholytic drugs such as labetalol [42] and alpha-methyl dopa [43,44] are currently employed in the treatment of both pre-eclampsia and eclampsia [45,46]. Whether these disorders are caused by an increase in the production of endothelium-derived catecholamines remains to be investigated.

5. Conclusion

The EFS-induced contractions of human umbilical vessels are abolished by the removal of the endothelium and inhibited by phentolamine. These findings indicate that endothelium-derived catecholamines modulate spasmogenic activity induced by EFS. The relevance of this study is to open a new window for studying the insight mechanisms for eclampsia and pre-eclampsia.

Author contributions

Conceptualization: José Britto-Junior, Gilberto De Nucci.
Data curation: Fabiola Z. Mónica, Edson Antunes, Gilberto De Nucci.
Formal analysis: Gilberto De Nucci.
Funding acquisition: Edson Antunes, Gilberto De Nucci.
Investigation: José Britto-Junior, Gilberto De Nucci.
Methodology: José Britto-Junior, Felipe Fernandes Jacintho, Guilherme Machado de Figueiredo Murari, Rafael Campos, Ronilson Agnaldo Moreno, Edson Antunes, Fabiola Z. Mónica, Gilberto De Nucci.
Project administration: Gilberto De Nucci.
Supervision: Fabiola Z. Mónica, Edson Antunes.
Visualization: Edson Antunes.
Writing – original draft: José Britto-Junior, Gilberto De Nucci.
Writing – review & editing: José Britto-Junior, Rafael Campos, Gilberto De Nucci.

The authors of this manuscript declare that have no conflicts of interest.

Acknowledgments

CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) awarded a PhD Grant to José B.J. CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) awarded a PhD Grant to Felipe F.J. for Grant 2018/24971-9. The authors thank Doctor Pedro Renato Guazzelli for providing the human umbilical cords. EA thanks FAPESP for Grant 2017/15175-1 and GDN thanks CNPq for Grant 303839/2019-8.

References

[1] T. Mori, K. Shinohara, A. Wakatsuki, K. Watanabe, A. Fujimaki, Adipocytokines and endothelial function in preeclamptic women, Hypertension Research: Official Journal of the Japanese Society of Hypertension. 33 (2010) 250–254, https://doi.org/10.1038/hr.2009.222.

[2] T. Mori, K. Watanabe, A. Iwasaki, C. Kimura, H. Matsushita, K. Shinohara, A. Wakatsuki, Differences in vascular reactivity between pregnant women with chronic hypertension and preeclampsia, Hypertension Research: Official Journal of the Japanese Society of Hypertension 37 (2014) 145–150, https://doi.org/10.1038/hr.2013.131.

[3] J.A. Bevan, Some characteristics of the isolated sympathetic nerve-pulmonary artery preparation of the rabbit, J. Pharmacol. Exp. Ther. 137 (1962) 213–218.

[4] G. Paterson, The response to transmural stimulation of isolated arterial strips and its modification by drugs, J. Pharm. Pharmacol. 17 (1965) 341–349.

[5] E.S. Darios, S.M. Barman, H.S. Orer, S.F. Morrison, R.P. Davis, B.M. Seitz, R. Burnett, S.W. Watts, 5-Hydroxytryptamine does not reduce sympathetic nerve activity or neuroeffector function in the splanchnic circulation, Eur. J. Pharmacol. 754 (2015) 140–147, https://doi.org/10.1016/j.ejphar.2015.02.032.

[6] D.A. Van Riper, J.A. Bevan, Electrical field stimulation-mediated relaxation of rabbit middle cerebral artery. Evidence of a cholinergic endothelium-dependent component, Circ. Res. 70 (1992) 1104–1112.

[7] E. Sastre, J. Blanco-Rivero, L. Caracuel, M. Callejo, G. Balfagon, Alterations in perivascular sympathetic and nitrergic innervation function induced by late pregnancy in rat mesenteric arteries, PLoS One 10 (2015) e0126017, , https://doi.org/10.1371/journal.pone.0126017.

[8] F.D. Reilly, P.T. Russell, Neurohistochemical evidence supporting an absence of adrenergic and cholinergic innervation in the human placenta and umbilical cord, Anat. Rec. 188 (1977) 277–286, https://doi.org/10.1002/ar.1091880302.

[9] S.B. Fox, T.Y. Khong, Lack of innervation of human umbilical cord. An immunohistological and histochemical study, Placenta. 11 (1990) 59–62.

[10] J.P. Ellison, The nerves of the umbilical cord in man and the rat, Am. J. Anat. 132 (1971) 53–60, https://doi.org/10.1002/aja.1001320107.

[11] M. Kawano, N. Mori, Prostacyclin producing activity of human umbilical blood vessels in adrenergic innervated and non-innervated portions, Prostaglandins Leukot. Essent. Fat. Acids 39 (1990) 239–245.

[12] D. Marzioni, L. Tamagnone, L. Capparuccia, C. Marchini, A. Amici, T. Todros, P. Bischof, S. Neidhart, G. Grenningloh, M. Castellucci, Restricted innervation of uterus and placenta during pregnancy: evidence for a role of the repelling signal Semaphorin 3A, Developmental Dynamics : An Official Publication of the American Association of Anatomists. 231 (2004) 839–848, https://doi.org/10.1002/dvdy.20178.

[13] R. Campos, A.F.O. Justo, F.Z. Monica, J.C. Cogo, R.A. Moreno, V.B. de Souza, A.A. Schenka, G. De Nucci, Electrical field-induced contractions on Crotalus durissus terrificus and Bothrops jararaca aortae are caused by endothelium-derived catecholamine, PLoS One 13 (2018) e0203573, , https://doi.org/10.1371/journal.pone.0203573.

[14] C. Bertrand, J. St-Louis, Reactivities to serotonin and histamine in umbilical and placental vessels during the third trimester after normotensive pregnancies and pregnancies complicated by preeclampsia, Am. J. Obstet. Gynecol. 180 (1999) 650–659, https://doi.org/10.1016/s0002-9378(99)70268-1.

[15] R.F. Furchgott, J.V. Zawadzki, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine, Nature. 288 (1980) 373–376, https://doi.org/10.1038/288373a0.

[16] F. Plane, T. Pearson, C.J. Garland, Multiple pathways underlying endothelium-dependent relaxation in the rabbit isolated femoral artery, Br. J. Pharmacol. 115 (1995) 31–38, https://doi.org/10.1111/j.1476-5381.1995.tb16316.x.

[17] H. Dong, G.J. Waldron, D. Galipeau, W.C. Cole, C.R. Triggle, NO/PGI2-independent vasorelaxation and the cytochrome P450 pathway in rabbit carotid artery, Br. J. Pharmacol. 120 (1997) 695–701, https://doi.org/10.1038/sj.bjp.0700945.

[18] L.J. Cruz, W.R. Gray, B.M. Olivera, R.D. Zeikus, L. Kerr, D. Yoshikami, E. Moczydlowski, Conus geographus toxins that discriminate between neuronal and muscle sodium channels, J. Biol. Chem. 260 (1985) 9280–9288.

[19] J.G. Parnavelas, W. Kelly, G. Burnstock, Ultrastructural localization of choline acetyltransferase in vascular endothelial cells in rat brain, Nature. 316 (1985) 724–725.

[20] C.J. Kirkpatrick, F. Bittinger, K. Nozadze, I. Wessler, Expression and function of the non-neuronal cholinergic system in endothelial cells, Life Sci. 72 (2003) 2111–2116.

[21] V.A. Pujol Lereis, F.J. Hita, M.D. Gobbi, M.G. Verdi, M.C. Rodriguez, R.P. Rothlin, Pharmacological characterization of muscarinic receptor subtypes mediating vasoconstriction of human umbilical vein, Br. J. Pharmacol. 147 (2006) 516–523, https://doi.org/10.1038/sj.bjp.0706654.

[22] N. Chen, J. Lv, L. Bo, N. Li, C. Wu, X. Yin, J. Li, J. Tao, J. Chen, Y. He, S. Huang, J. Xiao, C. Mao, Z. Xu, Muscarinic-mediated vasoconstriction in human, rat and sheep umbilical cords and related vasoconstriction mechanisms, BJOG: An International Journal of Obstetrics and Gynaecology. 122 (2015) 1630–1639, https://doi.org/10.1111/1471-0528.13144.

[23] C. Wilson, M.D. Lee, J.G. McCarron, Acetylcholine released by endothelial cells facilitates flow-mediated dilatation, J. Physiol. 594 (2016) 7267–7307, https://doi.org/10.1113/JP272927.

[24] A.G. Templeton, J.C. McGrath, M.J. Whittle, The role of endogenous thromboxane in contractions to U46619, oxygen, 5-HT and 5-CT in the human isolated umbilical artery, Br. J. Pharmacol. 103 (1991) 1079–1084, https://doi.org/10.1111/j.1476-5381.1991.tb12303.x.

[25] L.D. Montgomery, M.A. Belfort, G.R. Saade, K.J.J. Moise, Y.P. Vedernikov, Meconium inhibits the contraction of umbilical vessels induced by the thromboxane A2 analog U46619, Am. J. Obstet. Gynecol. 173 (1995) 1075–1078.

[26] D. Yang, M. Feletou, N. Levens, J.N. Zhang, P.M. Vanhoutte, A diffusible substance (s) mediates endothelium-dependent contractions in the aorta of SHR, Hypertension (Dallas, Tex: 1979) 41 (2003) 143–148, https://doi.org/10.1161/01.hyp.0000047651.45322.16.

[27] E.A. Ko, J. Han, I.D. Jung, W.S. Park, Physiological roles of K+ channels in vascular smooth muscle cells, Journal of Smooth Muscle Research = Nihon Heikatsukin Gakkai Kikanshi. 44 (2008) 65–81, https://doi.org/10.1540/jsmr.44.65.

[28] P. Martin, A. Rebolledo, A.R.R. Palomo, M. Moncada, L. Piccinini, V. Milesi, Diversity of potassium channels in human umbilical artery smooth muscle cells: a review of their roles in human umbilical artery contraction, Reproductive Sciences (Thousand Oaks, Calif). 21 (2014) 432–441, https://doi.org/10.1177/1933719113504468.

[29] V. Milesi, J. Raingo, A. Rebolledo, A.O. Grassi de Gende, Potassium channels in human umbilical artery cells, J. Soc. Gynecol. Investig. 10 (2003) 339–346, https://doi.org/10.1016/s1071-5576(03)00117-5.

[30] N. Teramoto, Physiological roles of ATP-sensitive K+ channels in smooth muscle, J. Physiol. 572 (2006) 617–624, https://doi.org/10.1113/jphysiol.2006.105973.

[31] M. Yanagisawa, H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, T. Masaki, A novel potent vasoconstrictor peptide produced by vascular endothelial cells, Nature. 332 (1988) 411–415, https://doi.org/10.1038/332411a0.

[32] A. Rizzi, G. Calo, B. Battistini, D. Regoli, Contractile activity of endothelins and their precursors in human umbilical artery and vein: identification of distinct endothelin-converting enzyme activities, J. Cardiovasc. Pharmacol. 31 (Suppl. 1) (1998) S58–S61.

[33] G. De Nucci, R. Thomas, P. D'Orleans-Juste, E. Antunes, C. Walder, T.D. Warner, J.R. Vane, Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 9797–9800, https://doi.org/10.1073/pnas.85.24.9797.

[34] D.A. Giussani, P.J. Moore, L. Bennet, J.A. Spencer, M.A. Hanson, Alpha 1- and alpha 2-adrenoreceptor actions of phentolamine and prazosin on breathing movements in fetal sheep in utero., J. Physiol. 486 (Pt 1) (1995) 249–255. https://doi.org/10.1113/jphysiol.1995.sp020807.

[35] R. Campos, F.Z. Monica, A.F.O. Justo, J.C. Cogo, E. de T. Oliveira, R.A. Moreno, E. Antunes, G. De Nucci, Electrical field stimulation-induced contractions on Pantherophis guttatus corpora cavernosa and aortae, PLoS One 13 (2018) e0196123, , https://doi.org/10.1371/journal.pone.0196123.

[36] D. Sorriento, G. Santulli, C. Del Giudice, A. Anastasio, B. Trimarco, G. Iaccarino, Endothelial cells are able to synthesize and release catecholamines both in vitro and in vivo, Hypertension (Dallas, Tex: 1979) (60) (2012) 129–136, https://doi.org/10.1161/HYPERTENSIONAHA.111.189605.

[37] G. Bodelsson, M. Stjernquist, Characterization of contractile adrenoceptors in the human umbilical artery, Eur. J. Pharmacol. 282 (1995) 95–101, https://doi.org/10.1016/0014-2999(95)00277-r.

[38] K. Starke, Alpha-adrenoceptor subclassification, Rev. Physiol. Biochem. Pharmacol. 88 (1981) 199–236.

[39] L. Zhang, D.C. Dyer, Characterization of alpha-adrenoceptors mediating contraction in isolated ovine umbilical vein, Eur. J. Pharmacol. 197 (1991) 63–67, https://doi.org/10.1016/0014-2999(91)90365-w.

[40] J.A. Ferreira-de-Almeida, L. Pereira-Leite, C. Cavallotti, A. Ricci, F. Amenta, Pharmacological characterization and autoradiographic localization of dopamine D1 receptors in the human umbilical artery, Eur. J. Pharmacol. 234 (1993) 209–214, https://doi.org/10.1016/0014-2999(93)90955-h.

[41] D.R. Burt, I. Creese, S.H. Snyder, Properties of [3H]haloperidol and [3H]dopamine binding associated with dopamine receptors in calf brain membranes, Mol. Pharmacol. 12 (1976) 800–812.

[42] P. Jouppila, J. Rasanen, Effect of labetalol infusion on uterine and fetal hemodynamics and fetal cardiac function, Eur. J. Obstet. Gynecol. Reprod. Biol. 51 (1993) 111–117.

[43] E. Ayitey-Smith, D.R. Varma, Mechanism of the hypotensive action of methyldopa in normal and immunosympathectomized rats, Br. J. Pharmacol. 40 (1970) 186–193, https://doi.org/10.1111/j.1476-5381.1970.tb09912.x.

[44] J. Cockburn, V.A. Moar, M. Ounsted, C.W. Redman, Final report of study on hypertension during pregnancy: the effects of specific treatment on the growth and development of the children, Lancet (London, England). 1 (1982) 647–649.

[45] G.M. Peres, M. Mariana, E. Cairrão, Pre-eclampsia and eclampsia: an update on the pharmacological treatment applied in Portugal, Journal of Cardiovascular Development and Disease. 5 (2018), https://doi.org/10.3390/jcdd5010003.

[46] G.D. Lamming, E.B. Symonds, Use of labetalol and methyldopa in pregnancy-induced hypertension, Br. J. Clin. Pharmacol. 8 (1979) 217S–222S.

4.2 Artigo 2 – Endothelium-derived dopamine modulates EFS-induced contractions of human umbilical vessels

Autores: José Britto-Júnior, David H A Pinheiro, Alberto F O Justo, Guilherme M Figueiredo Murari, Rafael Campos, Fernanda V Mariano, Valéria B de Souza, André A Schenka, Fabiola Z Mónica, Edson Antunes, Gilberto De Nucci

Revista: Pharmacology Research & Perspectives

Situação: Aceito a publicação em 06 de maio de 2020. Publicado on-line em 22 de junho de 2020

Received: 28 March 2020 | Revised: 2 May 2020 | Accepted: 6 May 2020

DOI: 10.1002/prp2.612

SHORT REPORT

Endothelium-derived dopamine modulates EFS-induced contractions of human umbilical vessels

José Britto-Júnior[1] | David H. A. Pinheiro[1] | Alberto F. O. Justo[1] | Guilherme M. Figueiredo Murari[1] | Rafael Campos[2] | Fernanda V. Mariano[3] | Valéria B. de Souza[1] | André A. Schenka[1] | Fabiola Z. Mónica[1] | Edson Antunes[1] | Gilberto De Nucci[1,4,5]

[1]Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, Brazil

[2]Superior Institute of Biomedical Sciences, Ceará State University (UECE), Fortaleza, Brazil

[3]Department of Pathology, Faculty of Medical Science, State University of Campinas (UNICAMP), Campinas, Brazil

[4]Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo (USP), São Paulo, Brazil

[5]Brazil University, Fernadopolis, Brazil

Correspondence
José Britto-Júnior, University of Campinas (Unicamp), Department of Pharmacology, Rua Tessália Vieira de Camargo, 126 – Cidade Universitária, 13083-887 – Campinas, São Paulo, Brazil.
Email: josebrittojr@gmail.com

Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: 303839/2019-8; Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant/Award Number: 2017/15175-1

Abstract

Electrical field stimulation (EFS) induces contractions of both snake aorta and human umbilical cord vessels (HUCV) which were dependent on the presence of the endothelium. This study aimed to establish the nature of the mediator(s) responsible for EFS-induced contractions in HUCV. Rings with or without endothelium from human umbilical artery (HUA) or vein (HUV) were mounted in organ bath chambers containing oxygenated, heated Krebs-Henseleit's solution. Basal release of dopamine (DA), noradrenaline, and adrenaline was measured by LC-MS-MS. Cumulative concentration-response curves were performed with dopamine in the absence and in the presence of L-NAME or of dopamine antagonists. EFS studies were performed in the presence and absence of L-NAME, the α-adrenergic blockers prazosin and idazoxan, and the dopamine antagonists SCH-23390 and haloperidol. Tyrosine hydroxylase (TH) and dopa-decarboxylase (DDC) were studied by immunohistochemistry and fluorescence in situ hybridizations. Basal release of dopamine requires an intact endothelium in both HUA and HUV. TH and DDC are present only in the endothelium of both HUA and HUV as determined by immunohistochemistry. Dopamine induced contractions in HUA only in the presence of L-NAME. Dopamine-induced contractions in HUV were strongly potentiated by L-NAME. The EFS-induced contractions in both HUA and HUV were potentiated by L-NAME and inhibited by the D2-like receptor antagonist haloperidol. The α-adrenergic antagonists prazosin and idazoxan and the D1-like receptor antagonist SCH-23390 had no effect on the EFS-induced contractions of HUA and HUV. Endothelium-derived dopamine is a major modulator of HUCV reactivity *in vitro*.

KEYWORDS

dopamine, EFS, endothelium, haloperidol, human umbilical artery, human umbilical vein, idazoxan, L-NAME, prazosin, tyrosine hydroxylase

Abbreviations: DDC, dopa decarboxylase; EFS, electric field stimulation; HUA, human umbilical artery; HUCV, human umbilical cord vessels; HUV, human umbilical vein; LC-MS-MS, Liquid chromatography/tandem mass spectrometry; L-NAME, Nω-nitro-L-arginine-methyl ester; TH, tyrosine hydroxylase.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2020 The Authors. *Pharmacology Research & Perspectives* published by John Wiley & Sons Ltd, British Pharmacological Society and American Society for Pharmacology and Experimental Therapeutics.

1 | INTRODUCTION

Electrical field stimulation (EFS) is a technique in which an electrical stimulus is applied uniformly to an isolated tissue in short pulse width waves.[1,2] It may cause tissue contraction or relaxation depending on the mediators released.[1,3,4] The proposed mechanism for EFS in isolated tissues is stimulation of intramural nerve endings.[5] The sodium channel blocker tetrodotoxin is classically used to block neural stimulation.[6] The EFS-induced contractions of the aortae of the snakes *Crotalus durissus terrificus* and *Bothrops jararaca*,[7] *Panterophis guttatus*,[8] and of the tortoise *Chelonoidis carbonaria*[9] are endothelium dependent and tetrodotoxin insensitive. The EFS-induced contractions of human umbilical cord vessels (HUCV) are also dependent on the presence of the endothelium and are not affected by tetrodotoxin,[10] the latter indicating lack of involvement of nerve terminals. Indeed, the umbilical cord has no innervation since no cholinergic or adrenergic nerve fibers have been identified by fluorescence.[11] The nonselective alpha-blocker phentolamine caused a significant inhibition of EFS-induced HUCV contractions. However, this inhibition was observed only at high concentrations, indicating that it may be acting on a different population of receptors.[10]

In this study, the nature of the mediator was identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS), followed by a pharmacological characterization of the EFS-induced contractions in both HUA and HUV *in vitro*.

2 | METHODS

2.1 | Study participants

Participants over the age of 18, undergoing the natural or cesarean delivery from Santa Casa de Vinhedo (Vinhedo-SP) and Campinas Maternity Hospital (Campinas-SP), were invited to take part in the study. The women were normotensive, and did not have preeclampsia, pregestational, or gestational diabetes mellitus and none were on regular medication. Written consent was obtained from those who agreed to participate. Umbilical cords from 67 volunteers aged 18-47 years were used (from 18-25, n = 23; from 26-35, n = 25; and from 36-47, n = 19 participants).

The investigation conformed to the principles outlined in the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Institute of Biomedical Sciences of the University of São Paulo—ICB/USP (protocol number 3.165.417).

2.2 | Reagents

Adrenaline, noradrenaline, dopamine, adenosine 5′-triphosphate (ATP), N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and SCH-23390 were purchased from Sigma-Aldrich Chemicals Co. (St Louis, Missouri, USA). Haloperidol was bought from Nallin Farmácia e Manipulação Ltda (Itatiba-SP, Brazil). Dopamine-d3 hydrochloride, DL-noradrenaline-d6 hydrochloride, and adrenaline-d6 hydrochloride were acquired from CDN Isotopes (Point Claire, Canada). Aluminum oxide and Harris' Hematoxilin were purchased from Dinamica Quimica Contemporanea Ltda (Indaiatuba-SP, Brazil). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$), potassium phosphate monobasic (KH_2PO_4), glucose, and entellan were bought from Merck KGaA (Darmstadt, Germany). Acetonitrile was obtained from J.T Baker (Phillipsburg, NJ, USA), and formic acid (HPLC grade) was purchased from Mallinckrodt (St Louis, MO, USA). Anti-human calretinin (code: IS627) was purchased from DAKO (Agilent, USA). Anti-tyrosine hydroxylase (code: ab76442), anti-dopa decarboxylase (code: ab211535), and the secondary antibody (a goat anti-chicken IgY [code: ab150169]) were purchased from Abcam (Cambridge, UK). The tertiary antibody, a rabbit anti-goat IgG (code: AP106P), was purchased from Sigma/Merck (Darmstadt, Germany). NovoLink™ Max Polymer Detection System (code: RE7280-k) and 3,3′ diaminobenzidine (DAB) were purchased from Leica Biosystems (UK). Citrate buffer, pepsin, 2XSSC, and DAPI (code: Z-2028-20) were bought from ZytoVision kit (Bremerhaven, Germany).

2.3 | LC-MS-MS analysis

This study was carried out on human umbilical cord specimens obtained from 12 different placentae/patients. A segment of the umbilical cord—10-20 cm from the insertion point in the placenta and 5 cm from the umbilicus—was removed by the obstetrician and placed in a container with Krebs-Henseleit's solution. The Wharton's jelly was removed and the umbilical arteries (HUA) and the umbilical vein (HUV) were dissected. Vessel rings (two HUA and one HUV; 15 mm each ring; with or without endothelium) were incubated in 10 mL organ baths containing Krebs-Henseleit's solution: (mM) NaCl (118), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (1.2), $NaHCO_3$ (25), KH_2PO_4 (1.2), and glucose (5.6) gassed with a mixture of 95%O_2: 5% CO_2 (pH 7.4) at 37°C. After a period of 30 minutes, an aliquot of 2 mL of the supernatant was transferred to an Eppendorf tube and stored at −20°C until the time for analysis.

The dopamine, noradrenaline, and adrenaline concentrations in the Krebs-Henseleit's solution were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The extraction procedure was similar to that described for extracting methyldopa from plasma.[12] Briefly, 100 µL of the internal standards (dopamine-d3, noradrenaline-d6, and adrenaline-d6 at 100 ng/mL) were added to the Krebs' solution (2 mL) followed by 1.5 mL of deionized water. After vortexing for 10 seconds, 100 mg of Al_2O_3 was added and left for incubation for 20 minutes in an orbital agitator (Centrifuge 5810/ 5810 R). The tubes were then centrifuged at 2000 g for 4 minutes at 4°C and the supernatant discarded. The residue was washed 4 times with 2 mL of deionized water. After the final wash, 200 µL of a solution containing trifluoroacetic acid 0.1% in HCN/H2O (60/40l; v/v) was added. After vortexing for 40 seconds, the Eppendorf tubes were centrifuged for 2000 g for 5 minutes and

TABLE 1 Mass spectrometry operating conditions

Analyte	MRM transition (m/z)	Q1 Prebias (V)	Collision energy	Q3 Prebias (V)	Retention time (min)
Dopamine	154.00 > 91.15	−12.00	−23.00	−18.00	3.12 ± 0.3
Noradrenaline	170.10 > 107.10	−12.00	−23.00	−18.00	2.97 ± 0.3
Adrenaline	184.20 > 107.00	−12.00	−23.00	−18.00	3.05 ± 0.3
Dopamine-d6	157.00 > 93.00	−12.00	−23.00	−18.00	3.12 ± 0.3
Noradrenaline-d3	176.10 > 158.10	−12.00	−23.00	−18.00	2.97 ± 0.3
Adrenaline-d6	190.00 > 171.95	−12.00	−23.00	−18.00	3.05 ± 0.3

the supernatant transferred to the vials for injection. The samples were analyzed by liquid chromatography coupled to a triple quadrupole mass spectrometer, LCMS-8050 (Shimadzu).

The separation of catecholamines was performed on a 100 × 4.6 mm Lichrospher RP-8 column (GL Sciences Inc) using acetonitrile/water (5/95, v/v) with 0.1% formic acid as mobile phase at a flow rate of 0.4 mL/min. The mass spectrometer operated in positive electrospray ionization mode (ES+) for catecholamine detection. The analyses were executed in selected Multiple Reaction Monitoring (MRM) detection mode. The transitions and retention times employed are described in the table below (Table 1).

2.4 | Immunohistochemistry

This assay was carried out on human umbilical cord specimens obtained from 8 different placentae/patients. The human umbilical cord samples were then fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Each block was cut into serial 4-µm-thick sections which were mounted on positively charged slides prior to H&E, immunohistochemical, or fluorescence in situ hybridization (FISH) stainings.

Tissue sections were deparaffinized and rehydrated in graded alcohols to distilled water, and then they were incubated in 3% hydrogen peroxide for 10 minutes to block the endogenous peroxidase. Antigen retrieval was performed by heating slides in citrate buffer (10 mm, pH 6.0) at 95°C for 20 minutes (in a steamer set). Subsequently, they were left to cool down at room temperature and rinsed with PBS. Each slide was then incubated for 2 hours at room temperature with one of the primary antibodies. The primary antibodies used were (1) anti-human calretinin (mouse monoclonal IgG1; clone: DAK-calret 1; catalog code: IS627; immunogen: purified recombinant protein, expressed from the human malignant mesothelioma cell line [Mero-41] calretinin encoding gene, in E. coli[1,2]; the epitope was not specified by the manufacturer; dilution: 1:200 in PBS; DAKO/Agilent, USA), (2) anti-tyrosine hydroxylase (chicken polyclonal IgY; catalog code: ab76442; immunogen: two synthetic peptide/keyhole limpet hemocyanin [KLH] conjugates—these synthetic peptides corresponded to different regions of the Tyrosine Hydroxylase gene product, but were shared between the human [P07101] and mouse [P24529] sequences; predicted reactivity: mouse, rat, and human, according to the manufacturer; dilution 1:1500 in PBS, Abcam, Cambridge, UK), and (3) anti-dopa decarboxylase (mouse monoclonal IgG1; clone: CL 2962; catalog code: ab211535; immunogen: recombinant fragment corresponding to Human DOPA Decarboxylase/DDC aa 114-221; sequence: LETVMMDWLGKMLEL PKAFLNEKAGEGGGVIQGSASEATLVALLAARTKVIHRLQAA SPELTQAAIMEKLVAYSSDQAHSSVERAGLIGGVKLKAIP SDGNFAMRASA; database link: P20711; epitope: binds to an epitope located within the peptide sequence MDWLGKMLEL (aa 119-128) as previously determined by the manufacturer using overlapping synthetic peptides; and dilution: 1:100 in PBS; Abcam, Cambridge, UK). Detection of tyrosine hydroxylase required the use of secondary and tertiary antibodies. The secondary antibody was a goat anti-chicken IgY (catalog code: ab150169; dilution 1:500 in PBS, Abcam, Cambridge, UK). The tertiary antibody was a rabbit anti-goat IgG (catalog code: AP106P, dilution 1:250 in PBS, Sigma/Merck, Germany). The slides were incubated for 1 hour at room temperature with the secondary antibody, followed by 1 hour incubation with the tertiary antibody. The detection system was the NovoLink™ Max Polymer Detection System (catalog code RE7280-k, Leica Biosystems, UK), following the protocol described by the manufacturer. Thereafter, 3,3′ diaminobenzidine (DAB) was used as chromogen. Finally, the slides were dehydrated, counterstained with Harris´ hematoxylin and cover slipped in Entellan (Sigma/Merck). Negative controls consisted of the omission of the primary antibody and incubation with the primary antibody diluents (as well as with the secondary/tertiary antibodies, where applicable, and the detection system) and were performed in all immunohistochemistry assays (one negative control per section) to identify any background staining. All solutions (including primary, secondary, and tertiary antibody stocks) were prepared for a single use on the same day of the immunohistochemistry assay, and kept at 4°C until use. All slides were examined and photomicrographed using a trinocular Eclipse 50i microscope (Nikon) coupled to a 10MP CMOS digital camera (AmScope, EUA). Positivity was assessed by an experienced MD, PhD pathologist (AAS), who was blind to the presence/absence of the primary antibody on the sample under examination (the observer did not know whether a test sample or an omission control was being assessed). Blinding was achieved by covering the slide labels with a removable occluding sticker.

For FISH analysis, sections from 5 randomly selected from the 8 human umbilical cords used for immunohistochemistry were deparaffinized with xylene and rehydrated in graded alcohols for

5 minutes each. Then, they were incubated in a 0.2 N HCl solution for 20 minutes, and subsequently treated with a citrate pH 6.0 buffer (ZytoVision kit, catalog code Z-2028-20, Germany) at 80°C for 1 hour. After this, they were incubated with pepsin for 8 minutes at room temperature. The slides were washed with 2XSSC (ZytoVision kit, catalog code Z-2028-20, Germany), dehydrated in a sequence of ethanols (75%, 80%, and 100% ethanol for 2 minutes each), and then air dried. The slides were incubated with 100μL of the TH mRNA probe (at a concentration of 100 μM, in RNAse-free water) for 10 minutes at 75°C and overnight in a Dako Hybridizer (Dako, Denmark) at 37°C. The TH mRNA probe sequence was as follows: 5′- AACCGCGGGGACATGATGGCCT-3′ (RNA Tm = 77.8°C) (catalog code: VC00021, Sigma/Merck, Germany). The probe was labeled with fluorescein 6-FAM in the 5' region. The next day, the slides were placed in a UREA/0,1Xssc solution at 45°C for 30 minutes, and then, they were washed with a 2xSSC solution for 2 minutes. After this, the slides were dehydrated in 75%, 85%, and 100% ethanols for 2 minutes each, and air dried. Finally, the slides were mounted with 15 μL of a DAPI containing mounting medium (from the ZytoVision kit) and cover slipped (the cover slip being sealed with a Fixogum Rubber Cement, from Marabu, Germany). Negative controls consisted of the omission of the probe and were performed in all FISH assays (one negative control per section) to control for any significant autofluorescence. All FISH slides were examined and photomicrographed using a trinocular DM4000 B LED microscope (Leica Microsystems, Wetzlar, Germany) coupled to a 1.4 MP DFC 310 FX camera (Leica, Switzerland).

2.5 | Pharmacological experiments

This study was carried out on human umbilical cord specimens obtained from 47 different placentae/patients. The Wharton's jelly was removed and the umbilical arteries (HUA) and the umbilical vein (HUV) were dissected. Vessels rings (3 mm) were suspended vertically between two metal hooks in 10 mL organ baths containing Krebs-Henseleit's, gassed with a mixture of 95%O_2:5% CO_2 (pH 7.4) at 37°C, and coupled to isometric transducer. The initial smooth muscle tension was set at 10 mN.[10] Tensioning force was recorded using a PowerLab 400 data acquisition system (Software Chart, version 7.0; ADInstruments, Colorado Springs, CO, USA).

Following a 90-min stabilization period, the rings were precontracted with 5-HT (1 μM), and the integrity of the endothelium in both HUA and HUV was evaluated by the addition of ATP to cause relaxation (10 μM).[10] Cumulative concentration-response curves to dopamine (10 nM to 3 mM) were performed in endothelium-intact HUA and HUV rings in the absence and in the presence of the NO synthesis inhibitor L-NAME (100 μM; for 60 minutes). The effect of D1-like receptor antagonist SCH-23390 (10 μM; for 30 minutes) and the D2-like receptor antagonist haloperidol (10 μM; for 30 minutes) in dopamine-induced contractions was investigated in endothelium-intact vessels treated with L-NAME.

The HUA and HUV rings were submitted to EFS at 60V for 30 seconds, at 8-16 Hz in square-wave pulses, 0.3 ms pulse width, and 0.1 ms delay, using a Grass S88 stimulator (Astro-Medical, Industrial Park, RI, USA). Electrical field simulations were performed in the presence and absence of L-NAME, and in the presence and absence of the dopamine D1-like receptor antagonist SCH-23390 (10 μM; for 30 minutes), of the dopamine D2-like receptor antagonist haloperidol (10 μM; for 30 minutes), of the adrenergic alpha-1 receptor antagonist prazosin (100 μM; for 30 minutes), and of the adrenergic alpha-2 receptor antagonist idazoxan (100 μM; for 30 minutes). The effect of the antagonists was evaluated always in the presence of L-NAME (100 μM).

2.6 | Data analysis

Data are expressed as mean ± (SEM) of the number of experiments (n = or>5). Paired Student's *t* test was used and a *P*-value < .05 was considered as significant. In the pharmacological experiments, the number of experiments is expressed as x/y, where x represents the number of umbilical vessels and y the number of rings employed in the experiment. For Emax analysis and pEC50, unpaired Student's *t* test was used and a *P*-value of < .05 was considered as significant.

Nonlinear regression analysis to determine the pEC_{50} was carried out using GraphPad Prism (GraphPad Software, version 6.0, San Diego, CA, USA) with the constraint that $\Phi = 0$. All concentration-response data were evaluated for a fit to a logistics function in the form: $E = Emax/([1 + (10^c/10^x)^n] + \Phi$. The values of pEC_{50} data represent the mean ± SEM. Values of Emax were represented by mN.

3 | RESULTS

3.1 | Determination of amine concentrations by LC-MS-MS

Dopamine, noradrenaline, and adrenaline calibration curves were linear for concentrations of 0.1-10.0 ng/mL, with a correlation coefficient higher than 0.99. The limit of quantification was 0.1 ng/mL. The method was fully validated, and the results reported elsewhere.[13] Only dopamine concentrations were above the limit of quantification, and were only observed in endothelium-intact HUA and HUV (Figure 1A and 1B).

3.2 | Umbilical cord vessels. Immunohistochemistry and fluorescence in situ hybridization (FISH) analysis

Tyrosine hydroxylase was detected by immunohistochemistry only in endothelial cells, in all samples of both HUA (n = 8; Figure 2A) and HUV (n = 8; Figure 2B). Negative controls were obtained by

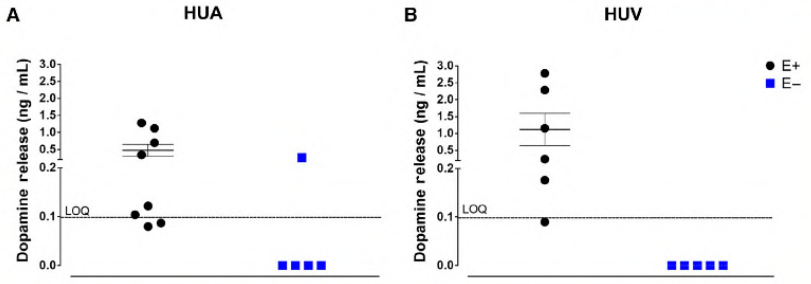


FIGURE 1 Panel A shows the basal release of dopamine in Krebs-Henseleit´s solution after 30 minutes incubation with human umbilical artery endothelium-intact rings (E+; n = 8) and with human umbilical artery endothelium-denuded rings (E-; n = 5). Panel B shows the basal release of dopamine in Krebs-Henseleit´s solution after 30 minutes incubation with human umbilical vein endothelium-intact rings (E+; n = 6) and with human umbilical vein endothelium-denuded rings (E-; n = 5). LOQ = limiti of quantitation

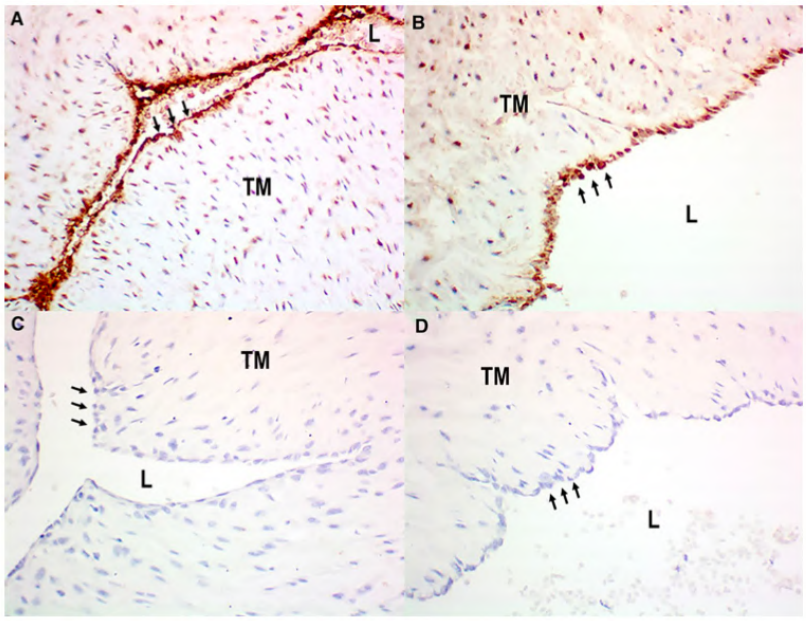


FIGURE 2 Detection of tyrosine hydroxylase by immunohistochemistry in human umbilical cords: positive endothelial staining in HUA (panel a [arrows]) and HUV (panel b [arrows]); negative control sections (omission of primary antibody) showing absence of positivity in both HUA (panel c [arrows]) and (panel d [arrows]) HUV endothelia. Immunoperoxidase (200X, original magnification). L = lumen; TM = Tunica Media

the omission of the primary antibody, as illustrated for HUA (n = 8; Figure 2C) and HUV (n = 8, Figure 2D).

Presence of tyrosine hydroxylase messenger RNA in HUA and HUV was assessed by fluorescence in situ hybridization analysis (FISH). Tyrosine hydroxylase mRNA was consistently detected in endothelial cells of all tested samples (n = 5), both in HUA (cytoplasmic green fluorescence in Figure 3B [cytoplasmic staining alone] and 3C [cytoplasmic and nuclear staining overlay]) and in HUV (cytoplasmic green fluorescence in Figure 3E [cytoplasmic staining alone] and 3F [cytoplasmic and nuclear staining overlay]). Endothelial nuclei are seen in blue (DAPI staining), both in HUA (Figure 3A [nuclear staining alone] and 3C [cytoplasmic and nuclear staining overlay]) and in HUV (Figure 3D [nuclear staining alone] and 3F [cytoplasmic and nuclear staining overlay]).

Dopa decarboxylase was detected by immunohistochemistry in the endothelia, in all samples of both HUA (n = 8; Figure 4A) and HUV (n = 8; Figure 4B). Negative controls were obtained by the omission of the primary antibody, as illustrated for HUA (n = 8; Figure 4C) and HUV (n = 8; Figure 4D). FISH could not be used to detect dopa decarboxylase mRNA because there were no commercially available probes at the time.

Using immunohistochemistry, we attempted to identify calretinin (a neural marker, commonly used to detect nerve fibers and neuronal cell bodies) in umbilical cord samples, with special attention to the vessel walls. Calretinin was not found in any samples of either HUA (n = 8;

Figure 5A) or HUV (n = 8; Figure 5B), which indicates lack of neural tissue within the vessels walls (thus, ruling out a neural origin for the vessel-derived catecholamines detected in the pharmacological assays).

3.3 | Effect of L-NAME

Dopamine alone induced contractions in L-NAME (100 μM)-treated HUA (Emax 7.5 ± 0.4 mN; pEC_{50} 3.8 ± 0.1 (n = 6/12; Figure 6A). Dopamine also induced contractions in ODQ (10 μM) pretreated HUA (Emax 6.9 ± 0.9 mN; pEC_{50} 4.1 ± 0.1; n = 5/10) and in endothelium-denuded HUA (Emax 7.2 ± 1.0 mN; pEC_{50} 2.9 ± 0.1; n = 5/10). There was no significant difference in the Emax, but the pEC_{50} 2.9 of dopamine-induced contractions in endothelium-denuded HUA presented a significant right shift when compared to either L-NAME- or ODQ-treated HUA.

Dopamine caused concentration-dependent contractions of HUV (Emax 5.9 ± 0.4 mN; pEC_{50} 4.8 ± 0.2 [n = 5/5]; Figure 6B), and those contractions were potentiated by previous incubation with L-NAME (Emax 12.8 ± 0.7 mN; pEC_{50} 4.6 ± 0.1 [n = 5/10]; Figure 6B).

Pretreatment with L-NAME (100 μM) significantly increased the EFS (8 Hz and 16 Hz)-induced contractions of both HUA (Figure 7A) and HUV (Figure 7B).

3.4 | Effect of alpha-adrenergic receptor antagonists

Incubation with prazosin (100 μM), a selective α_1-adrenoceptor antagonist, had no effect in the EFS-induced contraction of the HUA (4.3 ± 1.1 and 4.5 ± 1.4 mN for 8 Hz; 4.7 ± 1.0 and 4.8 ± 1.2 mN for 16 Hz; n = 5/5, for control and prazosin pretreated vessels, respectively; Figure 8A,C). Similar results were obtained in HUV (3.5 ± 0.5 and 3.6 ± 0.6 mN for 8 Hz; 5.5 ± 1.2 and 5.7 ± 1.3 for 16 Hz; n = 5/7, for control and prazosin pretreated vessels, respectively; Figure 8B,D).

Incubation with idazoxan (100 μM), a selective α_2-adrenoceptor antagonist, had no effect in the EFS-induced contraction of the HUA (3.4 ± 0.8 and 3.4 ± 1.0 mN for 8 Hz; 4.8 ± 1.2 and 4.9 ± 1.1 mN for 16 Hz; n = 5/5, for control and idazoxan pretreated vessels, respectively, Figure 9A,C). Similar results were obtained in HUV (4.4 ± 1.5 and 4.8 ± 1.4 mN for 8 Hz; 5.1 ± 1.4 and 5.2 ± 1.3 mN for 16 Hz; n = 5/6, for control and idazoxan pretreated vessels, respectively; Figure 9B,D).

3.5 | Effect of dopamine receptor antagonists

In L-NAME-treated vessels, the dopamine D1-like receptor antagonist SCH-23390 (10 μM) caused reduction in dopamine-induced contractions of HUA (Emax 7.6 ± 0.5 [n = 5/10] and 3.3 ± 0.5 mN [n = 5/10], without and with SCH-23390, respectively; pEC_{50} 3.9 ± 0.1 [n = 5/10] and 4.2 ± 0.3 mN [n = 5/10], $P < .05$, without and with SCH-23390, respectively; Figure 6C). Similar results were observed in HUV (Emax 12.8 ± 0.7 [n = 5/10] and 5.1 ± 0.9 mN [n = 5/10], without and with SCH-23390, respectively; pEC_{50} 4.6 ± 0.1 [n = 5/10] and 3.3 ± 0.3 mN [n = 5/10], $P < .05$, without and with SCH-23390, respectively; Figure 6D). The EFS (8 Hz and 16 Hz)-induced contractions of both HUA (Figure 7C; n = 5/5) and HUV (Figure 7D; n = 5/8) were not affected by incubation with SCH-23390 (10 μM).

In L-NAME-treated vessels, the dopamine D2-like receptor antagonist haloperidol (10 μM) abolished the contraction dependent of dopamine HUA (Emax 7.6 ± 0.5 mN and pEC_{50} 3.9 ± 0.1 without haloperidol [n = 5/10]; Figure 6E) and HUV (Emax 12.8 ± 0.7 mN and pEC_{50} 4.6 ± 0.1 [n = 5/10], without haloperidol; Figure 6D). The Emax data there were significant difference $P < .05$. Incubation with haloperidol (10 μM) caused significant reduction in EFS-induced

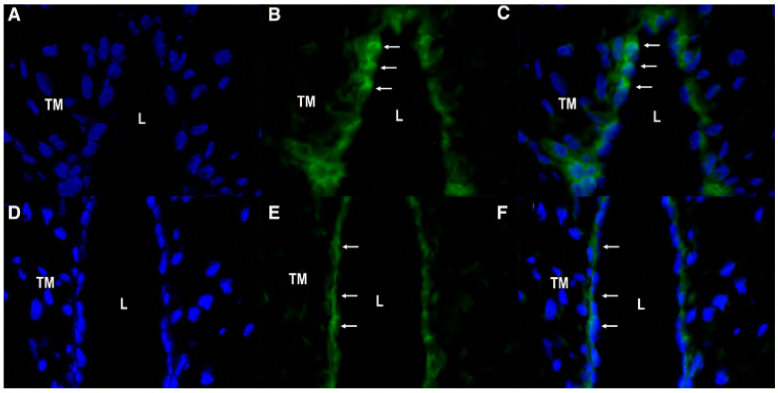


FIGURE 3 Detection of tyrosine hydroxylase mRNA by fluorescence in situ hybridization (FISH) in human umbilical cord artery (panel A-C) and vein (panel D-F); (panel A) HUA, DAPI staining in nuclei; (panel B) HUA, TH mRNA staining in the cytoplasm of endothelial cells (arrows); (panel C) HUA, overlay (DAPI + TH mRNA stainings [arrows]); (panel D) HUV, DAPI staining in nuclei; (panel E) HUV, TH mRNA staining in the cytoplasm of endothelial cells (arrows); (panel F) HUV, overlay (DAPI + TH mRNA stainings [arrows]). DAPI/FITC (400X, original magnification). L = lumen; TM = Tunica Media

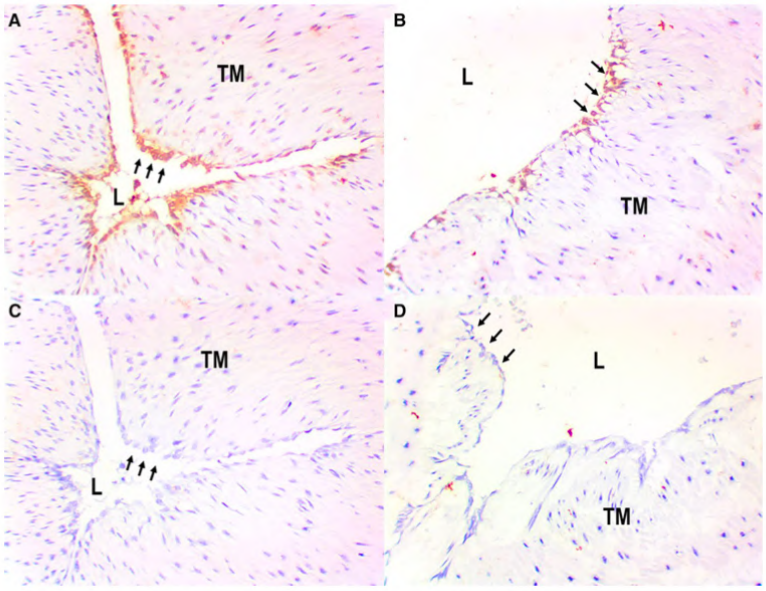
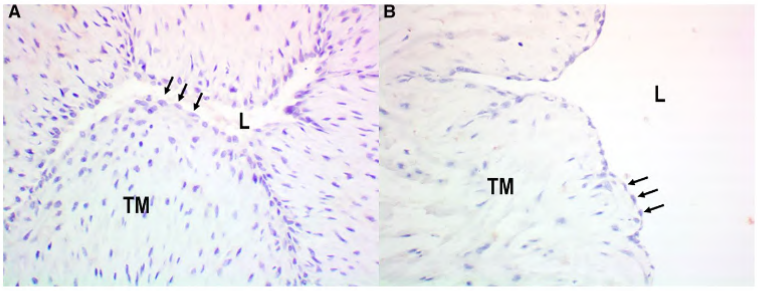


FIGURE 4 Detection of dopa decarboxylase by immunohistochemistry in human umbilical cords: positive endothelial staining in HUA (panel A [arrows]) and HUV (panel B [arrows]); negative control sections (omission of primary antibody) showing absence of positivity in both HUA (pane C [arrows]) and HUV (panel D [arrows]) endothelia. Immunoperoxidase (200X, original magnification). L = lumen; TM = Tunica Media

FIGURE 5 Detection of calretinin (CALRET) by immunohistochemistry in human umbilical cords: sections showing absence of positivity in both artery (panel A [arrows]) and vein (panel B [arrows]) endothelia. Calretinin is also negative in the tunica media (TM) of both vessels (panel A-B). Immunoperoxidase (400X, original magnification). L = lumen; TM = Tunica Media



contraction of the HUA (Figure 7E; n = 5/8) and HUV (Figure 7C; n = 5/9).

4 | DISCUSSION

The endothelium of umbilical cord vessels is capable of releasing mediators capable of modulating the contractile activity induced by EFS.[9] The results presented here clearly demonstrate, for the first time in human vessels, that HUA and HUV display a basal endothelium-derived dopamine release, as identified by tandem mass spectrometry. Furthermore, the enzymes involved in dopamine synthesis, tyrosine hydroxylase, which is the enzyme responsible for the conversion of tyrosine into L-dihydroxy-phenylalanine (L-DOPA)[14] and dopa-decarboxylase, also responsible for the conversion of L-DOPA into dopamine,[15] have been identified in the endothelial cells of both HUA and HUV by immunohistochemistry. The RNA messenger of tyrosine hydroxylase in the endothelial cells of both HUA and HUV was also characterized by fluorescence in situ hybridization. Cultured endothelial cells from bovine aorta[16] and from rat mesenteric artery[17] also express the enzymes involved in catecholamine synthesis. Thus, endothelium plays an obligatory role in dopamine release. In newborn Wistar rats has been demonstrated the non-neuronal origin of dopamine. Indeed, chemical sympathectomy with 6-hydroxydopamine caused a significant reduction in noradrenaline and adrenaline levels extracted from the aortae, while dopamine levels remained unaffected.[17] The absence of neural/neuronal tissue within HUCV walls, as indicated by the absence of calretinin at these sites, supports the hypothesis of a non-neuronal source for the vessel-released dopamine we described.

Dopamine acts on selective receptors, belonging to the G protein–coupled receptor family. Five genes encoding DA receptors (DRs) have been identified. These receptors are divided into two subfamilies: the D1-like receptor subtypes (D1R and D5R), coupled to

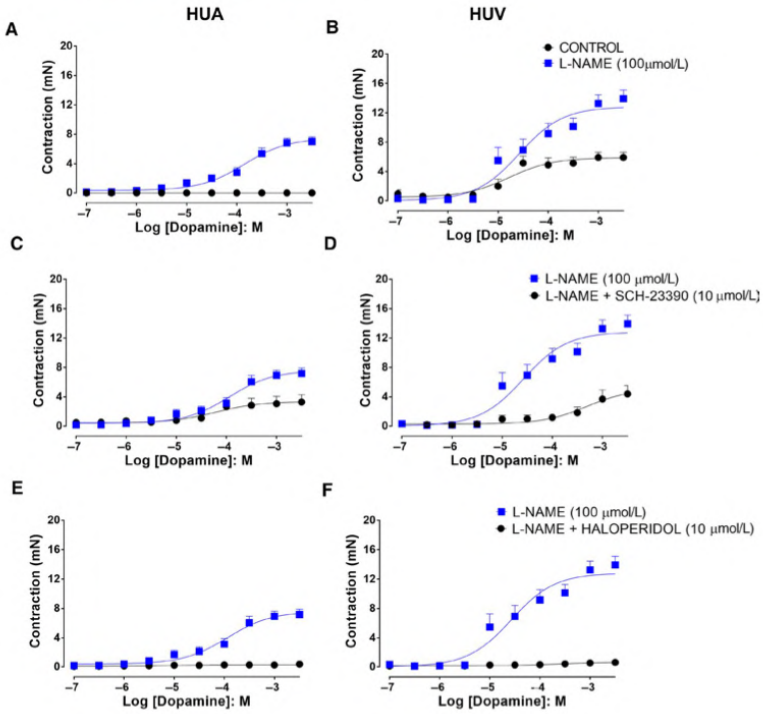


FIGURE 6 Dopamine concentration-response curves in the absence and presence of L-NAME in HUA rings (Panel A [control n = 6/6 and L-NAME 100 μM n = 6/12]) and in HUV rings (Panel B [control n = 5/5 and L-NAME 100 μM n = 5/10]). Effect of the D1-like receptor antagonist SCH-23390 on the dopamine concentration-response curves in HUA rings (Panel C [L-NAME 100 μM n = 5/10 and L-NAME + SCH-23390 10 μM n = 5/10]) and in HUV rings (Panel D [L-NAME 100 μM n = 5/10 and L-NAME + SCH-23390 10 μM n = 5/10]). Effect of the D2-like receptor antagonist haloperidol on the dopamine concentration-response curves in HUA rings (Panel E [L-NAME 100 μM n = 5/10 and L-NAME + haloperidol 10 μM n = 5/10]) and in HUV rings (Panel F [L-NAME 100 μM n = 5/10 and L-NAME + haloperidol 10 μM n = 5/10]). In the six panels, there was a significant difference in the Emax ($P < .05$)

Gs, activating adenylyl cyclase and the D2-like subfamily (D2R, D3R, and D4R) coupled to Gi, inhibiting adenylyl cyclase.[18] Dopaminergic receptors in vascular beds have been identified *in vitro* by radioligand-receptor binding and autoradiographic techniques. The localization of dopamine-1 (D_1)[19] and dopamine-2 (D_2) receptors has been assessed in smooth muscle tissue of rat cerebral, mesenteric and renal arteries.[19] In cerebral, coronary, pulmonary, and mesenteric arteries of rabbits, dopamine D_1 and D_2 receptors have been localized in the endothelium.[20] Immunohistochemical analysis has identified D_2 and D_4 subtypes in cerebral and mesenteric vascular bed and D_2 and D_3 receptors in renal vasculature, with the D_5 subtype predominantly residing as a smooth muscle receptor in the vascular beds of rats.[21] Similarly, in sections of HUA, the dopaminergic receptor D1 has been characterized by using the dopaminergic competitive antagonist SCH 23 390.[22,23] Dopamine is known to be able to cause endothelium-dependent relaxation of rabbit pulmonary artery.[24] Data assessing mRNA and/or protein expression of dopamine receptors in vessels seem to converge in showing D1-like receptors expressed in endothelial cell.[25] However, data on D1 signaling in endothelial cells are lacking.

The finding that dopamine could only contract HUA in the presence of the NO synthesis inhibitor L-NAME indicates a major interaction of dopamine with NO on this vessel. Indeed, similar results were obtained with the heme-site inhibitor of soluble guanylyl cyclase ODQ and in endothelium-denuded vessel. It is likely that the dopamine released in the circulation by EC would cause NO-dependent vasodilatation through the action on D1 receptors expressed by EC. Indeed, the hemodynamic effects of dopamine depend on the dose administered; with intravenous infusions ranging from 1 to 10 μg/kg/min, dopamine increased cardiac contractility,[26] cardiac output,[27] and renal blood flow[28] in normal subjects. The heart rate did not change and the mean arterial blood pressure was either unchanged or slightly decreased. When higher infusion rates were administered, arterial pressure increased and heart rate decreased.[29]

The inhibition of EFS-induced contractions by the D_2-like receptor antagonists haloperidol revealed another important modulator role of the endothelium-derived dopamine, acting as a vasoconstrictor through the D2-like receptor. The finding that SCH-23390 also had some inhibitory effect on dopamine-induced contractions of L-NAME-treated HUA and HUV was possibly due to the antagonistic effect of this compound on D2 receptors at higher concentration.[30] Indeed, SCH-23390 exhibits only 1/1,000th the potency of haloperidol as antagonist for the D2 receptor, which may explain the lack of effect observed in EFS-induced contractions of HUCV. The role of D2-like receptor as a modulator of vasoconstriction should not be restricted to the *in vitro* scenario. Domperidone and haloperidol applied as ophthalmic solutions in a rabbit ocular hypertensive model produced a marked increase in ocular blood flow.[31] Administration of the selective D2 receptor agonist pramiprexole to healthy male volunteers caused increased systolic blood pressure in both supine and standing positions.[32] In previous studies in the field of primary care, schizophrenic patients and nonschizophrenic patients treated with antipsychotics were strongly associated, after adjusted analysis, with a lesser

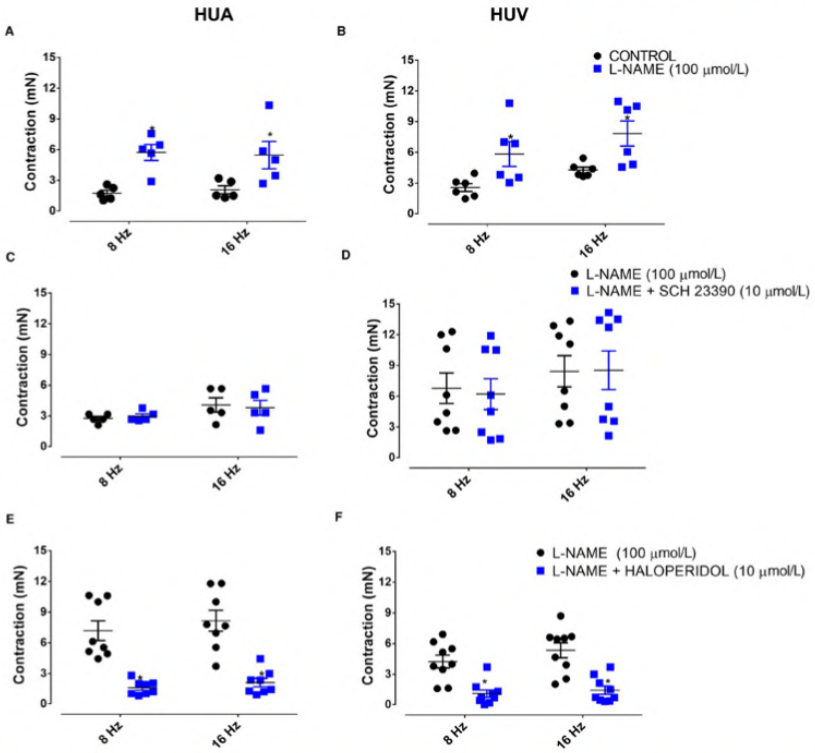


FIGURE 7 EFS caused a contraction in both HUA (panel A [control n = 5/5 and L-NAME 100 µM n = 5/5]) and HUV rings (panel B [control n = 6/6 and L-NAME 100 µM n = 6/6]). The response was significantly potentiated in both HUA and HUV by previous treatment with L-NAME. The incubation with SCH-23390 had no effect on the EFS-induced contractions in either HUA (panel C [L-NAME 100 µM n = 5/5 and L-NAME + SCH-23390 10 µM n = 5/5]) and HUV rings (panel D [L-NAME 100 µM n = 5/8 and L-NAME + SCH-23390 10 µM n = 5/8]). The treatment with haloperidol caused significant reduction in EFS-induced contractions in both HUA (panel E [L-NAME 100 µM n = 5/8 and L-NAME + haloperidol 10 µM n = 5/8]) and HUV rings (panel F [L-NAME 100 µM n = 5/9 and L-NAME + haloperidol 10 µM n = 5/9]). Data are expressed as mean ± SEM *P < .05. Vs control

presence of hypertension.[33] This was particularly unexpected since patients affected by schizophrenia have an increased cardiovascular morbidity and mortality.[34]

The alpha$_1$-adrenergic receptor antagonist prazosin[35] and the alpha$_2$-adrenergic receptor antagonist idazoxan[36] had no effect on EFS-induced contraction of HUCV, confirming that dopamine is the major catecholamine responsible for this phenomenon. The inhibition observed with phentolamine at higher concentration is possibly due to binding of phentolamine in D2-like receptors, as previously suggested.[10] Indeed, phentolamine at higher concentrations (>2 µM) displaces ^{3}H-haloperidol binding to dopamine receptors in calf brain membranes.[37]

The interaction between dopamine and NO should not be restricted to their pharmacological actions. Nitro-catecholamines such as nitro-dopamine, nitro-noradrenaline, and nitro-adrenaline have been found in rat brain.[38] Thus, it is possible that endothelium-derived dopamine may react with NO to form nitro-dopamine, and nitro-dopamine itself could be also an important mediator of cardiovascular reactivity. N-arachidonoyl dopamine (NADA) is a member of the N-acyl dopamine family; several lines of evidence identified NADA as an agonist of endo-vanilloid receptors with similar potency of capsaicin.[39] The finding that human vascular tissue displays basal release of endothelium-derived dopamine warrants further investigation on whether or not these and other dopamine derivatives may have a modulatory role on the cardiovascular system.

What is the possible physiological role of endothelial-derived dopamine? Although cardiac output is defined by the product of heart rate and systolic volume, it is known that the pumping function of the heart has a permissive role in the determination of cardiac output.[40] Indeed, the cardiac output was largely unaffected by heart rate when subjects were electrically paced.[41] The characteristics of the peripheral circulation such as capacitance and conductance/resistance play a major role in determining cardiac output. Most of the deductions in the role of sympathetic modulation of the circulation have been obtained by the use of either adrenergic agonists or antagonists, assuming that these mediators are coming from nerve terminals. The finding that human vascular tissue has basal release of dopamine should change this paradigm.

ACKNOWLEDGMENTS

JBJ thanks CAPES for PhD fellowship. EA, FM, AS, & GDN thank FAPESP (2017/15175-1 and 2016/04731-8). GDN thanks CNPq (303839/2019-8).

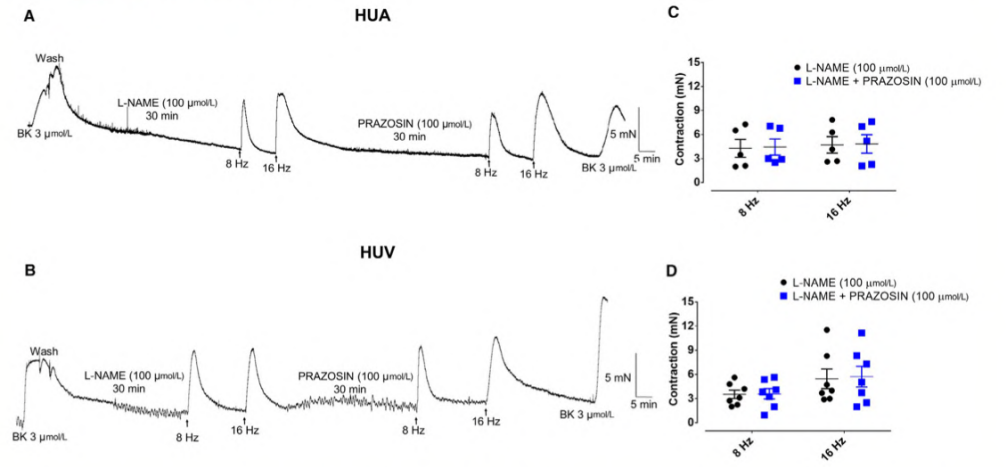


FIGURE 8 The incubation of the alpha1-adrenergic receptor antagonist prazosin has not affected the EFS-induced contractions of either HUA (panel A [L-NAME 100 µM n = 5/5] and [L-NAME 100 µM + prazosin 100 µM n = 5/5]) or HUV rings (panel B [L-NAME 100 µM n = 5/7] and [L-NAME 100 µM + prazosin 100 µM n = 5/7]). Scatter plot shows the individual values and mean ± SEM of the EFS-induced contractions in L-NAME (100 µM) pretreated HUA (panel C; n = 5/5 for 8 Hz and 16 Hz) and HUV rings (panel D; n = 5/7 for 8 Hz and 16 Hz) in the absence and presence of prazosin

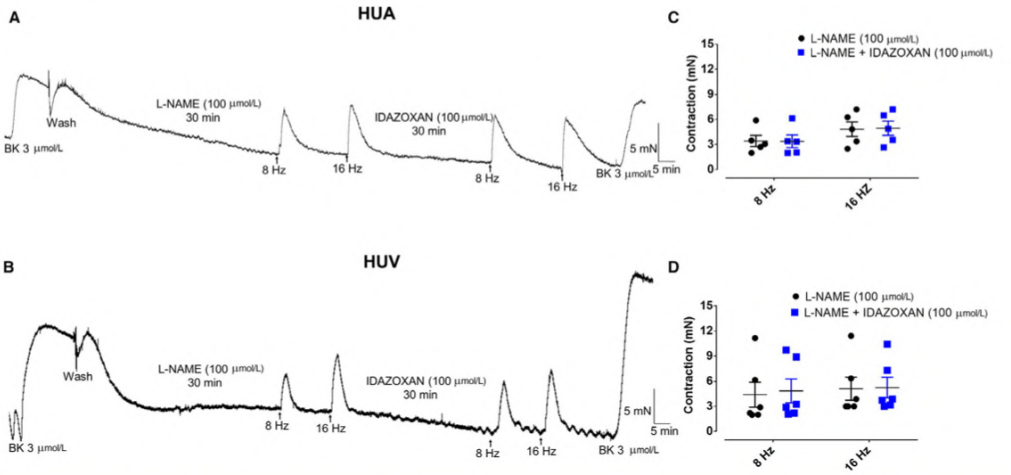


FIGURE 9 The incubation of the alpha2-adrenergic receptor antagonist idazoxan has not affected the EFS-induced contractions of either HUA (panel A [L-NAME 100 µM n = 5/5] and [L-NAME 100 µM + idazoxan 100 µM n = 5/5]) rings or HUV (panel B [L-NAME 100 µM n = 5/6] and [L-NAME 100 µM + idazoxan 100 µM n = 5/6]) rings. Scatter plot shows the individual values and mean ± SEM of the EFS-induced contractions in L-NAME (100 µM) pretreated HUA (panel C; n = 5/5 for 8 Hz and 16 Hz) and HUV (panel D; n = 5/6 for 8 Hz and 16 Hz) in the absence and presence of idazoxan

CONFLICT OF INTEREST

The authors of this manuscript declare that they have no conflicts of interest.

Research data are not shared.

AUTHOR CONTRIBUTIONS

Conceptualization: José Britto-Júnior, Gilberto De Nucci. **Data curation:** Fabiola Z. Mónica, Edson Antunes, Gilberto De Nucci. **Formal analysis:** Gilberto De Nucci. **Funding acquisition:** Edson Antunes,

Gilberto De Nucci. **Investigation:** José Britto-Júnior, Gilberto De Nucci. **Methodology**: José Britto-Júnior, David Halen Araújo Pinheiro, Alberto Fernando Oliveira Justo, Guilherme M. Figueiredo Murari, Rafael Campos, Fernanda Viviane Mariano, Valéria Barbosa de Souza, André Almeida Schenka, Edson Antunes, Fabiola Z. Mónica, Gilberto De Nucci. **Project administration:** Gilberto De Nucci. **Supervision:** Fabiola Z. Mónica, Edson Antunes. **Visualization:** Edson Antunes. **Writing – original draft:** José Britto-Júnior, Gilberto De Nucci. **Writing – review & editing:** José Britto-Júnior, Gilberto De Nucci.

OPEN RESEARCH BADGES

This article has earned Open Data and Open Materials badges for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data and materials are available at DOI: 10.22541/au.158456487.79594821.

ORCID

José Britto-Júnior https://orcid.org/0000-0003-0250-8468
David H. A. Pinheiro https://orcid.org/0000-0002-8661-9159
Alberto F. O. Justo https://orcid.org/0000-0001-5531-0485
Guilherme M. Figueiredo Murari https://orcid.org/0000-0002-1890-0723
Rafael Campos https://orcid.org/0000-0002-9816-2061
Fernanda V. Mariano https://orcid.org/0000-0003-3694-5025
Valéria B. de Souza https://orcid.org/0000-0002-6462-5718
André A. Schenka https://orcid.org/0000-0002-8162-8996
Fabiola Z. Mónica https://orcid.org/0000-0002-8449-6677
Edson Antunes https://orcid.org/0000-0003-2201-8247
Gilberto De Nucci https://orcid.org/0000-0002-4346-7941

REFERENCES

1. Paterson G. The response to transmural stimulation of isolated arterial strips and its modification by drugs. *J Pharm Pharmacol*. 1965;17:341-349.
2. Bevan JA. Some characteristics of the isolated sympathetic nerve-pulmonary artery preparation of the rabbit. *J Pharmacol Exp Ther*. 1962;137:213-218.
3. Van Riper DA, Bevan JA. Electrical field stimulation-mediated relaxation of rabbit middle cerebral artery. Evidence of a cholinergic endothelium-dependent component. *Circ Res*. 1992;70:1104-1112.
4. Buga GM, Ignarro LJ. Electrical field stimulation causes endothelium-dependent and nitric oxide-mediated relaxation of pulmonary artery. *Am J Physiol*. 1992;262:H973-H979.
5. Dail WG, McGuffee L, Minorsky N, et al. Responses of smooth muscle strips from penile erectile tissue to drugs and transmural nerve stimulation. *J Auton Pharmacol*. 1987;7:287-293.
6. Narahashi T, Moore JW, Scott WR. Tetrodotoxin blockage of sodium conductance increase in lobster Giant axons. *J Gen Physiol*. 1964;47:965-974.
7. Campos R, Justo AFO, Mónica FZ, et al. Electrical field-induced contractions on *Crotalus durissus terrificus* and *Bothrops jararaca* aortae are caused by endothelium-derived catecholamine. *PLoS One*. 2018;13:e0203573.
8. Campos R, Mónica FZ, Justo AFO, et al. Electrical field stimulation-induced contractions on *Pantherophis guttatus* corpora cavernosa and aortae. *PLoS One*. 2018;13:e0196123.
9. Campos R, Jacintho FF, Britto-Júnior J, et al. Endothelium modulates electrical field stimulation-induced contractions of *Chelonoidis carbonaria* aortic rings. *Comp Biochem Physiol C Toxicol Pharmacol*. 2020;233:108763.
10. Britto-Júnior J, Jacintho FF, Figueiredo Murari GM, et al. Electrical field stimulation induces endothelium-dependent contraction of human umbilical cord vessels. *Life Sci*. 2020;243:117257.
11. Reilly FD, Russell PT. Neurohistochemical evidence supporting an absence of adrenergic and cholinergic innervation in the human placenta and umbilical cord. *Anat Rec*. 1977;188:277-286.
12. Oliveira CH, Barrientos-Astigarraga RE, Sucupira M, et al. Quantification of methyldopa in human plasma by high-performance liquid chromatography-electrospray tandem mass spectrometry application to a bioequivalence study. *J Chromatogr B, Anal Technol Biomed life Sci*. 2002;768:341-348.
13. Britto-Júnior J, Antunes NJ, Campos R, et al. Determination of dopamine, noradrenaline and adrenaline in Krebs-Henseleit's solution by liquid chromatography coupled tandem mass spectrometry. Measurement of basal release from *Chelonoidis carbonaria* aortae *in vitro*. Biomedical Chromatography, 2020, in press.
14. Nagatsu T, Levitt M, Udenfriend S. Tyrosine hydroxylase. The initial step in norepineprine biosynthesis. *J Biol Chem*. 1964;239:2910-2917.
15. Bertoldi M. Mammalian Dopa decarboxylase: structure, catalytic activity and inhibition. *Arch Biochem Biophys*. 2014;546:1-7.
16. Sorriento D, Santulli G, Del Giudice C, et al. Endothelial cells are able to synthesize and release catecholamines both *in vitro* and *in vivo*. *Hypertens*. 2012;60:129-136.
17. Pfeil U, Kuncova J, Brüggmann D, et al. Intrinsic vascular dopamine - a key modulator of hypoxia-induced vasodilatation in splanchnic vessels. *J Physiol*. 2014;592:1745-1756.
18. Missale C, Nash SR, Robinson SW, et al. Dopamine receptors: from structure to function. *Physiol Rev*. 1998;78:189-225.
19. Amenta F, Collier WL, Ricci A. Autoradiographic localization of vascular dopamine receptors. *Am J Hypertens*. 1990;3:34S-36S.
20. Amenta F. Light microscope autoradiography of peripheral dopamine receptor subtypes. *Clin Exp Hypertens*. 1997;19:27-41.
21. Amenta F, Barili P, Bronzetti E, et al. Localization of dopamine receptor subtypes in systemic arteries. *Clin Exp Hypertens*. 2000;22:277-288.
22. Billard W, Ruperto V, Crosby G, et al. Characterization of the binding of 3H-SCH 23390, a selective D-1 receptor antagonist ligand, in rat striatum. *Life Sci*. 1984;35:1885-1893.
23. Antonio Ferreira-de-Almeida J, Pereira-Leite L, Cavallotti C, et al. Pharmacological characterization and autoradiographic localization of dopamine D1 receptors in the human umbilical artery. *Eur J Pharmacol*. 1993;234:209-214.
24. Yamauchi M, Kobayashi Y, Shimoura K, et al. Endothelium-dependent and -independent relaxation by dopamine in the rabbit pulmonary artery. *Clin Exp Pharmacol Physiol*. 1992;19:401-410.
25. Bucolo C, Leggio GM, Drago F, et al. Dopamine outside the brain: The eye, cardiovascular system and endocrine pancreas. *Pharmacol Ther*. 2019;203:107392.
26. Velasco M, Tjandramaga TB, McNay JL. Differential dose-related effects of dopamine on systemic and renal hemodynamics in hypertensive patients. *Clinical Research*. 1974;22:308A.
27. Horwitz D, Fox SM, Goldberg LI. Effects of Dopamine in man. *Circ Res*. 1962;10:237-243.
28. Mcdonald RHJ, Goldberg LI, Mcnay JL, Tuttle EPJ. Effect of dopamine in man: augmentation of sodium excretion, glomerular filtration rate, and renal plasma flow. *J Clin Invest*. 1964;43:1116-1124.
29. Goldberg LI. Dopamine–clinical uses of an endogenous catecholamine. *N Engl J Med*. 1974;291:707-710.

30. Grandy DK, Marchionni MA, Makam H, et al. Cloning of the cDNA and gene for a human D2 dopamine receptor. *Proc Natl Acad Sci USA*. 1989;86:9762-9766.
31. Chiou GC, Chen YJ. Effects of dopamine agonist, bromocriptine, and some dopamine antagonists on ocular blood flow. *J Ocul Pharmacol*. 1992;8:285-294.
32. Abou Farha K, Baljé-Volkers C, Tamminga W, et al. Dopamine D2R Agonist-Induced Cardiovascular Effects in Healthy Male Subjects: Potential Implications in Clinical Settings. *ISRN Neurol*. 2014;2014:956353.
33. Castillo-Sánchez M, Fàbregas-Escurriola M, Bergè-Baquero D, et al. Schizophrenia, antipsychotic drugs and cardiovascular risk: Descriptive study in primary care. *Eur Psychiatry*. 2015;30:535-541.
34. Fan Z, Wu Y, Shen J, Ji T, Zhan R. Schizophrenia and the risk of cardiovascular diseases: a meta-analysis of thirteen cohort studies. *J Psychiatr Res*. 2013;47:1549-1556.
35. Agrawal DK, Triggle CR, Daniel EE. Pharmacological characterization of the postsynaptic alpha adrenoceptors in vascular smooth muscle from canine and rat mesenteric vascular beds. *J Pharmacol Exp Ther*. 1984;229:831-838.
36. Doxey JC, Lane AC, Roach AG, et al. Comparison of the alpha-adrenoceptor antagonist profiles of idazoxan (RX 781094), yohimbine, rauwolscine and corynanthine. *Naunyn Schmiedebergs Arch Pharmacol*. 1984;325:136-144.
37. Burt DR, Creese I, Snyder SH. Properties of [3H]haloperidol and [3H]dopamine binding associated with dopamine receptors in calf brain membranes. *Mol Pharmacol*. 1976;12:800-812.
38. Tsunoda M, Uchino E, Imai K, et al. Determination of nitrocatecholamines in rat brain using high-performance liquid chromatography-peroxyoxalate chemiluminescence reaction detection. *J Chromatogr A*. 2007;1164:162-166.
39. Grabiec U, Dehghani F. N-Arachidonoyl Dopamine: A Novel Endocannabinoid and Endovanilloid with Widespread Physiological and Pharmacological Activities. *Cannabis cannabinoid Res*. 2017;2:183-196.
40. Guyton AC. The relationship of cardiac output and arterial pressure control. *Circulation*. 1981;64:1079-1088.
41. Ross JJ, Linhart JW, Brauwald E. Effects of changing heart rate in man by electrical stimulation of the right atrium. studies at rest, during exercise, and with isoproterenol. *Circulation*. 1965;32:549-558.

How to cite this article: Britto-JúniorJ, Pinheiro DHA, Justo AFO, et al. Endothelium-derived dopamine modulates EFS-induced contractions of human umbilical vessels. *Pharmacol Res Perspect*. 2020;e00612. https://doi.org/10.1002/prp2.612

5 DISCUSSÃO

Os vasos do cordão umbilical humano (HUCV) são capazes de contrair através de estimulação de campo elétrico (EFS) que são moduladas pela presença do endotélio. Nossos resultados mostram claramente que HUCV apresenta uma liberação basal de dopamina liberada pelo endotélio. Também identificado por imuno-histoquímica que a enzima responsável pela conversão da tirosina em L-dihidroxi-fenilalanina (L-DOPA) tirosina hidroxilase (76) e a enzima pela conversão de L-DOPA em dopamina dopa-descarboxilase (77), estão presente nas células endoteliais dos HUCV. O RNA mensageiro da tirosina hidroxilase foi identificado por hibridização *in situ* de fluorescência em no endotélio de ambos os vasos do cordão umbilical.

De fato, o endotélio desempenha um papel importante na liberação de dopamina a ausência de tecido neuronal nos vasos de HUC indicado pela ausência do marcado calretinina nesses locais sustenta a hipótese que a dopamina e liberada de tecidos não neuronais no HUCV e sim pelos próprios vasos.

A dopamina (DA) possui o seu próprio sitio de receptores seletivos, pertencendo à família de receptores acoplados à proteína G, foram descritos cincos receptores de DA (DRs) sendo duas grandes famílias: os subtipos $D_{1\text{-Like}}$ (D1R e D5R), acoplados a Gs, atua ativando a adenil ciclase (25), e os $D_{2\text{-Like}}$ (D2R, D3R e D4R), atua inibindo a adenil ciclase(26). Os receptores dopaminérgicos no leito vascular foram identificados por ligação radioligante-receptor e técnicas autorradiográficas. Os receptores de dopamina-1 (D1; (78)foram identificados em HUA e dopamina-2 (D1) foram identificados em musculatura lisa das artérias cerebrais, mesentéricas e renais de ratos(25) também foi identificado por imuno-histoquímica identificou os subtipos D2 e D4 no leito vascular cerebral e mesentérico e os receptores D2 e D3 na vasculatura renal, com o subtipo D5 residindo predominantemente como receptor de músculo liso nos leitos vasculares de ratos.

A inibição causada pelo antagonista de receptores $D_{2\text{Like}}$ Haloperidol nas contrações induzidas por EFS em HUCVs mostra a importante valor do endotélio na liberação de dopamina na modulação da contração no leito vascular do cordão umbilical.

O antagonista α_1-adrenorecptor prazosin (75) e o antagonista α_2-adrenorecptor idazoxan (76) não tiveram ação nas contrações induzidas por EFS em HUA e HUV. Confirmando que a dopamina e o mediador principal liberado pelo endotélio responsável pelo fenômeno. A inibição causada pela fentolamina em concentrações maiores possivelmente esta relacionada ao fato que a fentolamina atua em receptores dopaminérgicos do tipo D_{2like} no qual foi demostrado que (> 2 μM) desloca a ligação do 3H-haloperidol aos receptores de dopamina nas membranas do cérebro de bezerros (77).

Para finalizar vem a seguinte pergunta qual é o papel fisiológico da dopamina endotelial liberados dos vasos do cordão umbilical? Sabemos que embora o papel do débito cardíaco seja definido pelo resultado da frequência cardíaca pelo volume sistólico (78) e que se sabe que a função de bombeamento do coração tem papel permissivo na determinação do débito cardíaco. Em estudos demostram que indivíduos que passaram por foram estimulados eletricamente não foram afetados a amplitude da frequência cárdia (79). As características da circulação periférica, como capacitância e condutância/resistência, desempenham um papel importante na determinação do débito cardíaco. A maioria das deduções no papel da modulação simpática da circulação foi obtida pelo uso de agonistas ou antagonistas adrenérgicos, supondo-se que esses mediadores sejam provenientes de terminais nervosos. A descoberta de que o tecido vascular humano tem liberação basal de dopamina deve mudar esse paradigma.

6 CONCLUSÃO

O presente estudo demonstra, pela primeira vez, uma liberação basal da dopamina proveniente do cordão umbilical humano.

A presença das enzimas tirosina-hidroxilase e dopa-descarboxilase foi detectada no endotélio de HUA e HUV, conforme determinado por imuno-histoquímica. Além disso, a dopamina induziu a contração de HUA apenas na presença de L-NAME aumentando fortemente a contração induzida pela dopamina de HUA e HUV.

As contrações de HUA e HUV induzidas por EFS foram aumentadas por L-NAME e inibidas pelo haloperidol, antagonista do receptor semelhante a D2.

Os antagonistas α-adrenérgicos prazosin e idazoxan e o antagonista do receptor $D_{1\text{-like}}$ SCH - 23390 não tiveram efeito nas contrações induzidas por EFS de HUA e HUV. A dopamina derivada do endotélio é o principal modulador da reatividade do HUCV *in vitro*.

REFERÊNCIAS

1. Lakatta EG. Catecholamines and cardiovascular function in aging. Endocrinol Metab Clin North Am [Internet]. 1987 [cited 2022 Oct 16];16(4):877–91. Available from: https://pubmed.ncbi.nlm.nih.gov/3322821/

2. Alexopoulos GS. Mechanisms and treatment of late-life depression. [cited 2022 Oct 16]; Available from: https://doi.org/10.1038/s41398-019-0514-6

3. Brooks DJ. Functional imaging studies on dopamine and motor control. Journal of Neural Transmission 2001 108:11 [Internet]. 2001 [cited 2022 Oct 16];108(11):1283–98. Available from: https://link.springer.com/article/10.1007/s007020100005

4. Nagatsu T. The catecholamine system in health and disease —Relation to tyrosine 3-monooxygenase and other catecholamine-synthesizing enzymes—. Proc Jpn Acad Ser B Phys Biol Sci [Internet]. 2006 [cited 2022 Sep 19];82(10):388. Available from: /pmc/articles/PMC4338835/

5. Eisenhofer G, Kopin IJ, Goldstein DS. Catecholamine metabolism: a contemporary view with implications for physiology and medicine. Pharmacol Rev [Internet]. 2004 Sep [cited 2022 Sep 19];56(3):331–49. Available from: https://pubmed.ncbi.nlm.nih.gov/15317907/

6. Molinoff PB, Axelrod J. Biochemistry of catecholamines. Annu Rev Biochem [Internet]. 1971 [cited 2022 Sep 19];40:465–500. Available from: https://pubmed.ncbi.nlm.nih.gov/4399447/

7. Cosentino M, Marino F, Maestroni GJM. Sympathoadrenergic modulation of hematopoiesis: A review of available evidence and of therapeutic perspectives. Front Cell Neurosci. 2015 Aug 5;9(AUGUST):302.

8. Richter1 D. CCXLIX. ADRENALINE AND AMINE OXIDASE. 1937.

9. Kopin IJ, Br Li OH N CH LN. Acidic dopamine metabolites are actively extruded from PC12 cells by a novel sulfonylurea-sensitive transporter. Naunyn Schmiedebergs Arch Pharmacol. 2000; 361(6):654-64

10. Kopin IJ, Rundqvist B, Friberg P, Lenders J, Goldstein DS, Eisenhofer G. Different relationships of spillover to release of norepinephrine in human heart, kidneys, and forearm. 1998.

11. Black JW. Ahlquist and the development of beta-adrenoceptor antagonists. Postgrad Med J [Internet]. 1976 [cited 2022 Sep 19];52 Suppl 4(4 sup):11–3. Available from: https://pubmed.ncbi.nlm.nih.gov/9627/

12. AHLQUIST RP. A STUDY OF THE ADRENOTROPIC RECEPTORS. https://doi.org/101152/ajplegacy19481533586 [Internet]. 1948 Jun 1 [cited 2022 Oct 16];153(3):586–600. Available from: https://journals.physiology.org/doi/10.1152/ajplegacy.1948.153.3.586

13. Bylund DB, Eikenberg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP, et al. International Union of Pharmacology nomenclature of adrenoceptors. Pharmacol Rev [Internet]. 1994 Jun 1 [cited 2022 Oct 13];46(2):121–36. Available from: https://europepmc.org/article/med/7938162

14. Hieble JP, Bondinell WE, Ruffolo RR. MEDICINAL CHEMISTRY © Perspective a-and /?-Adrenoceptors: From the Gene to the Clinic. 1. Molecular Biology and Adrenoceptor Subclassification. [cited 2022 Oct 13];38. Available from: https://pubs.acs.org/sharingguidelines

15. Smeets WJAJ, González A. Catecholamine systems in the brain of vertebrates: new perspectives through a comparative approach. Brain Res Rev. 2000 Sep 1;33(2–3):308–79.

16. Costa KM, Schoenbaum G. Dopamine. Current Biology [Internet]. 2022 Aug 8 [cited 2022 Oct 13];32(15):R817–24. Available from: http://www.cell.com/article/S0960982222010223/fulltext

17. Hornykiewicz O. Dopamine miracle: From brain homogenate to dopamine replacement. Movement Disorders. 2002 May;17(3):501–8.

18. Zeng C, Zhang M, Asico LD, Eisner GM, Jose PA. The dopaminergic system in hypertension. Clin Sci [Internet]. 2007 Jun 1 [cited 2022 Oct 13];112(12):583–97. Available from: /clinsci/article/112/12/583/68255/The-dopaminergic-system-in-hypertension

19. Holtz P, Credner K. Dopahyperglykämie. Hoppe Seylers Z Physiol Chem [Internet]. 1944 Jan 1 [cited 2022 Oct 13];280(1–2):9–15. Available from: https://www.degruyter.com/document/doi/10.1515/bchm2.1944.280.1-2.9/html

20. Goldberg LI, McDonald RH, Zimmerman AM. SODIUM DIURESIS PRODUCED BY DOPAMINE IN PATIENTS WITH CONGESTIVE HEART FAILURE. N Engl J Med [Internet]. 1963 Nov 14 [cited 2022 Oct 13];269(20):1060–4. Available from: https://pubmed.ncbi.nlm.nih.gov/14085064/

21. McDonald RH, Goldberg LI. ANALYSIS OF THE CARDIOVASCULAR EFFECTS OF DOPAMINE IN THE DOG. Journal of Pharmacology and Experimental Therapeutics. 1963;140(1).

22. Beaulieu JM, Gainetdinov RR. The Physiology, Signaling, and Pharmacology of Dopamine Receptors. Pharmacol Rev [Internet]. 2011

Mar 1 [cited 2022 Oct 13];63(1):182–217. Available from: https://pharmrev.aspetjournals.org/content/63/1/182

23. Toda N, Goldberg LI. Dopamine-induced relaxation of isolated arterial strips. J Pharm Pharmacol [Internet]. 1973 [cited 2022 Oct 13];25(7):587–9. Available from: https://pubmed.ncbi.nlm.nih.gov/4147064/

24. Brown JH, Makman MH. Stimulation by Dopamine of Adenylate Cyclase in Retinal Homogenates and of Adenosine-3′:5′-Cyclic Monophosphate Formation in Intact Retina. Proceedings of the National Academy of Sciences [Internet]. 1972 Mar 1 [cited 2022 Oct 13];69(3):539–43. Available from: https://www.pnas.org/doi/abs/10.1073/pnas.69.3.539

25. Kebabian JW, Petzold GL, Greengard P. Dopamine-sensitive adenylate cyclase in caudate nucleus of rat brain, and its similarity to the “dopamine receptor.” Proceedings of the National Academy of Sciences [Internet]. 1972 Aug 1 [cited 2022 Oct 13];69(8):2145–9. Available from: https://www.pnas.org/doi/abs/10.1073/pnas.69.8.2145

26. Stoof JC, Kebabian JW. Opposing roles for D-1 and D-2 dopamine receptors in efflux of cyclic AMP from rat neostriatum. Nature 1981 294:5839 [Internet]. 1981 [cited 2022 Oct 13];294(5839):366–8. Available from: https://www.nature.com/articles/294366a0

27. de Camilli P, Macconi D, Spada A. Dopamine inhibits adenylate cyclase in human prolactin-secreting pituitary adenomas. Nature 1979 278:5701 [Internet]. 1979 [cited 2022 Oct 13];278(5701):252–4. Available from: https://www.nature.com/articles/278252a0

28. Zeng C, Sanada H, Watanabe H, Eisner GM, Felder RA, Jose PA. Functional genomics of the dopaminergic system in hypertension. Physiol Genomics [Internet]. 2005 Jan [cited 2022 Oct 13];19:233–46. Available from: https://journals.physiology.org/doi/10.1152/physiolgenomics.00127.2004

29. Grabiec U, Dehghani F. N-Arachidonoyl Dopamine: A Novel Endocannabinoid and Endovanilloid with Widespread Physiological and Pharmacological Activities. Cannabis Cannabinoid Res [Internet]. 2017 [cited 2022 Oct 13];2(1):183. Available from: /pmc/articles/PMC5627668/

30. BISOGNO T, MELCK D, BOBROV MYu, GRETSKAYA NM, BEZUGLOV V v., de PETROCELLIS L, et al. N-acyl-dopamines: novel synthetic CB1 cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity in vitro and in vivo. Biochemical Journal [Internet]. 2000 Nov 1 [cited 2022 Oct 13];351(3):817–24. Available from: /biochemj/article/351/3/817/38992/N-acyl-dopamines-novel-synthetic-CB1-cannabinoid

31. Britto-Júnior J, Coelho-Silva WC, Murari GF, Serpellone Nash CE, Mónica FZ, Antunes E, et al. 6-Nitrodopamine is released by human umbilical cord vessels and modulates vascular reactivity. Life Sci [Internet]. 2021 Jul 1

[cited 2022 Sep 19];276. Available from: https://pubmed.ncbi.nlm.nih.gov/33781827/

32. Campos R, Pinheiro DHA, Britto-Júnior J, de Castro HA, Mendes GD, Moraes MO, et al. Quantification of 6-nitrodopamine in Krebs-Henseleit's solution by LC-MS/MS for the assessment of its basal release from Chelonoidis carbonaria aortae in vitro. J Chromatogr B Analyt Technol Biomed Life Sci [Internet]. 2021 May 30 [cited 2022 Sep 19];1173. Available from: https://pubmed.ncbi.nlm.nih.gov/33819799/

33. Lima AT, dos Santos EX, Britto-Júnior J, de Souza VB, Schenka AA, Campos R, et al. Release of 6-nitrodopamine modulates vascular reactivity of Pantherophis guttatus aortic rings. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 2022 Dec 1;262:109471.

34. Britto-Júnior J, Ximenes L, Ribeiro A, Fregonesi A, Campos R, Ricardo de Almeida Kiguti L, et al. 6-Nitrodopamine is an endogenous mediator of rat isolated epididymal vas deferens contractions induced by electric-field stimulation. Eur J Pharmacol. 2021 Nov 15;911:174544.

35. Britto-Júnior J, da Silva-Filho WP, Amorim AC, Campos R, Moraes MO, Moraes MEA, et al. 6-nitrodopamine is a major endogenous modulator of human vas deferens contractility. Andrology [Internet]. 2022 [cited 2022 Oct 13]; Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/andr.13263

36. Britto-Júnior J, de Oliveira MG, dos Reis Gati C, Campos R, Moraes MO, Moraes MEA, et al. 6-NitroDopamine is an endogenous modulator of rat heart chronotropism. Life Sci. 2022 Oct 15;307:120879.

37. di Naro E, Ghezzi F, Raio L, Franchi M, D'Addario V. Umbilical cord morphology and pregnancy outcome. European Journal of Obstetrics and Gynecology and Reproductive Biology [Internet]. 2001 Jun 1 [cited 2022 Oct 13];96(2):150–7. Available from: http://www.ejog.org/article/S030121150000470X/fulltext

38. Benirschke K, Driscoll SG. The Pathology of the Human Placenta. Placenta [Internet]. 1967 [cited 2022 Oct 13];97–571. Available from: https://link.springer.com/chapter/10.1007/978-3-662-38455-8_2

39. di Naro E, Ghezzi F, Raio L, Franchi M, D'Addario V. Umbilical cord morphology and pregnancy outcome. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2001 Jun 1;96(2):150–7.

40. Arutyunyan I, Elchaninov A, Makarov A, Fatkhudinov T. Umbilical Cord as Prospective Source for Mesenchymal Stem Cell-Based Therapy. 2016 [cited 2022 Nov 22]; Available from: http://dx.doi.org/10.1155/2016/6901286

41. Weiss ML, Anderson C, Medicetty S, Seshareddy KB, Weiss RJ, VanderWerff I, et al. Immune Properties of Human Umbilical Cord Wharton's Jelly-Derived Cells. Stem Cells [Internet]. 2008 Nov 1 [cited 2022 Oct 13];26(11):2865–74. Available from: https://academic.oup.com/stmcls/article/26/11/2865/6415549

42. Xie H, Triggle CR. Endothelium-independent relaxations to acetylcholine and A23187 in the human umbilical artery. J Vasc Res. 1994;31(2):92–105.

43. Furchgott RF, Zawadzki J v. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980 Nov;288(5789):373–6.

44. Templeton AG, McGrath JC, Whittle MJ. The role of endogenous thromboxane in contractions to U46619, oxygen, 5-HT and 5-CT in the human isolated umbilical artery. Br J Pharmacol. 1991 May;103(1):1079–84.

45. Montgomery LD, Belfort MA, Saade GR, Moise KJJ, Vedernikov YP. Meconium inhibits the contraction of umbilical vessels induced by the thromboxane A2 analog U46619. Am J Obstet Gynecol. 1995 Oct;173(4):1075–8.

46. Rizzi A, Calo G, Battistini B, Regoli D. Contractile activity of endothelins and their precursors in human umbilical artery and vein: identification of distinct endothelin-converting enzyme activities. J Cardiovasc Pharmacol. 1998;31 Suppl 1:S58-61.

47. Starke K. Alpha-adrenoceptor subclassification. Rev Physiol Biochem Pharmacol. 1981;88:199–236.

48. Bodelsson G, Stjernquist M. Characterization of contractile adrenoceptors in the human umbilical artery. Eur J Pharmacol. 1995 Aug;282(1–3):95–101.

49. Furchgott RF, Zawadzki J v. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980 288:5789 [Internet]. 1980 [cited 2022 Oct 13];288(5789):373–6. Available from: https://www.nature.com/articles/288373a0

50. Joyner MJ, Limberg JK. Blood Pressure: Return of the Sympathetics? Curr Hypertens Rep [Internet]. 2016 Jan 1 [cited 2022 Oct 13];18(1):1–6. Available from: https://link.springer.com/article/10.1007/s11906-015-0616-3

51. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev. 1991;43(2).

52. Sandow SL. Factors, fiction and endothelium-derived hyperpolarizing factor. Clin Exp Pharmacol Physiol [Internet]. 2004 Sep 1 [cited 2022 Oct

13];31(9):563–70. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1440-1681.2004.04048.x

53. de Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD, et al. Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. Proceedings of the National Academy of Sciences [Internet]. 1988 Dec 1 [cited 2022 Oct 13];85(24):9797–800. Available from: https://www.pnas.org/doi/abs/10.1073/pnas.85.24.9797

54. Félétou M, Vanhoutte PM. Endothelium-Derived Hyperpolarizing Factor. Arterioscler Thromb Vasc Biol [Internet]. 2006 Jun 1 [cited 2022 Oct 13];26(6):1215–25. Available from: https://www.ahajournals.org/doi/abs/10.1161/01.ATV.0000217611.81085.c5

55. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J [Internet]. 2012 Apr [cited 2022 Oct 13];33(7):829. Available from: /pmc/articles/PMC3345541/

56. Kishimoto J, Spurr N, Liao M, Lizhi L, Emson P, Xu W. Localization of brain nitric oxide synthase (NOS) to human chromosome 12. Genomics. 1992 Nov 1;14(3):802–4.

57. Hecker M, Walsh DT, Vane JR. On the substrate specificity of nitric oxide synthase. FEBS Lett [Internet]. 1991 Dec 9 [cited 2022 Oct 13];294(3):221–4. Available from: https://onlinelibrary.wiley.com/doi/full/10.1016/0014-5793%2891%2981434-A

58. Mcdonald LJ, Murad F. Nitric Oxide and cGMP Signaling. Adv Pharmacol. 1995 Jan 1;34(C):263–75.

59. de Oliveira MG, Nadruz W, Mónica FZ. Endothelial and vascular smooth muscle dysfunction in hypertension. Biochem Pharmacol. 2022 Nov 1;205:115263.

60. Rubanyi GM, Vanhoutte PM. Hypoxia releases a vasoconstrictor substance from the canine vascular endothelium. J Physiol [Internet]. 1985 Jul 1 [cited 2022 Oct 13];364(1):45–56. Available from: https://onlinelibrary.wiley.com/doi/full/10.1113/jphysiol.1985.sp015728

61. Harder DR. Pressure-induced myogenic activation of cat cerebral arteries is dependent on intact endothelium. Circ Res [Internet]. 1987 [cited 2022 Oct 13];60(1):102–7. Available from: https://www.ahajournals.org/doi/abs/10.1161/01.res.60.1.102

62. Hickey KA, Rubanyi G, Paul RJ, Highsmith RF. Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. https://doi.org/101152/ajpcell19852485C550 [Internet]. 1985 [cited 2022

Oct 13];17(3). Available from: https://journals.physiology.org/doi/10.1152/ajpcell.1985.248.5.C550

63. Karwatowska-Prokopczuk E, Wennmalm. Endothelium-derived constricting factor(s): the last novelty—endothelin. Clinical Physiology [Internet]. 1990 Mar 1 [cited 2022 Oct 13];10(2):113–21. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1475-097X.1990.tb00245.x

64. Huang WQ, Zou Y, Tian Y, Ma XF, Zhou QY, Li ZY, et al. Mammalian Target of Rapamycin as the Therapeutic Target of Vascular Proliferative Diseases: Past, Present, and Future. J Cardiovasc Pharmacol. 2022 Apr 6;79(4):444–55.

65. Rubanyi GM, Vanhoutte PM. Hypoxia releases a vasoconstrictor substance from the canine vascular endothelium. J Physiol [Internet]. 1985 Jul 1 [cited 2022 Oct 13];364(1):45–56. Available from: https://onlinelibrary.wiley.com/doi/full/10.1113/jphysiol.1985.sp015728

66. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 1988 332:6163 [Internet]. 1988 [cited 2022 Oct 13];332(6163):411–5. Available from: https://www.nature.com/articles/332411a0

67. Hiley CR. Functional studies on endothelin catch up with molecular biology. Trends Pharmacol Sci. 1989 Feb 1;10(2):47–9.

68. Masashi Y, Tomoh M. Molecular biology and biochemistry of the endothelins. Trends Pharmacol Sci. 1989 Sep 1;10(9):374–8.

69. Campos R, Justo AFO, Mónica FZ, Cogo JC, Moreno RA, de Souza VB, et al. Electrical field-induced contractions on Crotalus durissus terrificus and Bothrops jararaca aortae are caused by endothelium-derived catecholamine. PLoS One [Internet]. 2018 Sep 1 [cited 2022 Oct 13];13(9):e0203573. Available from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0203573

70. Campos R, Jacintho FF, Britto-Júnior J, Mónica FZ, Justo AFO, Pupo AS, et al. Endothelium modulates electrical field stimulation-induced contractions of Chelonoidis carbonaria aortic rings. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 2020 Jul 1;233:108763.

71. Britto-Júnior J, Jacintho FF, Campos R, Pinheiro DHA, Figueiredo Murari GM, de Souza VB, et al. The basal release of endothelium-derived catecholamines regulates the contractions of Chelonoidis carbonaria aorta caused by electrical-field stimulation. Biol Open [Internet]. 2021 Jan 1 [cited 2022 Oct 13];10(1). Available from: /pmc/articles/PMC7847266/

72. Moncada S, Gryglewski R, Bunting S, Vane JR. An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. Nature 1976 263:5579 [Internet]. 1976 Oct 1 [cited 2022 Oct 13];263(5579):663–5. Available from: https://www.nature.com/articles/263663a0

73. Matsuyama T, Shiosaka S, Wanaka A, Yoneda S, Kimura K, Hayakawa T, et al. Fine structure of peptidergic and catecholaminergic nerve fibers in the anterior cerebral artery and their interrelationship: An immunoelectron microscopic study. Journal of Comparative Neurology [Internet]. 1985 May 8 [cited 2022 Oct 13];235(2):268–76. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/cne.902350209

74. Kadowitz PJ, Knight DS, Hibbs RG, Ellison JP, Joiner PD, Brody MJ, et al. Influence of 5- and 6-hydroxydopamine on adrenergic transmission and nerve terminal morphology in the canine pulmonary vascular bed. Circ Res [Internet]. 1976 [cited 2022 Oct 13];39(2):191–9. Available from: https://www.ahajournals.org/doi/abs/10.1161/01.RES.39.2.191

75. Britto-Júnior J, Antunes NJ, Campos R, Sucupira M, Mendes GD, Fernandes F, et al. Determination of dopamine, noradrenaline, and adrenaline in Krebs–Henseleit solution by liquid chromatography coupled with tandem mass spectrometry and measurement of their basal release from Chelonoidis carbonaria aortae in vitro. Biomedical Chromatography [Internet]. 2021 Feb 1 [cited 2022 Oct 13];35(2):e4978. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/bmc.4978

76. Nagatsu T, Levitt M, Udenfriend S. Tyrosine Hydroxylase THE INITIAL STEP IN NOREPINEPHRINE BIOSYNTHESIS. Journal of Biological Chemistry. 1964;239(S):2910–7.

77. Bertoldi M. Mammalian dopa decarboxylase: Structure, catalytic activity and inhibition. Arch Biochem Biophys. 2014 Mar 15;546:1–7.

78. Amenta F, Collier WL, Ricci A. Autoradiographic Localization of Vascular Dopamine Receptors. Am J Hypertens [Internet]. 1990 Jun 1 [cited 2022 Oct 15];3(6_Pt_2):34S-36S. Available from: https://academic.oup.com/ajh/article/3/6_Pt_2/34S/216672

7 ANEXOS e APÊNDICES

Anexo 1

Artigo 3 – Endothelium modulates electrical field stimulation-induced contractions of *Chelonoidis carbonaria* aortic rings

Autores: Campos R, Jacintho FF, Britto-Júnior J, Mónica FZ, Justo AFO, Pupo AS, Moreno RA, de Souza VB, Schenka AA, Antunes E, De Nucci G.

Revista: Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology

Situação: Aceito a publicação em 08 de março de 2020. Publicado on-line em 04 de abril de 2020

Comparative Biochemistry and Physiology, Part C 233 (2020) 108763

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Endothelium modulates electrical field stimulation-induced contractions of *Chelonoidis carbonaria* aortic rings

Rafael Campos[a,b,*], Felipe Fernandes Jacintho[b], José Britto-Júnior[b], Fabíola Z. Mónica[b], Alberto Fernando Oliveira Justo[b], André Sampaio Pupo[c], Ronilson Agnaldo Moreno[b], Valéria Barbosa de Souza[b], André Almeida Schenka[b], Edson Antunes[b], Gilberto De Nucci[b,d]

[a] Superior Institute of Biomedical Sciences, Ceará State University (UECE), Fortaleza, Brazil
[b] Faculty of Medical Sciences, Department of Pharmacology, University of Campinas (UNICAMP), Campinas, Brazil
[c] Department of Pharmacology, Institute of Biosciences of Botucatu, UNESP - São Paulo State University, Botucatu, Brazil
[d] Department of Pharmacology, Institute of Biomedical Sciences, USP – University of São Paulo, São Paulo, Brazil

ARTICLE INFO

Keywords:
Endothelium
Tetrodotoxin
Catecholamines

ABSTRACT

The role of endothelium in the electrical-field stimulation (EFS)-induced contractions of *Chelonoidis carbonaria* aorta was investigated. Contractions were evaluated in the presence and absence of L-NAME (100 μM), tetrodotoxin (1 μM), phentolamine (10 and 100 μM), phenoxybenzamine (1 and 10 μM), prazosin (100 μM), idazoxan (100 μM), atropine (10 μM), D-tubocurarine (10 μM) or indomethacin (10 μM). EFS-induced contraction was also carried out in endothelium-denuded rings. EFS-induced contraction was investigated by the sandwich assay. Concentration curves to endothelin-1 (0.1–100 nM) and U46619 (0.001–100 μM) were also constructed to calculate both Emax and EC_{50}. EFS at 16 Hz contracted *Chelonoidis* aorta, which was almost abolished by the endothelium removal. The addition of L-NAME increased the EFS response (2.0 ± 0.4 and 8.3 ± 1.9 mN). In L-NAME treated aortic rings, tetrodotoxin did not change the EFS-response (5.1 ± 1.8 and 4.9 ± 1.7 mN). Indomethacin, atropine and d-tubucurarine also did not affect the EFS-response. Phentolamine at 10 μM did not change the EFS-induced contraction; however, at 100 μM, reduced it (3.9 ± 1 and 1.9 ± 0.3 mN). Prazosin and idazoxan did not change EFS-induced contractions. Phenoxybenzamine at 1 μM reduced by 76% (9.6 ± 3.4 and 2.3 ± 0.8 mN) and at 10 μM by 90% the EFS response. Immunohistochemistry identified tyrosine hydroxylase in the endothelium and brain, whereas S100 protein was found only in brain. In conclusion, endothelium modulates EFS-induced contractions in *Chelonoidis* aortic rings and this modulation may be due to endothelium-derived catecholamines, possibly dopamine.

1. Introduction

Electrical field stimulation (EFS) is a technique in which the stimulus is applied uniformly to an isolated tissue in short pulse width waves, often used as a method of selectively stimulating intramural nerves (Bevan, 1962; Darios et al., 2015; Paterson, 1965). Electrical field stimulation (EFS) caused contractions of aortic rings of *Crotalus durissus terrificus* and *Bothrops jararaca* (Campos et al., 2018a). Interestingly, adrenergic nerve terminals were not observed in snake aorta (Campos et al., 2018a). The EFS-induced contractions of snake aorta were abolished by removal of endothelium or by pre-incubation with sympatholytic drugs such as phentolamine and guanethidine (Campos et al., 2018a, 2018b). These results indicated that EFS-induced contractions of snake aorta were mediated by catecholamine release by endothelial cells.

Interestingly, EFS-induced contractions of human umbilical cord vessels were also endothelium dependent, insensitive to tetrodotoxin and inhibited by the adrenergic antagonist phentolamine (Britto- Jr et al., 2020). Since this phenomenon was observed in both snake and human vessels, we have hypothesized that this event could be universal. The tortoise *Chelonoidis carbonaria* dorsal aorta releases nitric oxide (Campos et al., 2019), therefore we have investigated this vessel by functional protocols and immunohistochemical analysis on whether the endothelium could be a source of catecholamines.

* Corresponding author at: Superior Institute of Biomedical Sciences, Ceará State University (UECE), 60714-903 Fortaleza, Ceará, Brazil.
E-mail address: rafael.campos@uece.br (R. Campos).

https://doi.org/10.1016/j.cbpc.2020.108763
Received 28 November 2019; Received in revised form 8 March 2020; Accepted 4 April 2020
Available online 11 April 2020
1532-0456/ © 2020 Elsevier Inc. All rights reserved.

2. Material and methods

2.1. Animals

All experimental procedures using *Chelonoidis carbonaria* (male and female) were approved by the Institutional Animal Care and Use Committee (CEUA/UNICAMP: 3907-1) and were in compliance with the ARRIVE guidelines. The use of *Chelonoidis carbonaria* was authorized by the Brazilian Institute for Environment (Sisbio; number 20910) and the animals were provided by the Parque Ecológico do Tietê (São Paulo, SP, Brazil).

2.2. Chemical and reagents

Endothelin-1 was purchased from Americam Peptide Company (Sunnyvale, California). Acetylcholine, atropine, bradykinin, d-tubocurarine, indomethacin, N(w)-nitro-L-arginine methyl esther (L-NAME), phentolamine, phenoxybenzamine, prazosin, idazoxan, tetrodotoxin and U-46619 were bought from Sigma Aldrich Chemical Co (St Louis, Misouri). Chicken anti-tyrosine hydroxylase (ab76442) and goat anti-chicken gamma-immunoglobulin (ab150169) were obtained from Abcam (Cambridge, USA). Rabbit anti-S100 protein (MAB0791) and rabbit anti-goat (AP106P) gamma-immunoglobulin were obtained from Millipore (Temecula, USA). NovoLink Max Polymer Detection system was provided by Leica/Novocastra (Newcastle, UK).

2.3. Aortic ring preparation for isometric recording

Tortoises of either sex (weight range from 2 to 7 kg) were sedated with midazolam (2 mg/kg; IM), anesthetized with ketamine (40 mg/kg; IM) and propofol (15 mg/kg; IV), and euthanized by exsanguination. A segment of dorsal aorta was removed and immediately placed in Krebs-Henseleit solution at 27 °C. Subsequently, aortic rings (3 mm) were suspended vertically between two metal hooks in 10-mL organ baths containing Krebs-Henseleit solution (mM): NaCl (118), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (1.2), $NaCO_3$ (25), KH_2PO_4 (1.2) and glucose (5.6), gassed with a mixture of 95% O_2: 5% CO_2 (pH 7.4) at 27 °C, since it is the temperature often used for reptile tissue experiments (Stephens, 1984; Miller and Vanhoutte, 1986; Campos et al., 2019). Isometric force was recorded using a PowerLab 400TM data acquisition system (Software Chart, version 7.0, AD Instrument, MA, USA). The tissues were allowed to equilibrate for 1 h before starting the experiments.

2.4. Endothelial integrity assessment

Aortic rings were precontracted with either acetylcholine (ACh, 3 μM) or bradykinin (BK, 3 μM) and the integrity of the endothelium was assessed by a relaxation superior to 60% evoked by adenosine triphosphate (ATP; 10 μM) (Campos et al., 2019). In a separate set of experiments, the endothelium was removed with the aid of a thin stick, and the muscle integrity in denuded rings was assessed by the relaxation induced by sodium nitroprusside (SNP; 10 μM).

2.5. Electrical-field stimulation (EFS) of endothelium-intact aortic rings

Endothelium-intact aortic rings were submitted to EFS at 60 V for 30 s, subsequently, at 16 Hz in square-wave pulses; 0.5 ms pulse width; 0.2 ms delay, using a Grass S88 stimulator (Astro-Medical, RI, USA). The EFS-induced contractions of endothelium-intact aortic rings were performed in the absence and in the presence of adrenergic, cholinergic and sodium-channel antagonists and in the absence and in the presence of NO-synthesis and cyclo-oxygenase inhibitors. The effect of the antagonists and inhibitors were evaluated in the presence of L-NAME (100 μM). In a separate set of experiments to evaluate the obligatory role of the endothelium, the EFS were investigated in endothelium-denuded rings.

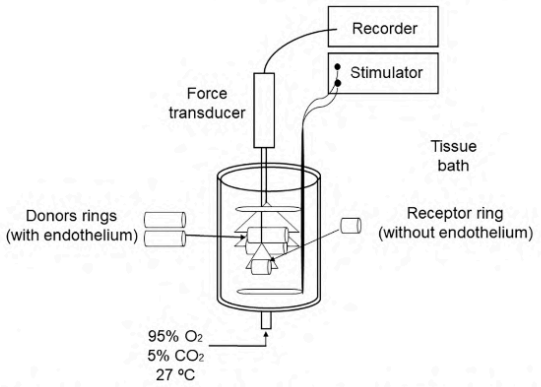


Fig. 1. Schematic illustration of the sandwich assay performed in *Chelonoidis aortic rings.*

2.6. Role of endothelial contracturant mediators on Chelonoidis carbonaria aortic rings tonus

Concentration response curves to U46619 (0.001–100 μM) and endothelin-1 (0.1–100 ηM) were performed on *Chelonoidis* aortic rings.

2.7. Sandwich assay

A "sandwich" assay was performed according to previous studies (Dong et al., 1997; Furchgott and Zawadzki, 1980; Plane et al., 1995). Briefly, three tissues (either three artery rings of *Chelonoidis carbonaria)* were placed in the 10-mL organ bath in Krebs-Henseleit solution, gassed with carbogenic mixture (O_2:CO_2, 95:5%) at a maintained temperature of 27 °C. The two rings with endothelium were referred as "donor tissue" (6 mm) and the ring without endothelium as "recipient tissue" (3 mm; Fig. 1). Each ring was maintained in the same organ bath for the same period of time and under the same conditions to investigate if factors released by the donor tissue could affect the recipient tissue during EFS. EFS-induced contractions were also performed in the presence and absence of phentolamine (100 μM).

2.8. Immunohistochemical analysis

Following euthanasia, samples of the *Chelonoidis* aorta (n = 4) were collected, fixed in 10% neutral buffered formalin for 24 h at 24 °C, dehydrated, embedded in paraffin wax and sectioned at 4-μm. Subsequently, these sections were stained for S-100 protein (S-100, a neural tissue marker) to investigate the presence of nerve fibers within aortic walls or for tyrosine hydroxylase (TH), using the following primary antibodies: (1) anti-S-100 (rabbit monoclonal antibody, Cat.# MAB0791, at 1:200, which reacts with bovine, human, rat and mouse S100 protein; Millipore) and (2) anti-tyrosine hydroxylase (chicken polyclonal, Cat.# ab76442, dilution 1:1500, which reacts with mouse, rat and human tyrosine hydroxylase; Abcam, Cambridge, USA).

Immunohistochemistry was performed manually. Briefly, the sections were deparaffinized in xylene and rehydrated in a series of ethanol baths of increasing concentration. They were then incubated in citrate buffer at pH 6.0 in a steamer set for 40 min (at approximately 95 °C). The sections were then incubated for 2hs at room temperature (25 °C) with the above-mentioned primary antibodies.

Tissue sections receiving the chicken anti-tyrosine hydroxylase antibody were sequentially incubated with a goat anti-chicken gamma immunoglobulin (IgG), and a rabbit anti-goat IgG, for 1 h each, before applying the anti-rabbit IgG detection system - NovoLink Max Polymer Detection System (Novocastra/Leica Biosystems), following the

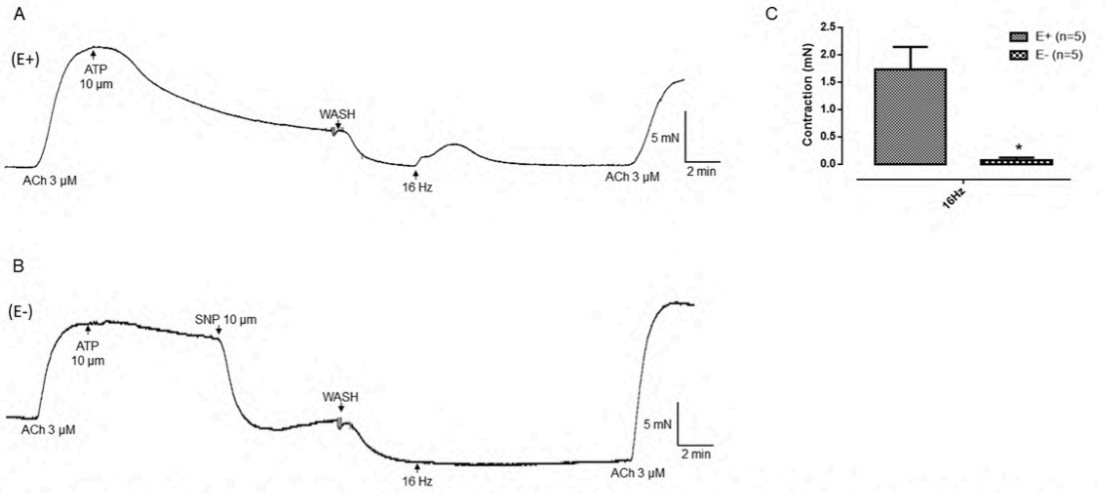


Fig. 2. Representative tracing of a n of 5 experiments for EFS-induced contraction in intact (A) and endothelium-denuded (B) *Chelonoids* aortic rings. Panel (C) shows the expressed as mean ± S.E.M. ($n = 5$, for each group; unpaired *t*-test (E^+ Vs E^-; * = $p < 0.05$).

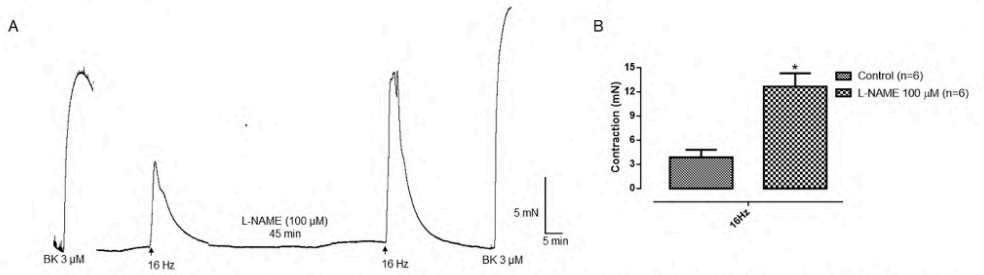


Fig. 3. Representative tracing of a n of 6 experiments of the effect of L-NAME in the EFS-induced contraction in endothelium-intact *Chelonoids* aortic rings (A). The histograms in Panel (B) represent the mean ± S.E.M ($n = 6$) for EFS-induced contractions in endothelium-intact aortic rings before and after incubation with L-NAME (100 mM). Paired t-test (Untreated vs treated; * = $p < 0.05$).

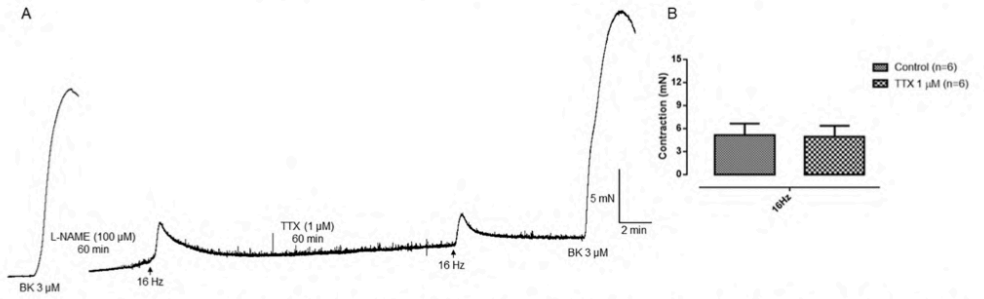


Fig. 4. Panel A shows a representative tracing of a n of 6 experiments of the effect of tetrodotoxin (TTX, 1 mM) in endothelium-intact *Chelonoids* aortic rings treated with L-NAME (100 mM). The histograms in Panel (B) represent the mean ± S.E.M (n = 6) for EFS-induced contractions in endothelium-intact aortic rings treated with L-NAME before and after incubation with tetrodotoxin. Paired t-test (untreated vs treated; $p = 0.90$).

manufacturer's instructions, and using diaminobenzidine (liquid DAB, DakoCytomation, Carpenteria, USA) as a chromogen (which renders a brown precipitate at the antibody binding site). Finally, the sections were counter-stained with Ehrlich's hematoxylin and cover-slipped in Entellan. Negative controls consisted of omission of the primary antibody and incubation with the primary antibody diluents (as well as with the secondary antibodies, where applicable). This was performed for all the immunohistochemical assays to identify any background staining. Furthermore, formalin-fixed, paraffin-embedded *Chelonoidis* brains ($n = 2$) were used as positive controls for the presence of both antigens (i.e., S-100 protein and tyrosine hydroxylase). All slides were examined and photomicrographed using a trinocular Eclipse 50i

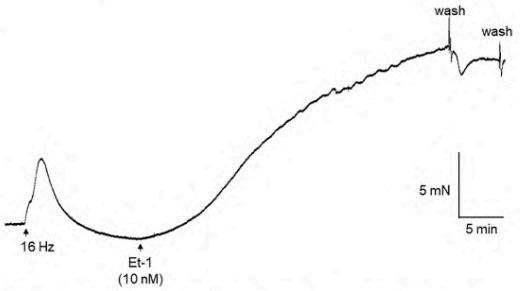


Fig. 5. Representative tracing of a n of 5 experiments comparing the contraction of the *Chelonoids* aortic rings induced by EFS and by ET-1 (10 nM).

microscope (Nikon, Tokyo, Japan) coupled to a 10MP CMOS digital camera (AmScope, EUA).

For the chromogranin A immunohistochemistry, the sections of *Chelonoidis carbonaria* aortic rings and positive controls tissues were incubated for 2hs at room temperature (25 °C) with a mouse monoclonal anti-chromogranin A antibody (clone DAK-A3, code M0869, 1:700, Dako/Agilent). Subsequently, these sections were incubated with the NovoLink Max Polymer Detection System (Novocastra/Leica Biosystems), following the manufacturer's instructions, and using diaminobenzidine (liquid DAB, DakoCytomation, Carpenteria, USA) as a chromogen (which renders a brown precipitate at the antibody binding site). Finally, the sections were counter-stained with Harris' hematoxylin and cover-slipped in Entellan. Formalin-fixed, paraffin-embedded sections of a neuroendocrine tumor and a sample of normal intestinal (colonic) mucosae were used as positive controls for the presence of chromogranin A. All slides were examined using a trinocular Eclipse E200 microscope (Nikon, Tokyo, Japan) coupled to a 10MP CMOS digital camera (Amscope, USA).

2.9. Data analysis

Data are expressed as mean ± standard error of mean (SEM) of the number of experiments. The contractions were quantified in milli-Newtons (mN). A p value < 0.05 was considered significant. When paired contractions were used, for example in the absence and in the presence of an antagonist/inhibitor (the first contaction being the controle response), Student's paired *t*-test was employed for statiscal analysis. When one ring was used as the control response, and another ring was incubated with an antagonist/inhibitor, Student's unpaired t-test was used.

3. Results

3.1. Role of endothelium on EFS-induced contraction Chelonoidis aortae

In pre-contracted rings with acetylcholine, the addition of ATP (10 μM) induced relaxation superior to 60% ($n = 5$) in endothelium-intact rings (Fig. 2A), which was not observed in endothelium-denuded rings ($n = 5$; Fig. 2B).

Electrical field stimulation at 16 Hz promoted contraction in endothelium-intact rings (1.7 ± 0.4 mN; $n = 5$; Fig. 2A and C). This response was not observed in endothelium-denuded rings (0.06 ± 0.0 mN;$p < 0.05$; n = 5) (Fig. 2B and C). The addition of L-NAME (100 μM) increased the EFS-induced contraction in endothelium-preserved rings (2.0 ± 0.4 mN and 8.3 ± 1.9 mN, before and after L-NAME incubation, respectively; Fig. 3A and B) ($p < 0.05$; $n = 6$).

3.2. Sodium channels

The addition of the sodium channel blocker tetrodotoxin (TTX, 1 μM) did not affect the EFS-induced contraction in endothelium-intact rings treated with L-NAME (6.6 ± 1.8 and 6.4 ± 1.6 mN, before and after TTX incubation, respectively) (Fig. 4A and B; $p = 0.9$; $n = 6$).

3.3. Cholinergic receptors

The EFS-induced contractions of endothelium-intact rings treated

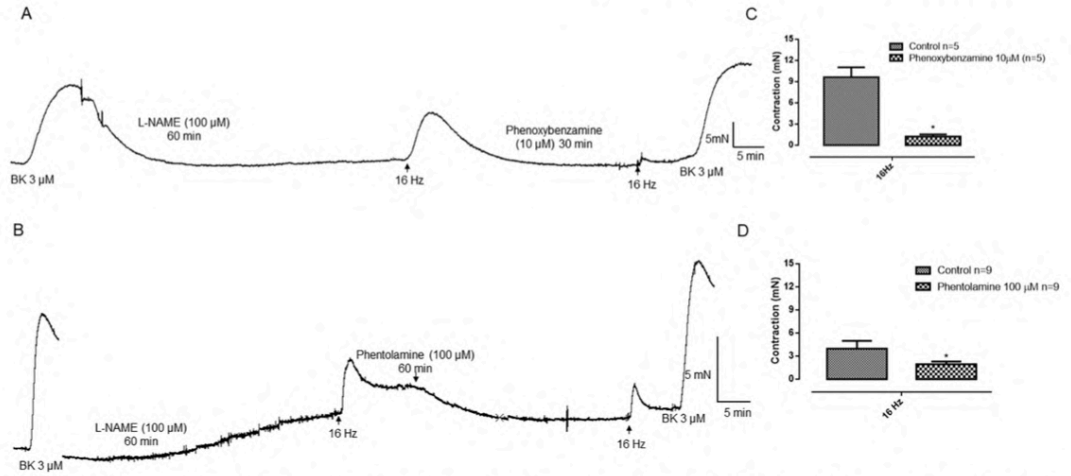


Fig. 6. Panel A shows a representative tracing of a n of 5 experiments of the effect of phenoxybenzamine (10 mM) in endothelium-intact *Chelonoids* aortic rings treated with L-NAME (100 mM). The histograms in Panel (C) represent the mean ± S.E.M (n = 5) for EFS-induced contractions in endothelium-intact aortic rings treated with L-NAME before and after incubation with phenoxybenzamine (10 mM). Paired t-test (Untreated vs treated; $p = 0.0064$). Panels B and D show a representative tracing and the data obtained before after incubation with phentolamine (100 mM; $n = 9$; paired t-test with $p = 0.0175$), respectively.

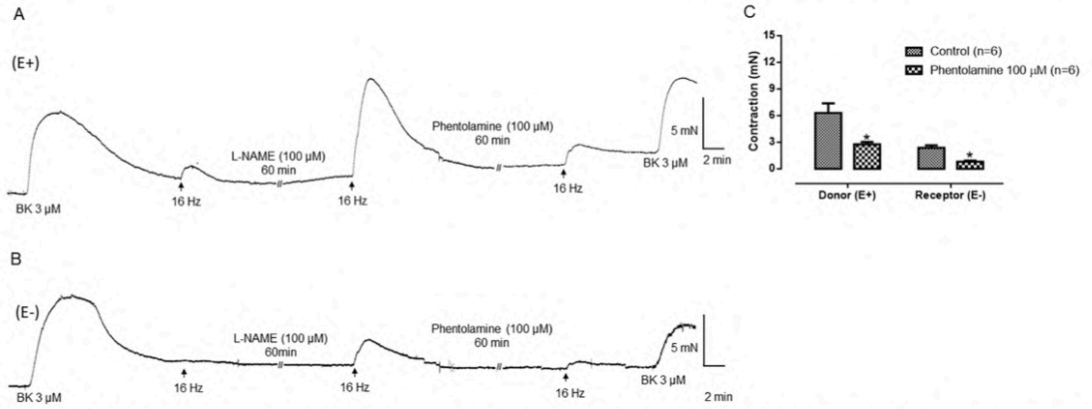


Fig. 7. The sandwich experiment. Two rings with endothelium-intact (E^+, donor tissues) and one ring with endothelium-removed (E^-, receptor tissue) were mounted in the same organ bath. Representative tracings of a n of 6 experiments of EFS-induced contractions in endothelium-intact (A) and endothelium denuded (B) aortic rings before and after L-NAME treatment (100 mM) and phentolamine(100 mM) treatment are shown. The histograms in Panels (C) represent the mean ± S.E.M (n = 6) for EFS-induced contractions in endothelium-intact (donor E^+) and endothelium-removed aortic rings (receptor E^-) treated with L-NAME, before and after incubation with phentolamine (100 mM). Paired t-test (phentolamine untreated vs phentolamine treated; * = $p < 0.05$).

Table 1
S100 protein and tyrosine hydroxylase immunodetection in *Chelonoidis carbonaria* tissues: frequency of positive cases and immunostaining intensity.

Antibody (dilution)	Aorta, *Chelonoidis* *Frequency*[a] *(Intensity*[b]*)*	Brain, *Chelonoidis* *Frequency*[a] *(Intensity*[b]*)*
Rabbit monoclonal anti-S100p (1:200)	–	2/2 (+ + +) in glial cells (cytoplasmic and nuclear), ependymal cells (cytoplasmic and nuclear) and neuropil
Chicken polyclonal anti-tyrosine hydroxylase (1:500)	4/4 (+ + − + + +) in luminal endothelia	2/2 (+ + +) in neurons (cytoplasmic) and neuropil

[a] Frequency: no. of positive samples/total no. of samples.
[b] Immunostaining intensity scale: (−): negative; (+): weak staining; (+ +): moderate staining; (+ + +): strong staining.

with L-NAME were not affected by pre-incubation with the muscarinic receptor antagonist atropine at 10 μM (4.8 ± 2.5 and 4.5 ± 2.3 mN, before and after atropine incubation, respectively; $n = 6$; p = 0.9). The EFS-induced contractions of endothelium-intact rings treated with L-NAME were not affected by pre-incubation with the niconitic receptor antagonist d-tubocurarine at 10 μM (4.5 ± 1.5 and 4.7 ± 1.4 mN, before and after d-tubocurarine incubation, respectively; $n = 6$; $p = 0.4$). Nicotine (100 nM-100 mM) caused concentration-dependent contraction of endothelium-intact rings (Emax 5.0 ± 0.3 mN and pEC_{50} 6 ± 0.1). D-tubocurarine (10 μM) abolished nicotine (1 mM)-induced contractions of endothelium-intact rings (6.18 ± 1.7 mN and 0 mN, untreated and treated rings, respectively; $n = 5$).

3.4. Cyclo-oxygenase products

The TXA_2 mimetic caused concentration-dependent contraction of endothelium-intact rings (Emax 12.9 ± 1.4 mN and EC_{50} 4.8 μM; n = 6). The cyclo-oxygenase inhibitor indomethacin (10 μM) did not affect EFS-induced contractions of aortic rings treated with L-NAME (3.1 ± 1.3 mN and 2.7 ± 1.1 mN); n = 6; $p = 0.19$).

3.5. Endothelin-1 receptors

Endothelin-1 (0.1–100 ηM) concentration dependently promoted a long lasting contraction on *Chelonoidis* endothelium-intact rings (Emax = 7.5 ± 3.4 mN and EC_{50} = 9 nM; n = 6), a profile that markedly contrasted with the short-acting contraction induced by EFS in endothelium-intact rings treated with L-NAME (Fig. 5; $n = 5$).

3.6. Adrenergic alfa-receptors

Phenoxybenzamine at 1 μM (9.6 ± 3.4 and 2.3 ± 0.8 mN, before and after phenoxybenzamime incubation, respectively; $n = 5$) at 10 μM (9.6 ± 1.5 and 1.2 ± 0.3 mN; $p < 0.05$; n = 5; Fig. 6A and C) caused significant reduction in the EFS-induced contractions of aortic rings treated with L-NAME. Phentolamine at 10 μM did not affect the EFS-induced contractions of endothelium-intact rings treated with L-NAME (6.8 ± 1.1 and 5.8 ± 1.5 mN; n = 5; $p = 0.04$). However, at a higher concentration (100 μM), the EFS-response was markedly reduced (3.9 ± 1 and 1.9 ± 0.3 mN $n = 9$; $p < 0.05$; Fig. 6B and D).

The addition of alfa-1 blocker prazosin (100 μM) did not affect the EFS-induced contractions of endothelium-intact rings treated with L-NAME (3.3 ± 0.6 and 2.7 ± 0.8 mN, before and after prazosin incubation, respectively;$n = 6$; $p = 0.35$).

Similar results were observed with the alfa-2 blocker idazoxan (100 μM; 3.8 ± 0.4 and 3.7 ± 0.8 mN, before and after idazoxan incubation, respectively; n = 6; $p = 0.78$).

3.7. The diffusible nature of the EFS-induced contractions in Chelonoidis aorta (sandwich protocol)

In the presence of L-NAME (100 μM), EFS induced contractions in donor (endothelium preserved) rings (6.3 ± 1.1 mN) (n = 6) and recipient rings (endothelium removed) (2.3 ± 0.3 mN; n = 6). The addition of phentolamine (100 μM) reduced the EFS-induced contractions in both donor (2.7 ± 0.3 mN; n = 6) and receptor tissues (0.8 ± 0.1 mN; n = 6; $p < 0.05$) (Fig. 7).

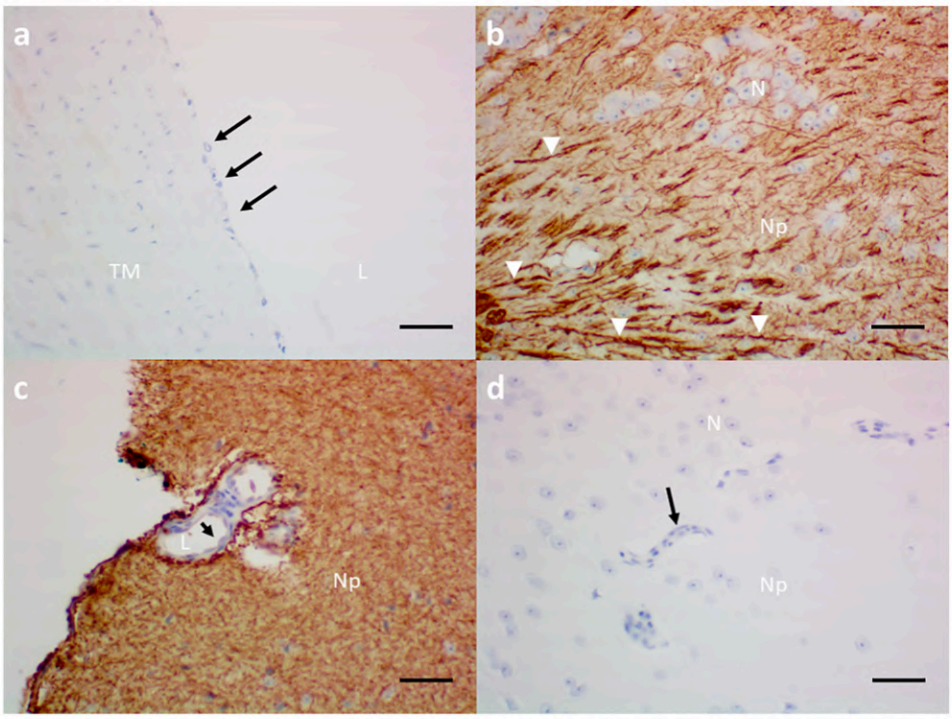


Fig. 8. Detection of S100 protein by immunohistochemistry in *Chelonoidis* aorta (a) and central nervous system (b-d): (a) negative endothelium (arrows) and absence of neural structures within aortic walls; (b) positive neurons (mostly dendrites and axons), and (c) negative vascular endothelium (arrow), in the central nervous system; (d): negative control section (omission of primary antibody) showing absence of positivity in neurons and vascular structures (arrows); N = a group of neurons (cell bodies), Np = neuropil, TM = tunica media, L = lumen, arrow head = axon. Immunoperoxidase (400×, original magnification), scale bar = 40 μm.

3.8. Immunohistochemical detection of tyrosine hydroxylase and S100 protein in Chelonoidis carbonaria aorta

Immunodetection of S100 protein and tyrosine hydroxylase (TH) in aortic specimens of *Chelonoidis carbonaria* are summarized in Table 1 and Figs. 8-10. S100 protein was consistently negative in all aortic tunicae from *Chelonoidis* investigated (4 out of 4 stained specimens), indicating the absence of nerve fibers in this vascular tissue (Table 1 and Fig. 8a). As expected, in positive controls (i.e., *Chelonoidis* brain sections), S100 protein was diffusely positive (Fig. 8b and c). Furthermore, no immunostaining for S100 protein was observed in endothelial cells from either the aorta (Fig. 8a) or the brain samples (Fig. 8c). Using the chicken anti-TH monoclonal antibody, TH presence was found to be moderately to strongly positive in neurons and endothelial cells from the positive control (*Chelonoidis* brain, Table 1 and Fig. 9a-b) and, most importantly, in aortic endothelial cells (Table 1 and Fig. 10a and b). Chromogranin A (a chromaffin cell marker) was consistently negative in all histological compartments of all tested *Chelonoidis* aortae; a strong positivity for this marker was observed in both positive controls, the neuroendocrine tumor and normal colonic cromaffin cells (data not shown).

4. Discussion

The proposed mechanism for EFS in isolated tissues is stimulation of intramural nerve endings (Dail et al., 1987). The sodium channel blocker tetrodotoxin is classically used to block neural stimulation (Campos et al., 2017; Narahashi et al., 1964). However, TTX did not affect the EFS-induced contractions of the tortoise aortic rings, suggesting that EFS is not acting on nerve terminals. Originally purified from bovine brain and long considered unique to the nervous system (Moore, 1965), S-100 protein is the most widely studied neuromarker (Wolf et al., 2013). S-100 proteins are only expressed in vertebrates (Donato et al., 2013); in reptilia it has been described in the forebrain and midbrain of the lizard *Gallotia galloti* (Romero-Alemán et al., 2003), in the intestinal tract of Chinese soft-shelled turtle *Pelodiscus sinensis* (Bao et al., 2011) and in the brain of the snake *Crotalus durissus terrificus* and *Bothops jararaca* (Campos et al., 2018a). Immunoreactivity for S-100 protein was found in *Chelonoidis carbonaria* brain, indicating that the rabbit antibody used recognizes the tortoise S-100 proteins. However, immunoreactivity for S-100 protein was absent in aortic tunicae of *Chelonoidis*, reinforcing that EFS-induced contraction of *Chelonoidis* is unrelated to nerve terminal stimulation. Another important piece of evidence that EFS is not stimulating nerve terminals is the finding that removal of the endothelium abolished EFS-induced contractions of the aortic rings. Similar results were also observed in *Crotalus durissus terrificus*, *Bothrops jararaca* and *Panterophis gutattus* snake aorta (Campos et al., 2018a, 2018b) and human umbilical vessels (Britto- Jr et al., 2020).

The endothelium-derived contracting factors (EDCF) identified so far include superoxide anions, endoperoxides, thromboxane A2, and endothelin-1. Endothelin (ET) is a potent vasoconstrictor both in vitro and in vivo (Yanagisawa et al., 1988). ET-1 potently contracts blood vessels from the turtle *Pseudymes scripta* (Poder et al., 1991) and

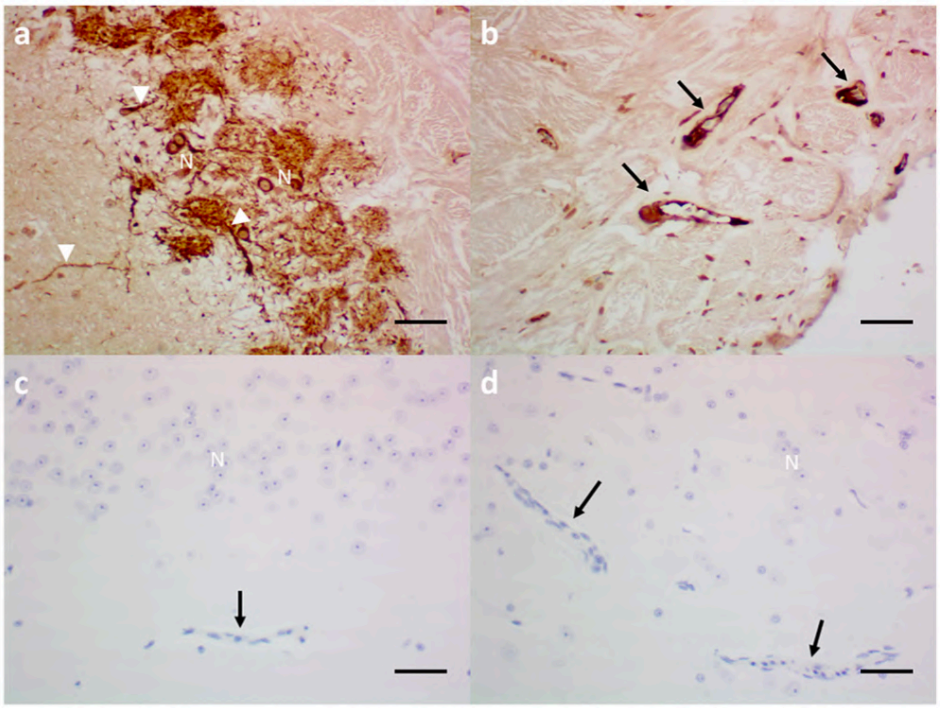


Fig. 9. Detection of tirosine hydroxylase by immunohistochemistry in *Chelonoidis carbonaria* (central nervous system): (a) neurons (cell body, dendrites and axons) and (b) vascular endothelia (arrows); (c) and (d): negative control sections (omission of primary antibody) showing absence of positivity in neurons and vascular structures (arrows). N = a group of neurons (cell bodies), arrow head = axon. Immunoperoxidase (400×, original magnification), scale bar = 40 μm.

Chelonoidis carbonaria aortic rings, as shown here. However, it is unlikely that the contraction induced by EFS is caused by ET release since ET-1-induced contractions of the tortoise aortic rings are long-lasting, as observed in other vascular tissues (De Nucci et al., 1988), whereas those caused by EFS are short-lasting.

Thromboxane A2 is produced by endothelial cells (Salzman et al., 1980) and the thromboxane mimetic U46619 contracts *Chelonoidis carbonaria* aortic rings, as shown here. Interestingly, an endothelium-derived contracting factor released by the aorta of spontaneous hypertensive rats requires activation of cyclo-oxygenase type 1 (Yang et al., 2003). Pre-incubation with indomethacin had no effect in EFS-induced contractions of *Chelonoidis* aorta, indicating that the mediator is not a cyclo-oxygenase metabolite.

The endothelium contains the enzymes necessary to synthesize, store and breakdown ACh (Kirkpatrick et al., 2003; Parnavelas et al., 1985). Acetylcholine contracts *Chelonoidis carbonaria* aortic rings via muscarinic receptors (Campos et al., 2019). In addition, a-subunits of nicotinic acetylcholine receptors in the rat arterial system in situ by means of RT-PCR and immunohistochemistry, indicating that both endothelial cells and arterial smooth muscle cells express various kinds of niconitic receptors (Brügmann et al., 2003). Activation of nicotinic receptors contributes to endothelium-dependent relaxations to acetylcholine in the rat aorta (Zou et al., 2012). Indeed, nicotine induced concentration-dependent contractions in the tortoise aorta, and the contractions were abolished by pre-treatment with the niconitic competitive antagonist d-tubocurarine (Karlin et al., 1986), indicating the presence of functional nicotinic receptors in this tissue. The relevance of this novel finding deserves further investigation. However, since neither atropine (a muscarinic antagonist) nor d-tubocurarine (a nicotinic antagonist) had effect on the EFS-induced contractions of aortic rings, it is unlikely that the cholinergic mediator could be responsible for the contractions induced by EFS.

Catecholamines are important mediators of vascular tonus (Ahlquist, 1948). Their production and release have been classically related to the presence of nerve endings on vessels (Kadowitz et al., 1976; Matsuyama et al., 1985). Our results extend previous observations obtained in isolated aortic rings of *Panterophis guttatus* (a non-venomous snake) (Campos et al., 2018b) and of *Crotalus durissus terrificus* and *Bothrops jararaca* (venomous snakes) (Campos et al., 2018a), indicating the endothelium as the main source of catecholamine release by the EFS-induced contractions. Indeed, synthesis of adrenergic catecholamines has been shown to occur in bovine aortic endothelial cells and in mouse femoral arteries (Sorriento et al., 2012). Indeed, as demonstrated here, the enzyme tyrosine hydroxylase was identified by immunohistochemistry in the endothelial cells of the tortoise aorta. Tyrosine hydroxylase is the enzyme responsible for the conversion of tyrosine to L-dihydroxy-phenylalanine (L-DOPA), the precursor of dopamine (Nagatsu et al., 1964) and tyrosine hydroxylase is considered the limiting step in the catecholamine biosynthesis (Ikeda et al., 1965).

Endothelium-derived catecholamines modulate EFS-induced contractions of snake aortic rings (Campos et al., 2018a, 2018b). These contractions are abolished by pre-incubation with the non-selective α-adrenergic antagonist phentolamine (Doxey et al., 1977; Giussani et al., 1995). Phentolamine and phenoxybenzamine (another non-selective α-adrenergic antagonist), markedly reduced the EFS-induced contractions of *Chelonoidis carbonaria* aortic rings, indicating that endothelium-

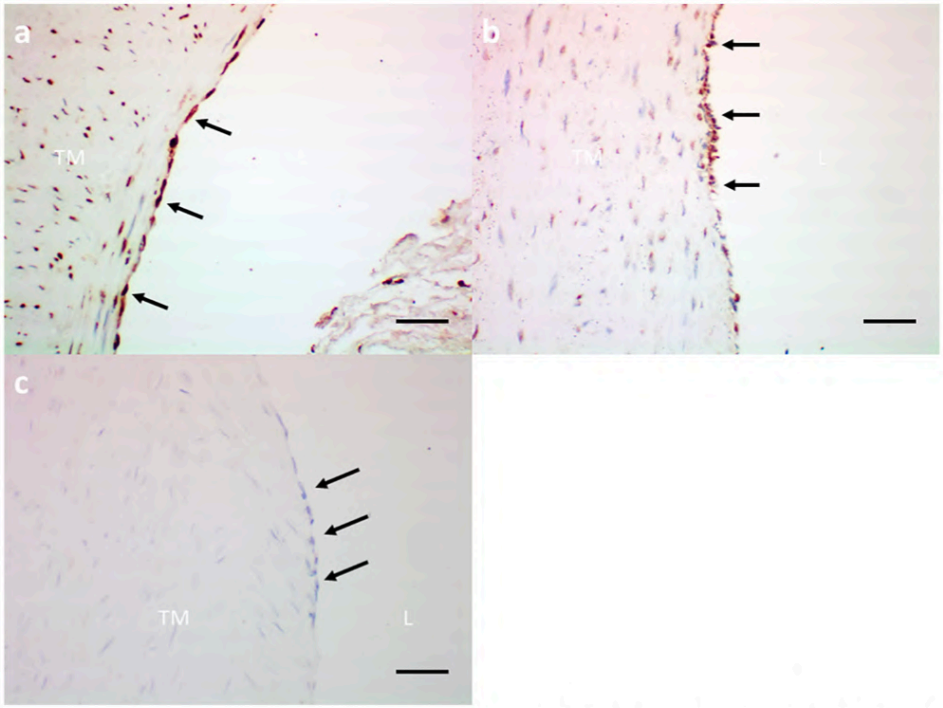


Fig. 10. Detection of tirosine hydroxylase by immunohistochemistry in *Chelonoidis carbonaria* (Aorta): positive aortic endothelium (arrows within a and b); (c): negative control sections (omission of primary antibody) showing absence of positivity in aortic endothelium (arrows). TM = tunica media, L = lumen. Immunoperoxidase (400×, original magnification); scale bar = 40 μm.

derived catecholamines account for the contractions. However, the alpha$_1$-adrenergic receptor antagonist prazosin (Agrawal et al., 1984) and the alpha$_2$-adrenergic receptor antagonist idazoxan (Doxey et al., 1984) had no effect on EFS-induced contraction tortoise aortic rings, indicating that alpha-adrenergic receptors are not involved. The inhibition observed with phentolamine was only observed at higher concentration, indicating that phentolamine must be acting in a different population of receptors. Indeed, phentolamine at higher concentrations (> 2 mM) displaces ^{3}H-haloperidol binding to dopamine receptors in calf brain membranes (Burt et al., 1976). Phenoxybenzamine is a b-haloalkylamine which alkylates chemically active radicals such as hydroxy, sulfhydryl, and amino groups. The alkylation by phenoxybenzamine selectively and irreversibly inactivates dopaminergic D2 receptors on primary cultured rat lactotrophs (Shin et al., 1992). Thus, it is likely that the main catecholamine released by the endothelial cells is dopamine.

In all mammals, the chromaffin (neuroendocrine) cells form discrete cell groups/collections called paraganglia which are closely associated with the autonomic nervous system and the gastrointestinal tract (Knottenbelt et al., 2015; La Perle and Dintzis, 2018). The chromaffin cells release catecholamines: ~80% of adrenaline (epinephrine) and ~20% of noradrenaline (norepinephrine) into systemic circulation. These cells have been described in other non-mammal vertebrates, not only in close association with the autonomic system, but also within cardiac tissue and some vascular structures, such as intercostal arteries and the azygous vein (Scheuermann, 1993) and cells in the heart and in the walls of arteries and veins of lungfish Nilsson, 2010). The chromaffin cells can be identified by a number of methods, immunohistochemistry being the most frequently used. Chromogranin A and synaptophysin are currently considered the most specific immunohistochemical markers of neuroendocrine (chromaffin) differentiation (Kyriakopoulos et al., 2018). Although strongly expressed in the positive controls (a neuroendocrine tumor and a normal intestinal mucosae), chromogranin A was consistently negative in all histologic compartments of all tested aortic rings. The lack of tyrosine hydroxylase and of chromogranin A in the walls (tunica media) of *Chelonoidis carbonaria* aorta, and the presence of tyrosine hydroxylase in endothelium, indicate that chromaffin cells are not present in this particular organ/species and that the primary source of aortic-derived catecholamines is more likely to be the endothelium.

5. Conclusion

The endothelium modulates EFS-induced contractions in *Chelonoidis* aortic rings and this modulation is at least in part due to endothelium-derived dopamine. The relevance of the endothelium-derived catecholamines in the vascular smooth muscle tonus regulation remains to be determined.

Declaration of competing interest

We declare that the content of this manuscript has not been published or submitted for publication elsewhere. We also declare that this manuscript has no conflicts of interests.

Acknowledgements

Rafael Campos is supported by CAPES fellowship. Felipe Fernandes Jacintho and Alberto Fernando Oliveira Justo are supported by FAPESP (2018/24971-9 and 2016/09539-8) André Almeida Schenka and Edson Antunes thank FAPESP (grant number 2016/04731-8 and 2017/15175-1, respectively) and Gilberto De Nucci is supported by a CNPq fellowship.

References

Agrawal, D.K., Triggle, C.R., Daniel, E.E., 1984. Pharmacological characterization of the postsynaptic alpha adrenoceptors in vascular smooth muscle from canine and rat mesenteric vascular beds. J. Pharmacol. Exp. Ther. 229, 831–838.

Ahlquist, R.P., 1948. A study of the adrenotropic receptors. Am. J. Phys. 153, 586–600. https://doi.org/10.1152/ajpendo.00664.2007.

Bao, H. jun, Liu, Y., Qin, J. hui, Xu, C. sheng, Hei, N. nan, Jaber, J.R., Chen, Q. sheng, 2011. An immunohistochemical study of S-100 protein in the intestinal tract of Chinese soft-shelled turtle, Pelodiscus sinensis. Res. Vet. Sci. 91, e16–e24. https://doi.org/10.1016/j.rvsc.2011.02.015.

Bevan, J.A., 1962. Some characteristics of the isolated sympathetic nerve-pulmonary artery preparation of the rabbit. J. Pharmacol. Exp. Ther. 137, 213–218.

Britto- Jr., J., Jacintho, F.F., Murari, G.M., Campos, R., Moreno, R.A., Antunes, E., Mónica, F.Z., De Nucci, G., 2020. Electrical field stimulation induces endothelium-dependent contraction of human umbilical cord vessels. Life Sci. 243, 117257.

Brügmann, D., Lips, K.S., Pfeil, U., Haberberger, R.V., Kummer, W., 2003. Rat arteries contain multiple nicotinic acetylcholine a-subunits. Life Sci. 72, 2095–2099.

Burt, D.R., Creese, I., Snyder, S.H., 1976. Properties of [3H]haloperidol and [3H]dopamine binding associated with dopamine receptors in calf brain membranes. Mol. Pharmacol. 12 (5), 800–812.

Campos, R., Mónica, F.Z., Rodrigues, R.L., Rojas-Moscoso, J.A., Moreno, R.A., Cogo, J.C., De Oliveira, M.A., Antunes, E., De Nucci, G., 2017. Tetrodotoxin-insensitive electrical field stimulation-induced contractions on crotalus durissus terrificus corpus cavernosum. PLoS One 12. https://doi.org/10.1371/journal.pone.0183766.

Campos, R., Justo, A.F.O., Monica, F.Z., Cogo, J.C., Moreno, R.A., de Souza, V.B., Schenka, A.A., De Nucci, G., 2018a. Electrical field-induced contractions on Crotalus durissus terrificus and Bothrops jararaca aortae are caused by endothelium-derived catecholamine. PLoS One 13, e0203573. https://doi.org/10.1371/journal.pone.0203573.

Campos, R., Mónica, F.Z., Oliveira Justo, A.F., Cogo, J.C., Oliveira, E. de T., Moreno, R.A., Antunes, E., De Nucci, G., 2018b. Electrical field stimulation-induced contractions on Pantherophis guttatus corpora cavernosa and aortae. PLoS One 13, 1–9. https://doi.org/10.1371/journal.pone.0196123.

Campos, R., Justo, A.F.O., Jacintho, F.F., Mónica, F.Z., Rojas-Moscoso, J.A., Moreno, R.A., Napolitano, M., Cogo, J.C., De Nucci, G., 2019. Pharmacological and transcriptomic characterization of the nitric oxide pathway in aortic rings isolated from the tortoise Chelonoidis carbonaria. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 222, 82–89. https://doi.org/10.1016/j.cbpc.2019.04.015.

Dail, W.G., McGuffee, L., Minorsky, N., Little, S., 1987. Responses of smooth muscle strips from penile erectile tissue to drugs and transmural nerve stimulation. J. Auton. Pharmacol. 7, 287–294. https://doi.org/10.1111/j.1474-8673.1987.tb00157.x.

Darios, E.S., Barman, S.M., Orer, H.S., Morrison, S.F., Davis, R.P., Seitz, B.M., Burnett, R., Watts, S.W., 2015. 5-Hydroxytryptamine does not reduce sympathetic nerve activity or neuroeffector function in the splanchnic circulation. Eur. J. Pharmacol. 754, 140–147. https://doi.org/10.1016/j.ejphar.2015.02.032.

De Nucci, G., Thomas, R., D'Orleans-Juste, P., Antunes, E., Walder, C., Warner, T.D., Vane, J.R., 1988. Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. Proc. Natl. Acad. Sci. U. S. A. 85, 9797–9800. https://doi.org/10.1073/pnas.85.24.9797.

Donato, R., Cannon, B.R., Sorci, G., Riuzzi, F., Hsu, K., Weber, D.J., Geczy, C.L., 2013. Functions of S100 proteins. Curr. Mol. Med. 13, 24–57.

Dong, H., Waldron, G.J., Galipeau, D., Cole, W.C., Triggle, C.R., 1997. NO/PGI2-independent vasorelaxation and the cytochrome P450 pathway in rabbit carotid artery. Br. J. Pharmacol. 120, 695–701. https://doi.org/10.1038/sj.bjp.0700945.

Doxey, J.C., Smith, C.F.C., Walker, J.M., 1977. Selectivity of blocking agents for pre- and postsynaptic α-adrenoceptors. Br. J. Pharmacol. 60, 91–96.

Doxey, J.C., Lane, A.C., Roach, A.G., Virdee, N.K., 1984. Comparison of the alpha-adrenoceptor antagonist profiles of idazoxan (RX 781094), yohimbine, rauwolscine and corynanthine. Naunyn Schmiedeberg's Arch. Pharmacol. 325, 136–144.

Furchgott, R.F., Zawadzki, J.V., 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288, 373–376. https://doi.org/10.1038/288373a0.

Giussani, D. a, Moore, P.J., Bennet, L., Spencer, J. a D., Hanson, M. a, 1995. α1- and α2-adrenoreceptor actions of pentolamine and prazosin on breathing movements in fetal sheep in utero. J. Physiol. 486, 249–255.

Ikeda, M., Levitt, M., Udenfriend, S., 1965. Hydroxylation of phenylalanine by purified preparations of adrenal and brain tyrosine hydroxylation. Biochem. Biophys. Res. Commun. 18, 1965.

Kadowitz, P.J., Knight, D.S., Hibbs, R.G., Ellison, J.P., Joiner, P.D., Brody, M.J., Hyman, A.L., 1976. Influence of 5- and 6-hydroxydopamine on adrenergic transmission and nerve terminal morphology in the canine pulmonary vascular bed. Circ. Res. 39, 191–199. https://doi.org/10.1161/01.RES.39.2.191.

Karlin, A., Cox, R.N., Dipaola, M., Holzman, E., Kao, P.N., Wang, L., Yodh, N., 1986. Functional domains of the niconitic acetylcholine receptor. Ann. N. Y. Acad. Sci. 463, 53–69.

Kirkpatrick, C.J., Bittinger, F., Nozadze, K., Wessler, I., 2003. Expression and function of the non-neuronal cholinergic system in endothelial cells. Life Sci. 72, 2111–2116. https://doi.org/10.1016/S0024-3205(03)00069-9.

Knottenbelt, D.C., Patterson-Kane, J.C., Snalune, K.L., 2015. Endocrine and neuroendocrine tumors. In: Clinical Equine Oncology, pp. 376–392. https://doi.org/10.1016/B978-0-7020-4266-9.00024-6.

Kyriakopoulos, G., Mavroeidi, V., Chatzellis, E., Kaltsas, G.A., Alexandraki, K.I., 2018. Histopathological, immunohistochemical, genetic and molecular markers of neuroendocrine neoplasms. Ann Transl Med 6, 252.

La Perle, K.M.D., Dintzis, S.M., 2018. Endocrine system. In: Comparative Anatomy and Histology (Second Edition), a Mouse, Rat, and Human Atlas, pp. 251–273. https://doi.org/10.1016/B978-0-12-802900-8.00015-4.

Matsuyama, T., Shiosaka, S., Wanaka, A., Yoneda, S., Kimura, K., Hayakawa, T., Emson, P.C., Tohyama, M., 1985. Fine structure of peptidergic and catecholaminergic nerve fibers in the anterior cerebral artery and their interrelationship: an immunoelectron microscopic study. J. Comp. Neurol. 235, 268–276. https://doi.org/10.1002/cne.902350209.

Miller, V.M., Vanhoutte, P.M., 1986. Endothelium-dependent responses in isolated blood vessels of lower vertebrates. Blood Vessels 23, 225–235.

Moore, B.W., 1965. A soluble protein characteristic of the nervous system. Biochem. Biophys. Res. Commun. 19, 739–744. https://doi.org/10.1016/0006-291X(65)90320-7.

Nagatsu, T., Levitt, M., Udenfriend, S., 1964. Tyrosine hydroxylase. The initial step in norepineprine biosynthesis. J. Biol. Chem. 239, 2910–2917. https://doi.org/10.1097/00005792-196405000-00019.

Narahashi, T., Moore, J.W., Scott, W.R., 1964. Tetrodotoxin blockage of sodium conductance increase in lobster Giant axons. J. Gen. Physiol. 47, 965–974. doi:https://doi.org/10.1016/0041-0101(67)90106–7.

Nilsson, S., 2010. On the autonomic nervous and chromaffin control systems of lungfish. Australian Zoologist 35, 363–368.

Parnavelas, J., Kelly, W., Burnstock, G., 1985. Ultrastructural localization of choline acetyltransferase in vascular endothelial cells in rat brain. Nature 336, 724–725.

Paterson, G., 1965. The response to transmural stimulation of isolated arterial strips and its modification by drugs. J. Pharm. Pharmacol. 17, 341–349.

Plane, F., Pearson, T., Garland, C.J., 1995. Multiple pathways underlying endothelium-dependent relaxation in the rabbit isolated femoral artery. Br. J. Pharmacol. 115, 31–38. https://doi.org/10.1111/j.1476-5381.1995.tb16316.x.

Poder, T.C., Silberberg, S.D., Rampe, D., 1991. Contraction of reptile, amphibian, and fish blood vessels by endothelin-1. Can. J. Physiol. Pharmacol. 69, 215–217. https://doi.org/10.1139/y91-032.

Romero-Alemán, M. del M., Monzón-Mayor, M., Yanes, C., Arbelo-Galván, J.F., Lang, D., Renau-Piqueras, J., Negrín-Martínez, C., 2003. S100 immunoreactive glial cells in the forebrain and midbrain of the lizard Gallotia galloti during ontogeny. J. Neurobiol. 57, 54–66. https://doi.org/10.1002/neu.10258.

Salzman, P.M., Salmon, J.A., Moncada, S., 1980. Prostacyclin and thromboxane A2 synthesis by rabbit pulmonary artery. J. Pharmacol. Exp. Ther. 215, 240–247.

Scheuermann, D.W., 1993. Comparative morphology, cytochemistry and innervation of chromaffin tissue in vertebrates. J. Anat. 183, 327–342.

Shin SH, McAssey K, Heisler RL, Szabo MS. 1992. Phenoxybenzamine selectively and irreversibly inactivates dopaminergic D2 receptors on primary cultured rat lactotrophs. Neuroendocrinology 56: 3970–406.

Sorriento, D., Santulli, G., Del Giudice, C., Anastasio, A., Trimarco, B., Iaccarino, G., 2012. Endothelial cells are able to synthesize and release catecholamines both in vitro and in vivo. Hypertension 60, 129–136. https://doi.org/10.1161/HYPERTENSIONAHA.111.189605.

Stephens, G.A., 1984. Angiotensin and norepinephrine effects on isolated vascular strips from a reptile. Gen. Comp. Endocrinol. 54, 175–180.

Wolf, H., Frantal, S., Pajenda, G.S., Salameh, O., Widhalm, H., Hajdu, S., Sarahrudi, K., 2013. Predictive value of neuromarkers supported by a set of clinical criteria in patients with mild traumatic brain injury: S100B protein and neuron-specific enolase on trial. J. Neurosurg. 118, 1298–1303. https://doi.org/10.3171/2013.1.JNS121181.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T., 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332, 411–415.

Yang, D., Félétou, M., Levens, N., Zhang, J.N., Vanhoutte, P.M., 2003. A diffusible substance(s) mediates endothelium-dependent contractions in the aorta of SHR. Hypertension 41, 143–148. https://doi.org/10.1161/01.HYP.0000047651.45322.16.

Zou, Q., Leung, S.W.S., Vanhoutte, P.M., 2012. Activation of nicotinic receptors can contribute to endothelium-dependent relaxations to acetyocholine in the rat aorta. J. Pharmacol. Exp. Ther. 341, 756–763. https://doi.org/10.1124/jpet.112.192229.

Anexo 2

Artigo 4 – Determination of dopamine, noradrenaline, and adrenaline in Krebs-Henseleit solution by liquid chromatography coupled with tandem mass spectrometry and measurement of their basal release from Chelonoidis carbonaria aortae in vitro

Autores: Britto-Júnior J, Antunes NJ, Campos R, Sucupira M, Mendes GD, Fernandes F, Moraes MO, Moraes MEA, De Nucci G.
Revista: Biomedical Chromatography

Situação: Aceito a publicação em 22 de abril de 2020. Publicado on-line em 14 de agosto de 2020

Received: 22 April 2020 | Revised: 14 August 2020 | Accepted: 27 August 2020
DOI: 10.1002/bmc.4978

RESEARCH ARTICLE

Biomedical Chromatography WILEY

Determination of dopamine, noradrenaline, and adrenaline in Krebs–Henseleit solution by liquid chromatography coupled with tandem mass spectrometry and measurement of their basal release from *Chelonoidis carbonaria* aortae *in vitro*

José Britto-Júnior[1] | Natalícia J. Antunes[1] | Rafael Campos[2,3] | Mauro Sucupira[3] | Gustavo Duarte Mendes[4,5] | Fernanda Fernandes[4] | Manoel Odorico Moraes[3] | Maria Elisabete A. Moraes[3] | Gilberto De Nucci[1,3,4,6]

[1]Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, SP, Brazil

[2]Superior Institute of Biomedical Sciences, Ceará State University (UECE), Fortaleza, CE, Brazil

[3]Clinical Pharmacology Unit, Drug Research and Development Center, Federal University of Ceará (UFC), Fortaleza, CE, Brazil

[4]Department of Pharmacology, Faculty of Medicine, São Leopoldo Mandic, Campinas, SP, Brazil

[5]Department of Pharmacology, Faculty of Medicine, Metropolitan University of Santos, Santos, SP, Brazil

[6]Institute of Biomedical Sciences, University of São Paulo (USP), São Paulo, Brazil

Correspondence
Natalícia de Jesus Antunes, State University of Campinas (UNICAMP), Faculty of Medical Sciences, Department of Pharmacology, Alexander Fleming Street, 105, 13083-881, Campinas. SP, Brazil.
Email: nataliciaja@gmail.com

Funding information
Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Grant/Award Number: 303839/2019-8; National Council for Scientific and Technological Development (CNPq), Grant/Award Number: 2016/22506-1; São Paulo Research Foundation (FAPESP)

Abstract

This study presented for the first time the development and validation of a sensitive method for quantification of dopamine, noradrenaline, and adrenaline in Krebs–Henseleit solution by LC–tandem mass spectrometry. Aliquots of 2.0 mL calibrators, quality controls, and samples of Krebs–Henseleit solution incubated with tortoise's aortic ring for 30 min were extracted by solid-phase extraction. Catecholamine separation was achieved on a 100 × 4.6 mm LiChrospher RP-8 column and the quantification was performed by a mass spectrometer equipped with an electrospray interface operating in positive ion mode. The run time was 4 min and the calibration curve was linear over the range of 0.1–20.0 ng/mL. The method was applied to the measurement of basal release of dopamine, noradrenaline, and adrenaline from the tortoise *Chelonoidis carbonaria* aortae *in vitro*. One aortic ring (30 mm) per tortoise ($n = 5$) was incubated for 30 min in a 5 mL organ bath filled with Krebs–Henseleit solution. The method demonstrated sensitivity, precision, and accuracy enough for its application in the measurement of basal release of these catecholamines from *C. carbonaria* aortic rings *in vitro*. The mean (standard deviation) concentrations of dopamine, noradrenaline, and adrenaline were 3.48 (2.55) ng/mL, 1.40 (0.57) ng/mL, and 1.87 (1.09) ng/mL, respectively.

KEYWORDS

catecholamines, *in vitro* release, LC–MS/MS, solid-phase extraction, tortoise

 © 2020 John Wiley & Sons, Ltd.

1 | INTRODUCTION

The endothelium of human umbilical cord vessels release mediator(s) capable of modulating the spasmogenic activity induced by electrical field stimulation, indicating a potential role of endothelium-derived catecholamines in modulating human umbilical arteries and the human umbilical vein reactivities (Britto-Júnior et al., 2020). Thus, the quantification of catecholamines in human umbilical cord vessels is of great importance in assessing their release.

The precise measurement of catecholamines in any biological sample is a challenge because they are found in low concentrations and they are easily oxidized (Xie, Chen, Gu, Wei, & Kang, 2018). Among the methods used for determination of catecholamines in different kinds of biological samples, such as plasma, urine, and peripheral blood mononuclear cells, LC coupled with tandem mass spectrometry (LC–MS/MS) is the most promising, because it has been demonstrating high sensitivity and specificity, besides being less time-consuming (He, Carballo-Jane, Tong, & Cohen, 2015; Ji, Walton, Su, & Tella, 2010; Li, Li, & Kellermann, 2016; Petteys, Graham, Parnás, Holt, & Frank, 2012; Saracino, Santarcangelo, Raggi, & Mercolini, 2015; Woo et al., 2016; Yu et al., 2019; Zhang et al., 2012; Zhang, Wu, Chow, Tam, & Rios, 2016; Zheng, Mandal, & Wishart, 2018).

This study presents, for the first time, the development and validation of a sensitive method to quantify dopamine, noradrenaline, and adrenaline in Krebs–Henseleit solution by LC–MS/MS with a lower limit of quantification (LLoQ) of 0.1 ng/mL for all analytes, which was adequate to the measurement of basal release of catecholamines from *Chelonoidis carbonaria* aortic rings *in vitro*.

2 | EXPERIMENTAL

2.1 | Animals

Five tortoises of both sexes, weighing from 2.0 to 7.0 kg, were provided by the Parque Ecológico do Tietê (São Paulo, SP, Brazil). All experimental procedures using the animals were approved by the Institutional Animal Care and Use Committee (CEUA/UNICAMP: 3907–1) and were in compliance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines. The use of *C. carbonaria* was authorized by the Brazilian Institute for Environment (Sisbio; number 20910).

2.2 | Chemicals and reagents

Adrenaline, noradrenaline, dopamine, ascorbic acid, and trifluoroacetic acid were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Dopamine-D3 hydrochloride, DL-noradrenaline-D6 hydrochloride, and adrenaline-D6 hydrochloride were acquired from CDN Isotopes (Point Claire, Canada). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$), potassium phosphate monobasic (KH_2PO_4), and glucose were purchased from Merck KGaA (Darmstadt, Germany). Acetonitrile and methanol were obtained from J. T. Baker (Phillipsburg, NJ, USA). The water was obtained from the purification system Synergy UV (Millipore, Molsheim, France). Aluminum oxide was bought from Dinâmica Química Contemporânea Ltda (Indaiatuba, SP, Brazil).

2.3 | Calibration standards and quality controls

Stock solutions of dopamine, noradrenaline, adrenaline, and the internal standards (ISs) dopamine-D3, noradrenaline-D6, and adrenaline-D6 were prepared in acetonitrile/water (20/80, *v/v*) 100%. Calibration curves of catecholamines (duplicate) were prepared by spiking blank Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5.6 mM glucose, and 3 mM ascorbic acid) with dopamine, noradrenaline, and adrenaline to generate calibrator concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 ng/mL for each catecholamine.

The quality control (QC) samples were prepared at low, middle 1, middle 2, and high concentrations of 0.3, 1.5, 9.0, and 15.0 ng/mL of dopamine, noradrenaline, and adrenaline, respectively in blank Krebs–Henseleit solution.

2.4 | Tissue preparation

The tortoises were sedated with midazolam (2 mg/kg; intramuscularly), anesthetized with ketamine (40 mg/kg; intramuscularly) and propofol (15 mg/kg; intravenously), and euthanized by exsanguination. A segment of dorsal aortae was removed and immediately placed in Krebs–Henseleit solution at 27°C. Subsequently, one aortic ring per animal (30 mm) was suspended vertically between two metal hooks in 5 mL organ baths containing Krebs–Henseleit solution [NaCl (118 mM), KCl (4.7 mM), $CaCl_2$ (2.5 mM), $MgSO_4$ (1.2 mM), $NaCO_3$ (25 mM), KH_2PO_4 (1.2 mM), and glucose (5.6 mM)] and then gassed with a mixture of 95% O_2 and 5% CO_2 (pH 7.4) at 27°C, because this temperature is often used for reptile tissue experiments (Britto-Júnior et al., 2020; Campos et al., 2018). After 30-min incubation, an aliquot of 2 mL of the supernatant was transferred to a tube and stored at −20°C until analysis.

2.5 | Sample preparation

Catecholamines were extracted from 2 mL Krebs–Henseleit solution. Calibrators and QCs prepared in blank Krebs–Henseleit solution (section 2.3) and samples of Krebs–Henseleit solution incubated with tortoise's aortic ring for 30 min (section 2.4) were spiked with 100 µL IS solution containing 100 ng/mL dopamine-d3, noradrenaline-d6, and adrenaline-d6. Then, to each solution mixture 1.5 mL deionized water was added. The samples were homogenized by vortexing for 10 s and

then 100 mg aluminum oxide (Al_2O_3) was added. The tubes containing these mixtures were incubated on an orbital shaker for 20 min and centrifuged (5810/5810 R) at 2000*g* for 4 min at 4°C. The tubes were washed four times with 2 mL deionized water and the mixtures were subjected to centrifugation (2000*g* for 4 min at 4°C). Then, 200 μL of a solution containing acetonitrile/H_2O (60/40, *v*/*v*) + 0.1% trifluoroacetic acid was added. The samples were homogenized by vortexing for 40 s and centrifuged at 2000*g* for 5 min at 4°C. The supernatants were transferred to vials and submitted to chromatographic analysis.

2.6 | Chromatographic analysis

The dopamine, noradrenaline, and adrenaline concentrations were determined in Krebs–Henseleit solution using a Nexera liquid chromatography (HPLC) system coupled to a 8060 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan).

The HPLC system consisted of an autoinjector (model SIL-30AC), an LC-30 AD binary pump, and a CTO-20AC column oven. Catecholamines were separated on a 100 × 4.6 mm LiChrospher RP-8 column (GL Sciences Inc., Tokyo, Japan) using acetonitrile/water (90/10, *v*/*v*) + 2.5 mM ammonium hydroxide as the mobile phase at a flow rate of 1.3 mL/min with a split ratio of 1:5. The temperatures of the column and the autosampler were maintained at 65 and 8°C, respectively.

The MS/MS detection system was equipped with an electrospray interface operating in positive ion mode. The ion spray capillary voltage was set at 4.0 kV, desolvation temperature at 250°C, nebulizer gas flow at 3.0 L/min, heat block temperature at 400°C, and drying gas flow at 10 L/min. The analyses were performed in the multiple reaction monitoring mode. The ion transitions, cone voltages, collision energies, and retention time are presented in Table 1. Data acquisition and quantification were performed using LabSolutions software, version 5.0 (Shimadzu Corporation, Kyoto, Japan).

2.7 | Validation

The method for analysis of catecholamines was validated according to the recommendations of the United States Food and Drug Administration (Food and Drug Administration, 2018) and the Brazilian National Sanitary Surveillance Agency (Angência Nacional de Vigilância Sanitária., 2011).

2.7.1 | Selectivity

Blank Krebs–Henseleit solution samples ($N = 6$) were individually tested for interferences using the described sample preparation methods and chromatographic conditions and compared with the results obtained from the LLoQ samples.

2.7.2 | Carryover

To assess carryover, extracted double banks (triplicate) were injected immediately following the upper limit of quantification (ULQ) prepared in the analyte-free Krebs–Henseleit solution (20.0 ng/mL of each catecholamine). Carryover was assessed by comparing the signal in the analyte-free Krebs–Henseleit solution samples injected after the ULQ sample with the signal in the LLoQ sample. The signal limits may be ≤20% for analytes and ≤5% for ISs.

2.7.3 | Linearity

The linearity of the calibration curves (0.1–20.0 ng/mL in duplicate) was determined by plotting the peak area ratio of each catecholamine/IS *versus* nominal concentration of each catecholamine. The calibration curves were constructed by weighted (1/x) least squares linear regression. The linear correlation coefficient must be ≥0.98.

2.7.4 | Accuracy and precision

Seven aliquots of LLoQ and every QC sample (0.3, 1.5, 9.0, and 15.0 ng/mL) were analyzed in three validation lots on 3 different days. Inter- and intra-day precisions were determined as coefficient of variation (% CV) and the accuracy as the percentage relative error (% RE). The CV values must not exceed 15% for each QC sample and 20% for LLoQ and the accuracy must be within the range of 85%–115% of the actual values for QCs and within the range of 80%–120% for LLoQ samples.

TABLE 1 Mass spectrometry operating conditions

Analyte	Multiple reaction monitoring transition (*m*/*z*)	Q1 Pre-bias (V)	Q3 Pre-bias (V)	Retention time (min)
Dopamine	154 > 91	12.00	18.00	3.12 ± 0.3
Noradrenaline	170 > 107	12.00	18.00	2.97 ± 0.3
Adrenaline	184 > 107	12.00	18.00	3.05 ± 0.3
Dopamine-D3	157 > 93	12.00	18.00	3.12 ± 0.3
Noradrenaline-D6	176 > 158	12.00	18.00	2.97 ± 0.3
Adrenaline-D6	190 > 172	12.00	18.00	3.05 ± 0.3

2.7.5 | Matrix effect and recovery

The matrix effect was evaluated by comparing the peak area of catecholamines (0.3 and 15.0 ng/mL) and IS injected directly into the mobile phase with the peak areas generated from the standard solutions added to blank Krebs–Henseleit solution extracts ($N = 6$). Each sample was obtained by a matrix factor normalized by IS (MFN) according to the following formula: MFN = (response of each catecholamine in matrix/IS response matrix)/(response of each catecholamine in solution/response of the IS solution). The CV of the MFNs for all samples should be <15%.

The analytical extraction recovery was determined by comparing the catecholamine mean peak area ratio (analyte: IS) response of two extracted QC samples (0.3 and 15.0 ng/mL) with the mean peak area ratio response of blank Krebs–Henseleit solution samples spiked after extraction at same concentrations as the QC samples in three replicates.

2.7.6 | Stability

To evaluate the stability of the method, QC samples (0.3 and 15.0 ng/mL) were subjected to stability test under different conditions:

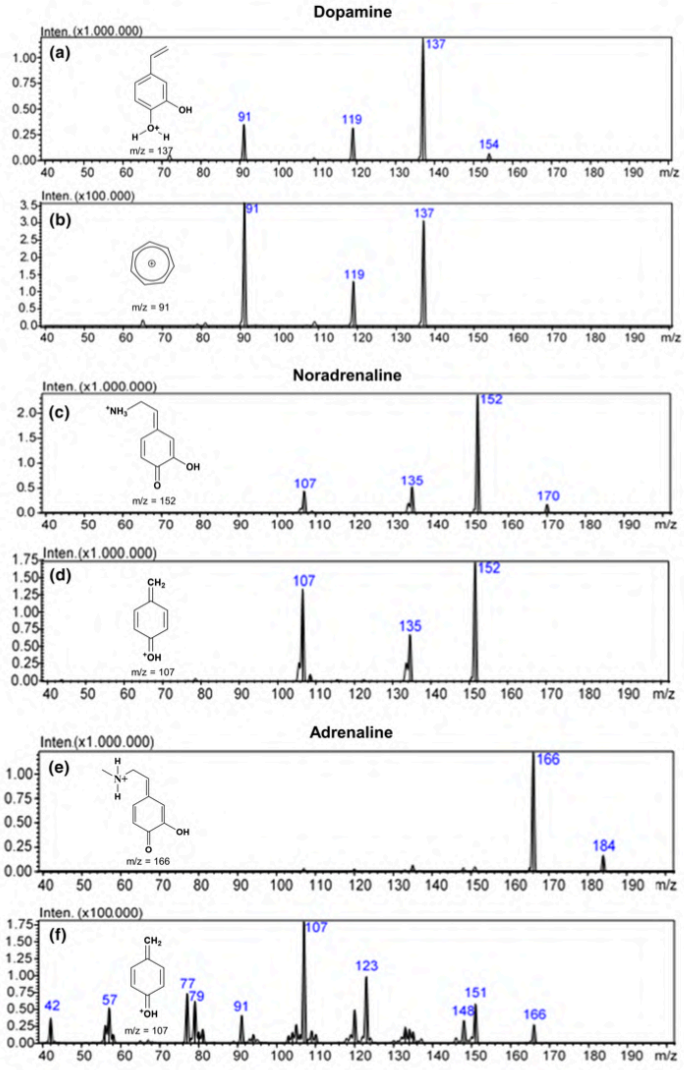


FIGURE 1 Product-ion spectra of the protonated molecular ion of dopamine with collision energy (CE) (a) 15 V and (b) 30 V; noradrenaline with CE (c) 10 V and (d) 15 V; and adrenaline with CE (e) 10 V and (f) 25 V acquired in positive-mode electrospray ionization

short-term stability (storage at room temperature for 60 h), three freeze–thaw (−80 to 25°C) cycles, processing on an autosampler (for 7 days at 6°C), and long-term storage stability (31 days at −20°C) in triplicate.

3 | RESULTS AND DISCUSSION

This study shows, for the first time, a selective and sensitive method for the analysis of dopamine, noradrenaline, and adrenaline in Krebs–Henseleit solution samples after incubation of *C. carbonaria* aortic rings using LC–MS/MS and solid-phase extraction (SPE).

3.1 | Chromatographic analysis and sample preparation

The mass spectra for catecholamines and ISs are presented in Figures 1 and 2, respectively, and the ion transitions, as well their respective retention times, are presented in Table 1. The total run time was 4.0 min (Figure 3).

Figure 3 shows the chromatograms of blank Krebs–Henseleit solution and Krebs–Henseleit solution spiked with dopamine, noradrenaline, and adrenaline (0.1 ng/mL).

Catecholamines are unstable and readily oxidized. Plasma levels of adrenaline and noradrenaline were reduced to 64.8% and 85.0% of nominal concentration, respectively, at room temperature after 24 h without stabilizers. However, these catecholamines remained stable for at least 24 h when treated with sodium metabisulfite or citric acid and ascorbic acid solution (Zhang et al., 2012). In the present study, 3 mM ascorbic acid was added to Krebs–Henseleit solution to stabilize catecholamines (Van Oene, Sminia, Mulder, & Horn, 1983).

Catecholamines were extracted by SPE, a method commonly used for extracting catecholamines from diverse samples such as serum, urine, and plasma, which has shown acceptable results (Ji et al., 2010; Li et al., 2016; Petteys et al., 2012; Yu et al., 2019). Noradrenaline and adrenaline were quantified in human plasma by

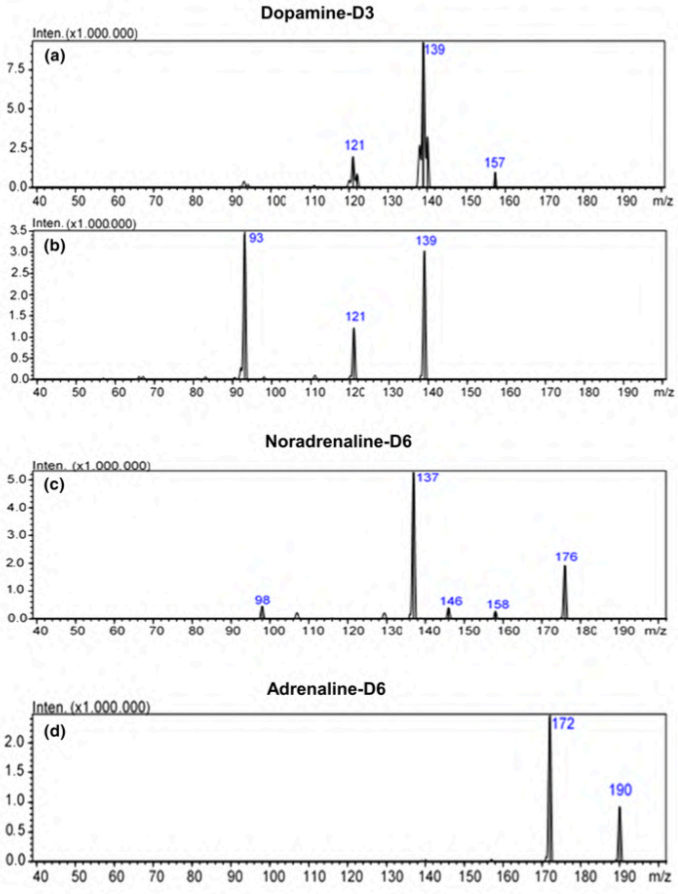


FIGURE 2 Product-ion spectra of the protonated molecular ion of dopamine-D3 with collision energy (CE) (a) 10 V and (b) 25 V; noradrenaline-D6 with (c) CE 5 V and adrenaline-D6 with CE (d) 5 V acquired in positive-mode electrospray ionization

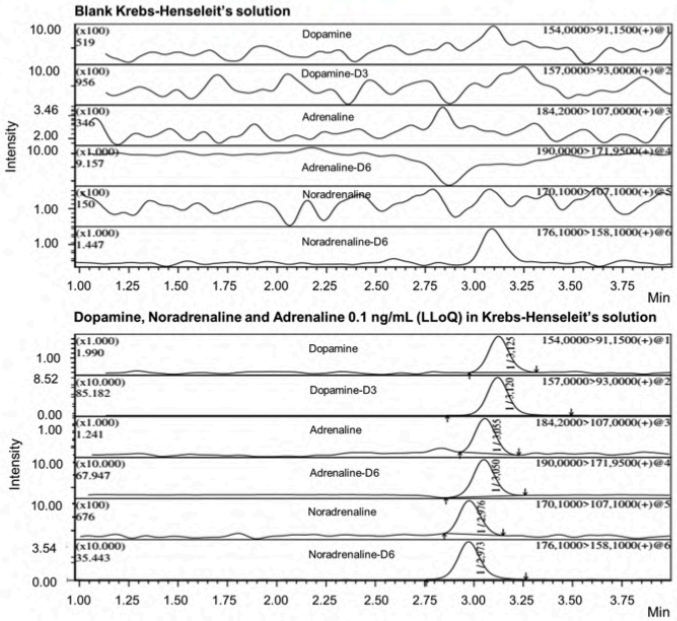


FIGURE 3 Chromatograms obtained in the analysis of dopamine, noradrenaline, and adrenaline. Blank Krebs–Henseleit solution and blank Krebs–Henseleit solution spiked with 0.1 ng/mL of every analyte and their respective internal standard. LLoQ, lower limit of quantification

LC–MS/MS in combination with alumina-based SPE and a derivatization procedure with LLoQ of 20.0 pg/mL for noradrenaline and 5.0 pg/mL for adrenaline (Zhang et al., 2012). Dopamine was measured in human neonate plasma by LC–MS/MS and strong cation exchange SPE, and subsequently derivatized with propionic anhydride. The LLoQ of dopamine was 10.0 pg/mL (Zhang et al., 2016). Dopamine, adrenaline, and noradrenaline, and their metabolites metanephrine, normetanephrine, and 3-methoxytyramine were determined in human plasma by isotope dilution LC–tandem mass spectrometry and SPE. The reported LLoQ was 5 pg/mL for dopamine, 10 pg/mL for noradrenaline, and 1 pg/mL for adrenaline, metanephrine, normetanephrine, and 3-methoxytyramine (Yu et al., 2019). Free catecholamines were measured in urine by LC–MS/MS and SPE, with LLoQ of 5.4, 7.4, and 3.8 ng/mL for dopamine, noradrenaline, and adrenaline, respectively (Woo et al., 2016). Catecholamines were also determined in rat and mini-pig plasma and urine using LC–MS/MS and a weak cation exchange SPE, presenting an LLoQ of 25 pg/mL for all three analytes in plasma and 250 pg/mL in urine (He et al., 2015).

There are no data in the literature reporting the measurement of dopamine, noradrenaline, and adrenaline from *C. carbonaria* aortic rings in Krebs–Henseleit solution. Catecholamines were previously extracted from aortae and mesenteric arteries using HCl and their concentrations were determined by radioimmunoassay (Sorriento et al., 2012).

3.2 | Validation

3.2.1 | Selectivity

The method demonstrated selectivity through the absence of endogenous interference in the quantification of catecholamines across the samples tested.

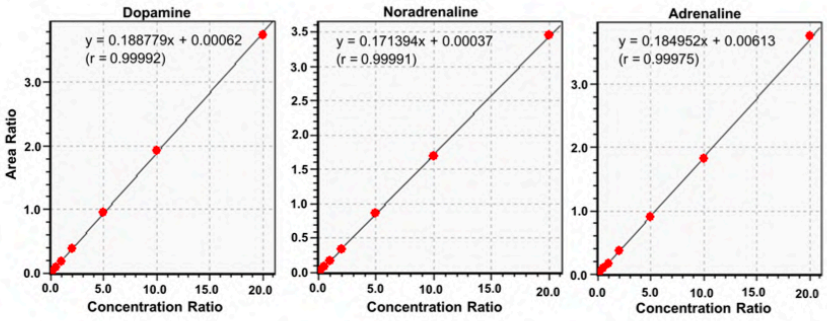


FIGURE 4 Calibration curve of dopamine, noradrenaline, and adrenaline

3.2.2 | Carryover

No carryover was observed (no peaks were observed in the analyte-free samples analyzed directly after analysis of the high-concentration sample). No significant interference was found in the retention time of the compounds analyzed.

3.2.3 | Linearity

The calibration curve of all catecholamines showed linearity in the concentration range of 0.1–20.0 ng/mL. The correlation coefficient was higher than 0.99 (Figure 4).

3.2.4 | Accuracy and precision

Within-batch and between-batch precision (% CV) and accuracy (% RE) were <10.1% and 95.9%–108.9%, respectively (Table 2), ensuring the reproducibility and repeatability of the results.

3.2.5 | Matrix effect and recovery

No significant matrix effect on ionization of catecholamines and IS was observed (Table 3).

TABLE 3 Matrix effect for dopamine, noradrenaline, adrenaline, and their internal standards (ISs) in Krebs–Henseleit solution

Catecholamine	FMN[a]	CV (%)[b]
Dopamine		
0.3 ng/mL	0.877	13.0
15.0 ng/mL	0.872	5.3
Noradrenaline		
0.3 ng/mL	0.855	1.2
15.0 ng/mL	0.878	4.3
Adrenaline		
0.3 ng/mL	0.901	11.9
15.0 ng/mL	0.840	5.8

[a]FMN = matrix factor normalized by internal standard [(response of the analyte in matrix/internal standard response matrix)/(response of the analyte in solution/response of the internal standard solution)].
[b]CV = coefficient of variation [(standard deviation FMN/mean FMN) × 100].

Dopamine recoveries in Krebs–Henseleit solution samples ranged from 74.97% to 97.10% with a mean of 87.67% and 87.16% in low and high QC concentrations, respectively. Recoveries of noradrenaline ranged from 83.75% to 91.24% with a mean of 85.55% and 87.84% in low and high QC concentrations, respectively. For adrenaline, recoveries ranged from 79.02% to 101.43% with a mean of 90.09% and 83.96% in low and high QC concentrations, respectively.

TABLE 2 Precision and accuracy data from dopamine, adrenaline, and noradrenaline in Krebs–Henseleit solution

Catecholamine	Sample		Within-assay				Between-assay			
		Nominal (ng/mL)	N	Mean (ng/mL)	Precision (% CV)[a]	Accuracy (%)[b]	N	Mean (ng/mL)	Precision (% CV)[a]	Accuracy (%)[b]
Dopamine	LLoQ	0.1	21	0.11	6.5	108.9	3	0.11	8.1	108.9
	QC1	0.3	21	0.32	4.3	106.8	3	0.3	4.1	106.8
	QC2	1.5	21	1.51	1.9	100.7	3	1.5	1.9	100.6
	QC3	9.0	21	9.00	1.9	100.0	3	9.0	2.2	100.0
	QC4	15.0	21	14.73	3.2	98.3	3	14.7	3.4	98.3
Noradrenaline	LLoQ	0.1	21	0.11	9.0	105.8	3	0.11	9.4	105.8
	QC1	0.3	21	0.31	4.9	103.3	3	0.3	5.7	103.4
	QC2	1.5	21	1.50	4.1	99.9	3	1.5	4.4	99.9
	QC3	9.0	21	8.9	2.3	99.0	3	8.9	2.4	99.0
	QC4	15.0	21	14.6	4.2	97.7	3	14.7	4.2	97.7
Adrenaline	LLoQ	0.1	21	0.11	9.8	106.3	3	0.11	10.1	106.3
	QC1	0.3	21	0.30	8.2	101.7	3	0.3	8.7	101.7
	QC2	1.5	21	1.44	4.9	96.0	3	1.4	6.4	96.0
	QC3	9.0	21	8.6	4.3	96.0	3	8.6	4.8	96.0
	QC4	15.0	21	14.4	5.4	95.9	3	14.4	5.6	95.9

[a]CV% = [(SD/M) × 100].
[b]Accuracy % = [(E – T)/T] × 100
CV, coefficient of variation; E, experimentally determined concentration; LLoQ, lower limit of quantification; M, mean; QC, quality control; SD, standard deviation of M; T, theoretical concentration.

3.2.6 | Stability

All catecholamines showed stability in Krebs–Henseleit solution after 60 h at room temperature, three freeze–thaw cycles, processing in the autoinjector for 7 days, and after long-term (31 days) storage at −20°C (Table 4), as shown by CV and accuracy lower than 10.9%.

Over the storage period of 31 days at −80°C, no degradation was observed for catecholamines and the IS stock solutions. The mean peak area of dopamine, adrenaline, and noradrenaline for the previously prepared stock solutions was 104.5%, 100.2%, and 95.2% of the peak area of the recently prepared stock solution, respectively. For dopamine-d3, adrenaline-d6, and noradrenaline-d6, the mean peak area for the previously prepared stock solutions was 102.7%, 97.5%,

TABLE 4 Summary stability data of dopamine, noradrenaline, and adrenaline in Krebs–Henseleit solution using LC–MS/MS

Catecholamine	Sample	Nominal (ng/mL)	N	Mean (ng/mL)	Precision (% CV)[a]	Accuracy (% RE)[b]
Freshly prepared						
Dopamine	QC1	0.3	3	0.32	2.7	106.7
	QC4	15.0	3	15.00	5.1	100.0
Noradrenaline	QC1	0.3	3	0.31	4.1	102.0
	QC4	15.0	3	14.60	2.5	97.3
Adrenaline	QC1	0.3	3	0.31	8.4	103.0
	QC4	15.0	3	14.40	4.4	96.0
	Short term (60.0 h at room temperature)					
Dopamine	QC1	0.3	3	0.32	2.9	106.3
	QC4	15.0	3	14.80	1.9	98.7
Noradrenaline	QC1	0.3	3	0.29	1.6	96.3
	QC4	15.0	3	14.8	4.1	98.7
Adrenaline	QC1	0.3	3	0.29	10.9	98.3
	QC4	15.0	3	14.20	3.5	94.7
	Post-processing (7 days at 6°C)					
Dopamine	QC1	0.3	3	0.32	2.9	106.3
	QC4	15.0	3	14.8	1.9	98.7
Noradrenaline	QC1	0.3	3	0.31	5.2	104.0
	QC4	15.0	3	15.30	3.0	102.0
Adrenaline	QC1	0.3	3	0.29	7.0	97.3
	QC4	15.0	3	15.0	2.8	100.0
	Freeze–thaw (3 × freeze–thaw cycles)					
Dopamine	QC1	0.3	3	0.31	3.2	105.0
	QC4	15.0	3	14.9	2.7	99.3
Noradrenaline	QC1	0.3	3	0.32	7.7	106.3
	QC4	15.0	3	14.90	4.3	99.3
Adrenaline	QC1	0.3	3	0.30	7.5	99.7
	QC4	15.0	3	14.90	1.0	99.3
	Long term (31 days at −20°C)					
Dopamine	QC1	0.3	3	0.30	2.9	100.0
	QC4	15.0	3	15.10	2.1	100.7
Noradrenaline	QC1	0.3	3	0.29	3.2	95.3
	QC4	15.0	3	15.30	1.6	102.0
Adrenaline	QC1	0.3	3	0.28	10.7	94.7
	QC4	15.0	3	14.7	3.3	98.0

[a]CV % = coefficient of variation [(SD/mean) × 100].
[b]Accuracy % = [(E/T) × 100].
CV, coefficient of variation; E, experimentally determined concentration; MS/MS, tandem mass spectrometry; QC, quality control; RE, relative error; SD, standard deviation; T, theoretical concentration.

TABLE 5 Concentration of dopamine, adrenaline, and noradrenaline in Krebs–Henseleit modification solution samples after incubation of *Chelonoidis carbonaria* aortic rings

Sample number	Dopamine (ng/mL)	Noradrenaline (ng/mL)	Adrenaline (ng/mL)
1	1.70	2.32	3.34
2	3.09	1.22	2.51
3	5.98	0.91	1.69
4	6.19	1.02	1.31
5	0.46	1.54	0.50
Mean	3.48	1.40	1.87
Standard deviation	2.55	0.57	1.09

and 93.5% of the peak area of the newly prepared sulindac stock solution, respectively.

3.3 | Method application

The developed and validated method was applied to the measurement of basal release of dopamine, adrenaline, and noradrenaline from *C. carbonaria* aortic rings *in vitro*. The mean (SD) concentrations of dopamine, noradrenaline, and adrenaline were 3.48 (2.55), 1.40 (0.57), and 1.87 (1.09) ng/mL, respectively (Table 5). Dopamine [mean (SD) 13.7 (11.4) pg/mg] was found in the endothelial cells isolated from the porcine pulmonary trunk. Noradrenaline and adrenaline concentrations were below LLoQ (Pfeil et al., 2014). In a previous study, endothelial cells also have demonstrated to be able to synthesize and release catecholamines in response to hypoxia when evaluated *in vitro* and to ischemia *in vivo* (Sorriento et al., 2012).

4 | CONCLUSION

The method developed and validated for quantification of dopamine by LC–MS/MS in Krebs–Henseleit solution was sensitive, precise, and sufficiently accurate for its application in the measurement of basal release of these catecholamines from *C. carbonaria* aortic rings *in vitro*. The mean (SD) concentrations of dopamine, noradrenaline, and adrenaline determined were 3.48 (2.55) ng/mL, 1.40 (0.57) ng/mL, and 1.87 (1.09) ng/mL, respectively.

ACKNOWLEDGEMENTS

This study was funded by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Grant n. 303839/2019-8 National Council for Scientific and Technological Development (CNPq) and Grant n. 2016/22506-1 São Paulo Research Foundation (FAPESP).

CONFLICT OF INTEREST

Authors have no conflict of interest to declare on the contents of the manuscript.

ORCID

Natalícia J. Antunes https://orcid.org/0000-0002-7820-552X
Gustavo Duarte Mendes https://orcid.org/0000-0001-8700-5709

REFERENCES

Angência Nacional de Vigilância Sanitária (ANVISA). (2011). Resolution - RE N° 899. *29th*, (May 2003) Retrieved April 22, 2020, from. http://www.anvisa.gov.br/legis/resol/2003/re/899_03re.htm

Britto-Júnior, J., Fernandes, F., Figueiredo, G. M., Campos, R., Agnaldo, R., Antunes, E., … Nucci, G. D. (2020). Electrical field stimulation induces endothelium-dependent contraction of human umbilical cord vessels. *Life Sciences*, *243*(November 2019), 117257. https://doi.org/10.1016/j.lfs.2020.117257

Campos, R., Mónica, F. Z., Justo, A. F. O., Cogo, J. C., Oliveira, E. D. T., Moreno, R. A., … De Nucci, G. (2018). Electrical field stimulation-induced contractions on Pantherophis guttatus corpora cavernosa and aortae. *PLoS ONE*, *13*(4), 1–9. https://doi.org/10.1371/journal.pone.0196123

Food and Drug Administration (FDA). (2018). Bioanalytical Method Validation Guidance. Retrieved April 22, 2020, from https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf

He, H., Carballo-Jane, E., Tong, X., & Cohen, L. H. (2015). Measurement of catecholamines in rat and mini-pig plasma and urine by liquid chromatography-tandem mass spectrometry coupled with solid phase extraction. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *997*, 154–161. https://doi.org/10.1016/j.jchromb.2015.05.014

Ji, C., Walton, J., Su, Y., & Tella, M. (2010). Simultaneous determination of plasma epinephrine and norepinephrine using an integrated strategy of a fully automated protein precipitation technique, reductive ethylation labeling and UPLC-MS/MS. *Analytica Chimica Acta*, *670*(1–2), 84–91. https://doi.org/10.1016/j.aca.2010.04.051

Li, X. S., Li, S., & Kellermann, G. (2016). An integrated liquid chromatography-tandem mass spectrometry approach for the ultra-sensitive determination of catecholamines in human peripheral blood mononuclear cells to assess neural-immune communication. *Journal of Chromatography a*, *1449*, 54–61. https://doi.org/10.1016/j.chroma.2016.04.039

Petteys, B. J., Graham, K. S., Parnás, M. L., Holt, C., & Frank, E. L. (2012). Performance characteristics of an LC-MS/MS method for the determination of plasma metanephrines. *Clinica Chimica Acta*, *413*(19–20), 1459–1465. https://doi.org/10.1016/j.cca.2012.06.006

Pfeil, U., Kuncova, J., Brüggmann, D., Paddenberg, R., Rafiq, A., Henrich, M., … Kummer, W. (2014). Intrinsic vascular dopamine - a key modulator of hypoxia-induced vasodilatation in splanchnic vessels. *Journal of Physiology*, *592*(8), 1745–1756. https://doi.org/10.1113/jphysiol.2013.262626

Saracino, M. A., Santarcangelo, L., Raggi, M. A., & Mercolini, L. (2015). Microextraction by packed sorbent (MEPS) to analyze catecholamines in innovative biological samples. *Journal of Pharmaceutical and Biomedical Analysis*, *104*, 122–129. https://doi.org/10.1016/j.jpba.2014.11.003

Sorriento, D., Santulli, G., Del Giudice, C., Anastasio, A., Trimarco, B., & Iaccarino, G. (2012). Endothelial cells are able to synthesize and release catecholamines both in vitro and in vivo. *Hypertension*, *60*(1), 129–136. https://doi.org/10.1161/HYPERTENSIONAHA.111.189605

Van Oene, J. C., Sminia, P., Mulder, A. H., & Horn, A. S. (1983). The purported dopamine agonist DPI inhibits [3H]noradrenaline release from rat cortical slices but not [3H]dopamine and [14C]acetylcholine release from rat striatal slices in-vitro. *Journal of Pharmacy and Pharmacology*, *35*(12), 786–792. https://doi.org/10.1111/j.2042-7158.1983.tb02897.x

Woo, H. I., Yang, J. S., Oh, H. J., Cho, Y. Y., Kim, J. H., Park, H. D., & Lee, S. Y. (2016). A simple and rapid analytical method based on solid-phase extraction and liquid chromatography-tandem mass spectrometry for the simultaneous determination of free catecholamines and metanephrines in urine and its application to routine clinical analysis. *Clinical Biochemistry*, *49*(7–8), 573–579. https://doi.org/10.1016/j.clinbiochem.2016.01.010

Xie, L., Chen, L., Gu, P., Wei, L., & Kang, X. (2018). A convenient method for extraction and analysis with high-pressure liquid chromatography of catecholamine neurotransmitters and their metabolites. *Journal of Visualized Experiments*, *2018*(133), 1–10. https://doi.org/10.3791/56445

Yu, S., Yin, Y., Li, Q., Yu, J., Liu, W., Wang, D., … Qiu, L. (2019). Validation of an improved liquid chromatography tandem mass spectrometry method for rapid and simultaneous analysis of plasma catecholamine and their metabolites. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *1129*(1), 121805. https://doi.org/10.1016/j.jchromb.2019.121805

Zhang, D., Wu, L., Chow, D. S. L., Tam, V. H., & Rios, D. R. (2016). Quantitative determination of dopamine in human plasma by a highly sensitive LC-MS/MS assay: Application in preterm neonates. *Journal of Pharmaceutical and Biomedical Analysis*, *117*, 227–231. https://doi.org/10.1016/j.jpba.2015.09.003

Zhang, G., Zhang, Y., Ji, C., McDonald, T., Walton, J., Groeber, E. A., … Lin, Z. (2012). Ultra sensitive measurement of endogenous epinephrine and norepinephrine in human plasma by semi-automated SPE-LC-MS/MS. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *895–896*, 186–190. https://doi.org/10.1016/j.jchromb.2012.03.026

Zheng, J., Mandal, R., & Wishart, D. S. (2018). A sensitive, high-throughput LC-MS/MS method for measuring catecholamines in low volume serum. *Analytica Chimica Acta*, *1037*, 159–167. https://doi.org/10.1016/j.aca.2018.01.021

How to cite this article: Britto-Júnior J, Antunes NJ, Campos R, et al. Determination of dopamine, noradrenaline, and adrenaline in Krebs–Henseleit solution by liquid chromatography coupled with tandem mass spectrometry and measurement of their basal release from *Chelonoidis carbonaria* aortae *in vitro*. *Biomedical Chromatography*. 2020;e4978. https://doi.org/10.1002/bmc.4978

Anexo 3

Artigo 5 – The basal release of endothelium-derived catecholamines regulates the contractions of *Chelonoidis carbonaria* aorta caused by electrical-field stimulation

Autores: Britto-Júnior J, Fernandes Jacintho F, Campos R, Pinheiro DHA, Figueiredo Murari GM, de Souza VB, Schenka AA, Mónica FZ, Moreno RA, Antunes E, De Nucci G.

Revista: Biology Open

Situação: Aceito a publicação em 17 de novembro de 2020. Publicado on-line em 20 de janeiro de 2021

© 2021. Published by The Company of Biologists Ltd | Biology Open (2021) 10, bio057042. doi:10.1242/bio.057042

RESEARCH ARTICLE

The basal release of endothelium-derived catecholamines regulates the contractions of *Chelonoidis carbonaria* aorta caused by electrical-field stimulation

José Britto-Júnior[1,*], Felipe Fernandes Jacintho[1], Rafael Campos[2], David Halen Araújo Pinheiro[1], Guilherme M. Figueiredo Murari[1], Valéria B. de Souza[1], André A. Schenka[1], Fabíola Z. Mónica[1], Ronilson Agnaldo Moreno[1], Edson Antunes[1] and Gilberto De Nucci[1,3]

ABSTRACT

The contractions of *Chelonoidis carbonaria* aortic rings induced by electrical field stimulation (EFS) are not inhibited by blockade of the voltage-gated sodium channels by tetrodotoxin but almost abolished by the α1/α2-adrenoceptor antagonist phentolamine. The objective of this study was to identify the mediator(s) responsible for the EFS-induced contractions of *Chelonoidis carbonaria* aortic rings. Each ring was suspended between two wire hooks and mounted in isolated 10 ml organ chambers filled with oxygenated and heated Krebs-Henseleit's solution. Dopamine, noradrenaline and adrenaline concentrations were analysed by liquid chromatography coupled to tandem mass spectrometry. The contractions caused by dopamine and EFS were done in absence and presence of the nitric oxide (NO) synthesis inhibitor L-NAME, the NO-sensitive guanylyl cyclase inhibitor ODQ, the D1-like receptor antagonist SCH-23390, the D2-like receptor antagonists risperidone, quetiapine, haloperidol, and the tyrosine hydroxylase inhibitors salsolinol and 3-iodo-L-tyrosine. Basal concentrations of dopamine, noradrenaline and adrenaline were detected in Krebs-Henseleit solution containing the aortic rings. The catecholamine concentrations were significantly reduced in endothelium-denuded aortic rings. L-NAME and ODQ significantly potentiated the dopamine-induced contractions. The D2-like receptor antagonists inhibited the EFS-induced contractions of the aortic rings treated with L-NAME, whereas SCH 23390 had no effect. Similar results were observed in the contractions induced by dopamine in L-NAME treated aortic rings. These results indicate that catecholamines released by endothelium regulate the EFS-induced contractions. This may constitute a suitable mechanism by which reptilia modulate specific organ blood flow distribution.

[1]Faculty of Medical Sciences, Department of Pharmacology, University of Campinas (UNICAMP), Campinas 13083-894, Brazil. [2]Department of Physiology, Superior Institute of Biomedical Sciences, Ceará State University (UECE), Fortaleza 60714-903, Brazil. [3]Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo 05508-060, Brazil.

*Author for correspondence (josebrittojr@gmail.com)

J.B-J., 0000-0003-0250-8468; F.F.J., 0000-0003-1379-6450; R.C., 0000-0002-9816-2061; D.H.A.P., 0000-0002-8661-9159; G.M.F.M., 0000-0002-1890-0723; V.B.d.S., 0000-0002-6462-5718; A.A.S., 0000-0002-8162-8996; F.Z.M., 0000-0002-8449-6677; R.A.M., 0000-0001-6692-1011; E.A., 0000-0003-2201-8247; G.D.N., 0000-0002-4346-7941

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

Received 30 September 2020; Accepted 17 November 2020

This paper has an associated First Person interview with the first author of the article.

KEY WORDS: LC-MS-MS, Tortoise, Vessel, ODQ, L-NAME, Tyrosine hydroxylase

INTRODUCTION

It is well established that endothelial cells modulate vascular reactivity through the release of mediators such as prostacyclin (Moncada et al., 1976), nitric oxide (Furchgott and Zawadzki, 1980) and endothelin (Yanagisawa et al., 1988). Catecholamines modulate vascular tonus through the actions on α- and β-adrenoceptors (Ahlquist, 1948); however, the production and release of catecholamines are associated with the existence of nerve terminals on vessels (Kadowitz et al., 1976; Matsuyama et al., 1985).

Electrical-field stimulation (EFS) is a technique in which an electrical stimulus is applied uniformly to an isolated tissue in short pulse widthwaves (Paterson, 1965; Bevan, 1962). EFS is commonly used in protocols evaluating adrenergic (Campos et al., 2019a,b; Dail et al., 1987), cholinergic (De Oliveira et al., 2019) and non-adrenergic non-cholinergic events (Ignarro et al., 1990; De Oliveira et al., 2003). Tetrodotoxin is considered an inhibitor of nerve terminal stimulation, since it blocks voltage-sensitive sodium channels (Narahashi et al., 1964).

Electrical-field stimulation causes aortic contractions of the tortoise *Chelonoidis carbonaria*, but these responses are not inhibited by tetrodotoxin, indicating they are not due to nerve terminal stimulation (Campos et al., 2020). Interestingly, these EFS-induced aortic contractions are reduced by either the α-adrenoceptor antagonist phentolamine or by endothelium removal (Campos et al., 2020), suggesting a potential modulatory role for endothelium-derived catecholamines. Similar observations have been reported for EFS-induced aortic contractions of the snakes *Crotalus durissus terrificus*, *Bothrops jararaca* (Campos et al., 2018a) and *Panterophis guttatus* (Campos et al., 2018b), as well as of the human umbilical cord vessels (Britto-Júnior et al., 2020a). Since immunohistochemistry failed to identify nerve terminals in *Chelonoidis carbonaria* aortae (Campos et al., 2020), the results indicate a non-neuronal source of catecholamine synthesis. Interestingly, the enzyme tyrosine hydroxylase, responsible for catalyzing the conversion of L-tyrosine to L-DOPA, was identified only in the endothelial cells from *Chelonoidis carbonaria* aorta (Campos et al., 2020) and from both human umbilical artery and human umbilical vein (Britto-Junior et al., 2020b). The inhibition by phentolamine of EFS-induced contractions in both tortoise (Campos et al., 2020) and umbilical cord vessels (Britto-Júnior

Biology Open (2021) 10, bio057042. doi:10.1242/bio.057042

et al., 2020a) was observed only at high concentrations of this adrenoceptor antagonist, suggesting that it may be acting on a different population of receptors. In addition, a basal endothelium-derived dopamine release was identified by tandem mass spectrometry in human umbilical cord vessels and use of the dopamine D2-like receptor antagonist haloperidol reduced the EFS-induced contraction in human umbilical cord artery and vein (Britto-Junior et al., 2020b).

In this manuscript, the nature of the mediators released by endothelial cells of aortic rings of *Chelonoidis carbonaria* was identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS), followed by a pharmacological characterization of the EFS-induced contractions in *Chelonoidis carbonaria* aortic rings *in vitro*.

RESULTS

Determination of catecholamine concentrations by LC-MS-MS

Dopamine, noradrenaline and adrenaline calibration curves were linear for concentrations of 0.1-10.0 ng/ml, with a correlation coefficient greater than 0.99. The lower limit of quantification was 0.1 ng/ml. Dopamine, noradrenaline and adrenaline concentrations were above the limit of quantification in the Krebs-Henseleit solution of all six of the aortic rings with endothelium intact. The basal releases of catecholamines were significantly reduced in endothelium-denuded aortic rings (n=6/6; Fig. 1).

Effect of L-NAME and ODQ in aortic rings

Dopamine caused concentration-dependent contractions of endothelium-intact aortic rings (E_{max} 13.2±1.6 mN; pEC_{50} 4.0±0.1, n=4/5; Fig. 2A). Incubation with L-NAME (100 µM) caused a significant leftward shift of the concentration-response curves to dopamine (pEC_{50} 5.1±0.2, P<0.05) accompanied by an increase in E_{max} value (16.1±1.6 mN, n=5/6; Fig. 2A, P<0.05). Likewise, incubation of the preparations with ODQ (100 µM) caused a significant (P<0.05) leftward shift (pEC_{50} 5.1±0.1) and a significant increase of the E_{max} value (14.6±1.4 mN, n=5/5; Fig. 2A).

Evaluation of dopamine receptors in aortic rings

In L-NAME (100 µM)-treated aortic rings, the dopamine D1-like receptor antagonist SCH-23390 caused no significant shifts in the dopamine-induced aortic contractions (pEC_{50} 4.9±0.2, 4.6±0.1 and 4.7±0.1 for 0.3, 1 and 3 µM, respectively, n=4/5) compared with L-NAME alone (pEC50 5.1±0.2; Fig. 2B).

The dopamine D2-like receptor antagonist risperidone caused significant concentration-dependent rightward shifts of the concentration-dependent dopamine contractions in L-NAME-treated aortic rings (pEC_{50} 4.1±0.1, 3.6±0.1 and 3.1±0.3 for 0.3, 1 and 3 µM, respectively; n=4/5) compared with L-NAME alone (pEC_{50} 5.1±0.2, n=5/6; Fig. 2C). The E_{max} values were not significantly changed by risperidone (Fig. 2C).

The dopamine D2-like receptor antagonists quetiapine (0.3, 1 and 3 µM) and haloperidol (1 and 3 µM) also caused significant rightward shifts of the concentration-dependent dopamine contractions in L-NAME-treated aortic rings without affecting the E_{max} values (Table 1; Fig. 2D,E, respectively). In L-NAME-treated aortic rings, the dopamine D1-like receptor antagonist SCH-23390 (1 µM, n=4/6) had no significant effect on the EFS-induced contractions of aortic rings (2.4±0.7 and 2.5±0.7 mN at 16 Hz, for control and SCH-23390, respectively; Figs 3A and 4A).

The dopamine D2-like receptor antagonist risperidone (1 µM, n=4/6) significantly (P<0.05) reduced the EFS (16 Hz)-induced

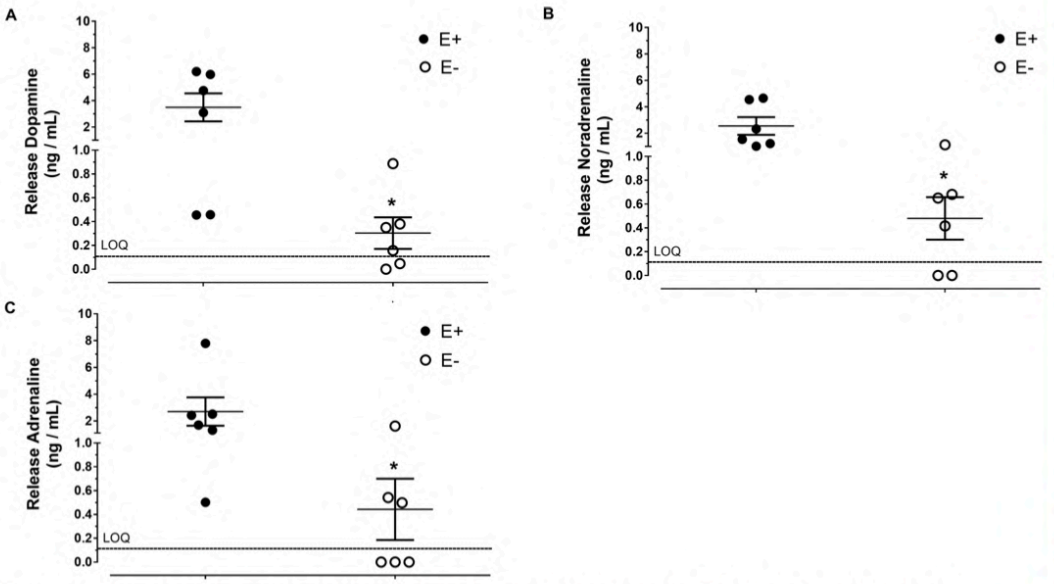


Fig. 1. Basal release of catecholamines in endothelium-intact aortic rings of *Chelonoidis carbonaria*. The basal release of dopamine (A), noradrenaline (B) and adrenaline (C) in Krebs-Henseleit solution after 30 min incubation with endothelium-intact (E+; n=6/6) and endothelium-denuded aortic rings (E- n=6/6). P<0.05 compared with E- preparations.

Biology Open (2021) 10, bio057042. doi:10.1242/bio.057042

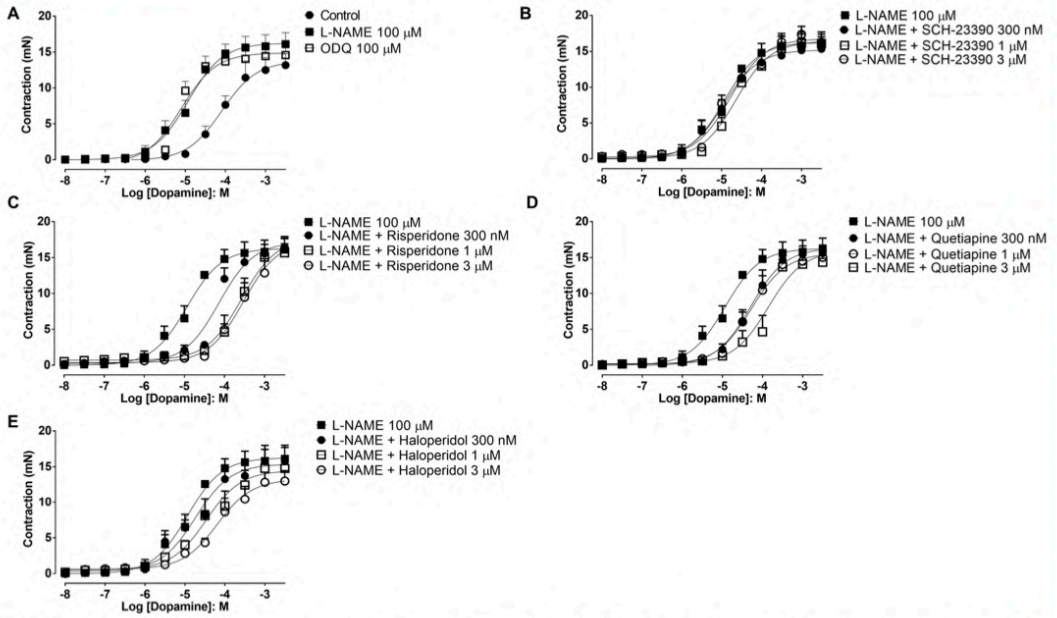


Fig. 2. Concentration-response curves to dopamine in *Chelonoidis carbonaria* aortic rings. Cumulative concentration-response curves to dopamine in *Chelonoidis carbonaria* aortic rings was performed in presence and absence of L-NAME (100 µM; *n*=5/6) and ODQ (100 µM; *n*=5/5; panel (A). Concentration-response curves to dopamine in L-NAME-pretreated preparations (100 µM; *n*=5/6) were also performed in presence and absence of the D1-like receptors antagonist SCH 23390 (0.3, 1 and 3 µM; *n*=4/5; B) and the D2-like receptors antagonists risperidone (0.3, 1 and 3 µM; *n*=4/5; C), quetiapine (0.3, 1 and 3 µM; *n*=4/5; D) and haloperidol (0.3, 1 and 3 µM; *n*=4/5; E). Data are expressed as mean±s.e.m.

contractions in L-NAME-treated aortic rings (3.1±0.8 and 1.6±0.4 mN for control and risperidone, respectively; Fig. 3B). Quetiapine (1 µM, *n*=4/5) also significantly reduced ($P<0.05$) the EFS-induced contractions (2.8±0.3 and 1.2±0.2 mN for control and quetiapine, respectively; Fig. 3C). Similar reductions were observed with haloperidol at both 1 µM (3.1±0.4 and 1.8±0.3 mN for control and test, respectively; Figs 3D and 4B) and 3 µM (2.5±0.5 and 1.0±0.2 for control and test, respectively, *n*=5/7; Fig. 3E).

Table 1. The potency (pEC_{50}) and maximum response (E_{max}) of the dopamine D1-like receptor antagonists SCH-23390 and of the dopamine D2-like receptor antagonists risperidone, quetiapine and haloperidol in L-NAME-treated aortic rings

Antagonist	pEC_{50}	E_{max}	*n*
L-NAME (100 µM)	5.1±0.2	16.1±1.6	5/6
L-NAME+SCH-23390 (300 nM)	4.9±0.2	15.2±0.6	4/5
L-NAME+SCH-23390 (1 µM)	4.6±0.1	16.4±0.7	4/5
L-NAME+SCH-23390 (3 µM)	4.7±0.1	16.8±0.5	4/5
L-NAME+risperidone (300 nM)	4.1±0.1**	16.4±1.2	4/5
L-NAME+risperidone (1 µM)	3.6±0.1*	15.7±1.3	4/5
L-NAME+risperidone (3 µM)	3.1±0.3*	16.2±1.7	4/5
L-NAME+quetiapine (300 nM)	4.3±0.2*	15.6±1.1	4/5
L-NAME+quetiapine (1 µM)	4.2±0.1*	15.0±1.2	4/5
L-NAME+quetiapine (3 µM)	3.9±0.2*	14.3±1.4	4/5
L-NAME+haloperidol (300 nM)	4.8±0.3	15.8±2.2	4/5
L-NAME+haloperidol (1 µM)	4.4±0.3*	14.8±1.3	4/5
L-NAME+haloperidol (3 µM)	4.1±0.3*	13.0±1.5	4/5

Data are expressed as mean±s.e.m. *$P<0.05$ versus control.

Effect of L-NAME and risperidone on basal tonus of aortic rings

In L-NAME (100 µM)-treated aortic rings, the basal tonus was increased in 10/18 out of 10/32 aortic rings. The elevated basal tonus induced by L-NAME (5.5±1.6 mN) was promptly reversed by risperidone (1 µM; reduction to 3.6±1.0 mN) in all aortic rings tested (*n*=5/5; Fig. 5).

Effect of tyrosine hydroxylase inhibition with salsolinol and 3-Iodo-L-tyrosine

The EFS (16 Hz)-induced aortic contractions in L-NAME (100 µM)-treated preparations were significantly reduced ($P<0.05$) by incubation with the tyrosine hydroxylase inhibitor salsolinol (100 µM; 3.1±0.4 and 1.0±0.2 mN for control and salsolinol, respectively; Fig. 6A,B; *n*=4/6). On the other hand, salsolinol (100 µM, *n*=4/5) had no effect on the dopamine-induced contractions of L-NAME-treated aortic rings (E_{max} 16.1±1.6 and 16.9±1.2 mN and pEC_{50} 5.1±0.2 and 4.7±0.1 for L-NAME alone and salsolinol, respectively; Fig. 6C).

The EFS (16 Hz)-induced contractions of L-NAME-treated aortic rings were also significantly reduced ($P<0.05$) by incubation with the tyrosine hydroxylase inhibitor 3-Iodo-L-tyrosine (1 mM; Fig. 7A,B; *n*=3/5). The 3-iodo-L-tyrosine had no effect on the dopamine-induced contractions of L-NAME-treated aortic rings (E_{max} 16.5±0.8 and 15.8±1.0 mN; pEC_{50} 4.6±0.1 and 4.9±0.1 for 0.1 and 1 mM of iodo-L-tyrosine, respectively; *n*=5/5) compared with L-NAME alone (E_{max} 16.1±1.6 mN, pEC_{50} 5.1±0.2, *n*=5/6; Fig. 7C).

Biology Open (2021) 10, bio057042. doi:10.1242/bio.057042

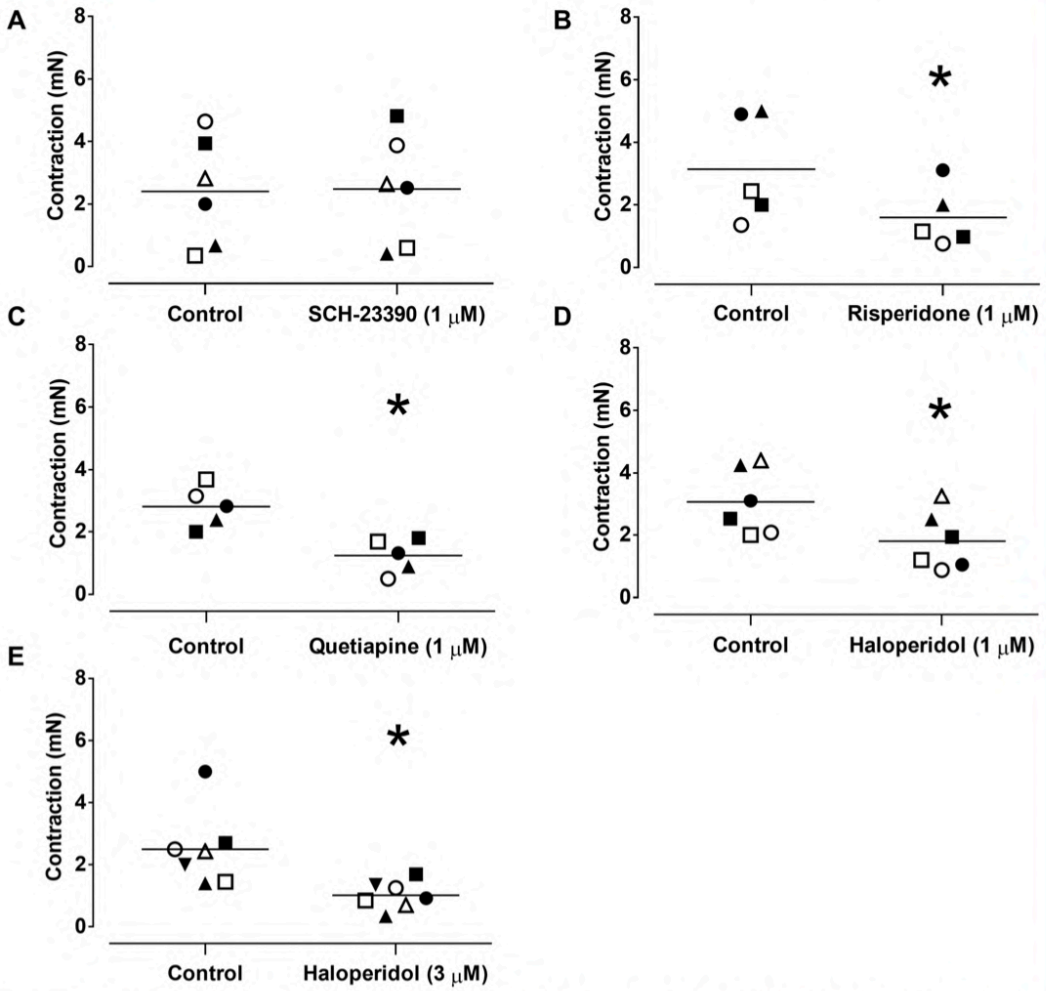


Fig. 3. Effects of D1-like and D2-like receptor antagonists on EFS-induced contractions of aortic rings of *Chelonoidis carbonaria*. Scatter plots show the individual values of the effects of the D1-like receptor antagonist SCH-23390 (*n*=4/6; 1 μM; A) and the D2-like receptor antagonists risperidone (1 μM; *n*=4/5; B), quetiapine (1 μM; *n*=4/5; C) and haloperidol (1 μM; *n*=4/6; D and 3 μM; *n* = 5/7; E) on EFS (16 Hz)-induced contractions of aortic rings pretreated with L-NAME (100 μM). **P*<0.05. Each individual symbol represents a ring before and after treatment.

Immunohistochemistry

Fig. 8A and B show that there was an absence of Chromogranin A staining (a biomarker for chromaffin cells) in all sections of Chelonoidis aortae that were tested. Positive controls demonstrated the presence of Chromogranin A staining in neuroendocrine tumor and normal chromaffin cells from the colon (Fig. 8C,D).

DISCUSSION

The results presented here clearly demonstrate, for the first time in the tortoise, that *Chelonoidis carbonaria* aortae have a basal release of dopamine, noradrenaline and adrenaline, as identified by tandem mass spectrometry, and the amount released is significantly reduced by endothelium-removal. Basal release of endothelium-derived catecholamines also occur in human umbilical vessels (Britto-Júnior et al., 2020b).

The contractions induced by EFS in the aortic rings were only inhibited by the non-selective α-adrenergic blocker phentolamine at high concentrations. The finding that the α1 antagonist prazosin (Agrawal et al., 1984) and the α2 antagonist idazoxan (Doxey et al., 1984) had no effect on the contractions of *Chelonoidis carbonaria* aortic rings induced by EFS indicated that the inhibition by phentolamine is unlikely to be due to its action on α-adrenoceptors (Campos et al., 2020). Phentolamine also acts as an antagonist of dopaminergic receptors, since it displaces ^{3}H-haloperidol binding at

Biology Open (2021) 10, bio057042. doi:10.1242/bio.057042

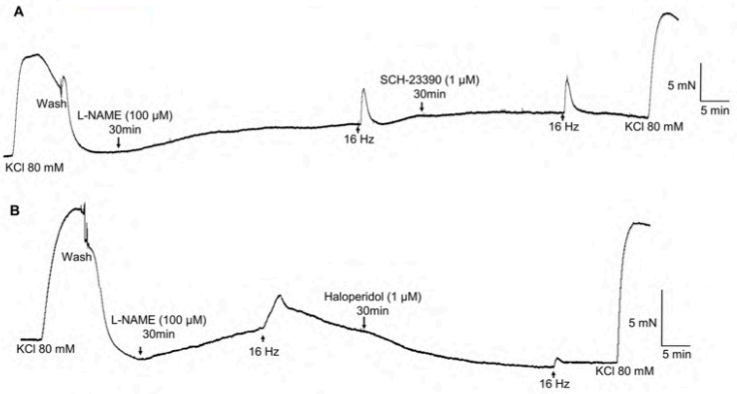


Fig. 4. Representative tracing of the effect of the D1-like receptor antagonist SCH 23390 (1 µM; *n*= 4/6) and the D2-like receptor antagonist haloperidol (1 µM; *n*=4/6) on EFS (16 Hz)-induced contractions of aortic rings of *Chelonoidis carbonaria* pretreated with L-NAME (100 µM).

concentrations above 2 µM in calf brain membranes (Burt et al., 1976). In our study, the contractions induced by EFS were inhibited by the D_2-like receptor antagonists risperidone, quetiapine and haloperidol, but not affected by the D1-like receptor antagonist SCH-23390 (Billard et al., 1984). Dopaminergic receptors in vascular beds have been identified *in vitro* by radioligand-receptor binding and autoradiographic techniques. The localization of dopamine-1 (D_1) (Amenta and Ricci, 1990) and dopamine-2 (D_2) receptors have been assessed in smooth muscle tissue of rat cerebral, mesenteric and renal arteries (Amenta et al., 1990). The contraction of *Chelonoidis carbonaria* aortic rings induced by dopamine was blocked by D_2-like antagonists, indicating the presence of D_2-like receptors. Furthermore, the EFS-induced contractions were also blocked by D2-like receptor antagonists, indicating that release of dopamine plays a major role on this phenomenon. The contractions induced by EFS in human umbilical artery and vein are also blocked by D_2-like receptor antagonists, but not affected by the D_1-receptor antagonist SCH-23390 (Britto-Junior et al., 2020b). The inhibition of EFS-induced contractions by the D_2-like receptor antagonist haloperidol reveals an important modulatory role of the endothelium-derived dopamine, acting as a vasoconstrictor through the D2-like receptors. It is interesting that both domperidone and haloperidol applied as ophthalmic solutions in a rabbit ocular hypertensive model produced a marked increase of ocular blood flow (Chiou and Chen, 1992). It is important to mention that although endothelial cells are not considered excitable cells, they do express voltage-gated potassium channels (Félétou, 2011). Adams and Hill (2004) report that in endothelial cells (including in human capillaries), a fast-activating transient outward potassium current has been observed similar to that of vascular smooth muscle cells showing the characteristics of A-type potassium currents. Our results indicate that endothelial cells present a basal release of catecholamines but whether EFS induces further release of these mediators, remains to be further investigated and the data presented here only provides evidence that endothelial catecholamines modulate EFS-induced contractions. Although the heart output is defined as the product of heart rate and stroke volume, the pumping function of the heart has been considered to have a minor role in the determination of cardiac output (Guyton, 1981). The systemic outflow is primarily controlled by a balance of arterial vasodilatation (regulation of systemic conductance) and venous constriction (regulation of vascular capacitance; Joyce and Wang, 2020). Indeed, the heart output was largely unaffected by increase in the heart rate of electrically paced subjects (Ross et al., 1965). Patients who where subjected to heart transplantation present extrinsic heart denervation caused by axonal Wallerian degeneration due to surgical interruption of the parasympathetic vagal neurons and the intrinsic post-ganglionic sympathetic nerve fibers traveling from the stellate ganglia to the myocardium (Awad et al., 2016). Afferent and efferent denervation of the transplanted organs in heart-lung transplanted patients is caused by the interruption of the central connections from the low-pressure receptors in the atria and pulmonary veins (Jamieson et al., 1984). In a study comparing eight healthy heart-lung transplant recipients with eight normal subjects matched for age and sex revealed that the transplant group had significantly higher heart frequency and diastolic blood pressure (Banner et al., 1990). Interestingly, the increase of both heart frequency and diastolic pressure during head-up tilt were similar in the two groups. Baseline levels of noradrenaline and adrenaline were also similar in the two groups; however, during head-up tilt, plasma noradrenaline levels increased to a significantly greater extent in the transplant group as compared

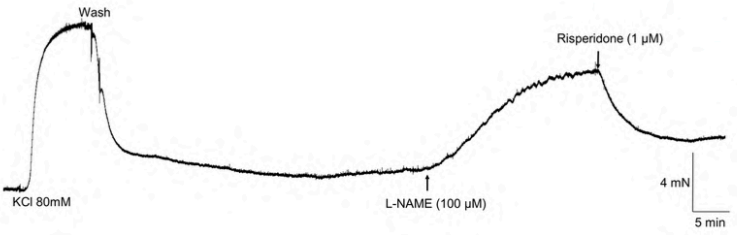


Fig. 5. Representative tracing showing the reversal by risperidone (1 µM; *n*=5/5 of the elevated tonus induced by L-NAME (100 µM) in aortic rings of *Chelonoidis carbonaria*.

Biology Open (2021) 10, bio057042. doi:10.1242/bio.057042

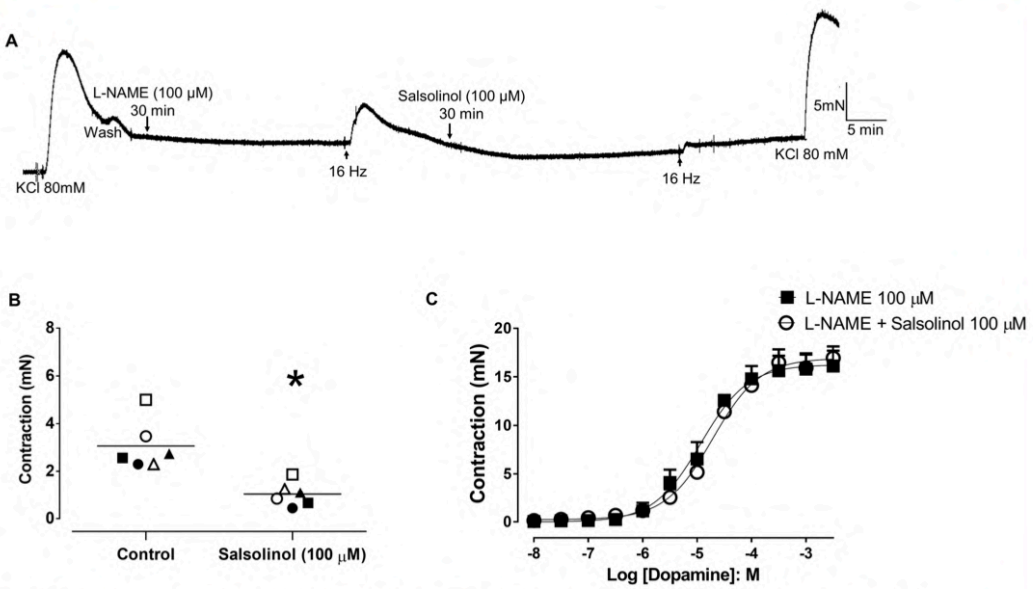


Fig. 6. Effect of the tyrosine hydroxylase inhibitor salsolinol on EFS-induced contraction of aortic rings of *Chelonoidis carbonaria*.
(A) Representative tracing displaying the inhibitory effect of salsolinol (100 µM) on EFS (16 Hz)-induced contraction of aortic rings pretreated with L-NAME (100 µM; *n*=4/6). (B) Scatter plots of individual values and mean values ±s.e.m. of the EFS-induced contractions of L-NAME (100 µM)-treated preparations in the presence and the absence of salsolinol (100 µM; *n*=4/6). (C) Cumulative concentration-response curves to dopamine in aortic rings pretreated with L-NAME (100 µM; *n*=5/6) in the presence and absence of the salsolinol (100 µM; *n*=5/5). **P*<0.05 compared with control. Each individual symbol represents a ring before and after treatment.

to the control group. It is clear from the above that the catecholamines are doing their job, and that they are not coming from the denervated adrenergic branches in the heart.

What is the possible physiological role(s) of endothelium-derived catecholamines in reptilia? Acute anoxic exposure of the turtle heart *Chrysemys scripta in situ* is accompanied by a weak negative chronotropic effect at both 5°C and 15°C (Farrell et al., 1994). An elevation of plasma catecholamine levels has been also associated to anoxia (Wasser and Jackson, 1991). The remarkable cardiovascular down-regulation that accompanies long periods of cold anoxia is characterized by a substantial increase in the systemic peripheral resistance, probably reflecting a prioritization of regional blood flow distribution (Hicks and Farrell, 2000). Indeed, α-adrenergic vasoactivity does contribute to blood flow regulation to the liver and shell during anoxic submergence at 5°C in the turtle *Trachemys scripta* (Stecyk et al., 2004). The differential release of catecholamines may be a suitable mechanism by which reptilia have specific organ blood flow distribution.

The basal release of dopamine, noradrenaline and adrenaline by *Chelonoidis carbonaria* aorta endothelial cells modulates EFS-induced contractions and endothelium-derived catecholamines acting on D2-like receptors may constitute a suitable mechanism for local control of blood flow in reptilia. It is known that large arteries, although capable of constricting and dilating, serve virtually no role in the regulation of pressure and blood flow under normal physiological conditions (Goodwill et al., 2017). However, what is being proposed is that endothelium-derived catecholamines will do that; endothelium-derived catecholamines should occur in all vessels, including the microcirculation. It is interesting that D2-receptors have been identified in rabbit pulmonary capillary microcirculation (Bruzzone et al., 2002).

Another possible source of extra-neuronal catecholamines is chromaffin cells. Chromaffin cells (neuroendocrine cells) grouped together make up paraganglia and are linked to both the visceral nervous system and the digestive tract. They can be distinguished into two categories: adrenal (i.e. the adrenal medulla) and extra-adrenal (Knottenbelt et al., 2015; La Perle and Dintzis, 2018). Interestingly, other non-mammal vertebrates have been shown to possess these cells associated with the autonomic system alongside the presence being in cardiac and vascular tissues, including the intercostal arteries and the azygous vein (Scheuermann, 1993; Nilsson, 2010). Nilsson, in particular, reported histological and histochemical evidence of chromaffin cells in lungfish heart and vascular walls (Nilsson, 2010). Until now, Chromogranin A and synaptophysin are considered reliable immunohistochemical markers for neuroendocrine/chromaffin differentiation (Kyriakopoulos et al., 2018). Despite positive controls undoubtedly showing the presence of chromogranin A, no chromogranin A staining was observed in any of the aortic ring tissues tested, indicating that these cells are not present in *Chelonoidis carbonaria* aortae, and thus cannot be responsible for the catecholamine release detected in this study.

MATERIALS AND METHODS

Animals

The experimental protocol using *Chelonoidis carbonaria* of either sex (weight varied from 2 to 7 kg) were authorized by the Institutional Animal

RESEARCH ARTICLE Biology Open (2021) 10, bio057042. doi:10.1242/bio.057042

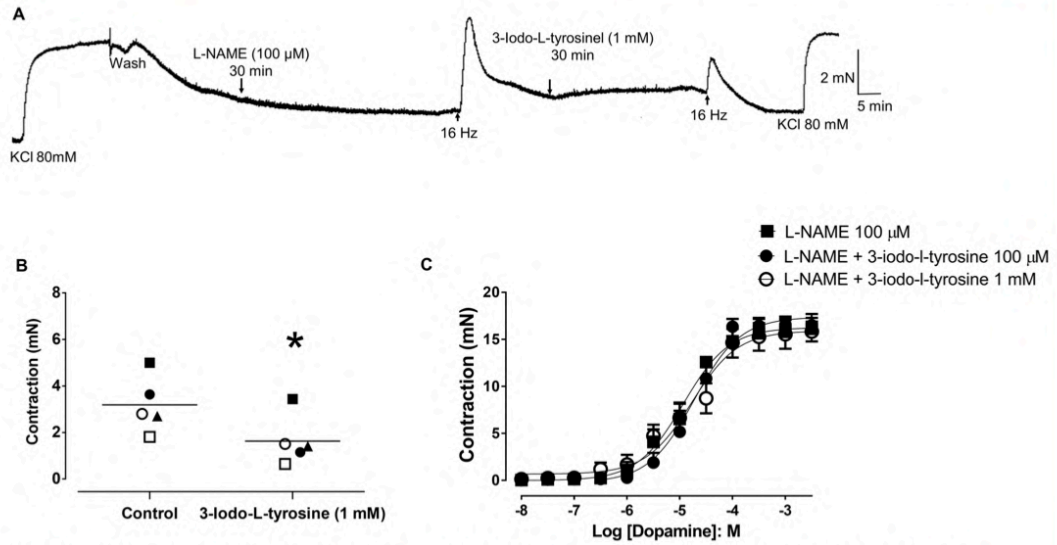


Fig. 7. Effect of the tyrosine hydroxylase inhibitor 3-iodo-tyrosine on EFS-induced contraction of aortic rings of *Chelonoidis carbonaria*.
(A) Representative tracing of the inhibitory effect of 3-iodo-L-tyrosine (1 mM) on EFS (16 Hz)-induced contraction of aortic rings pretreated with L-NAME (100 µM; *n*=3/5). (B) shows scatter plots of individual values and mean values ±s.e.m. of the EFS-induced contraction in aortic rings pretreated with L-NAME (100 µM) in the presence and the absence of 3-iodo-L-tyrosine (1 mM; *n*=3/5). (C) Cumulative concentration-response curve to dopamine in aortic rings pretreated with L-NAME (100 µM; *n*=5/6) in the presence and absence of the 3-iodo-L-tyrosine (0.1 and 1 mM; *n*=5/5 for each curve). **P* <0.05 compared with control. Each individual symbol represents a ring before and after treatment.

Care and Use Committee (CEUA/UNICAMP: 3907-1, respectively) in compliance with the ARRIVE guidelines. The use of *Chelonoidis carbonaria* was approved by the Brazilian Institute for Environment (Sisbio; number 20910), and the tortoises were supplied by the Tietê Ecological Park (São Paulo, SP, Brazil).

Chemical and reagents

Adrenaline, acetylcholine, noradrenaline, dopamine, adenosine 5′-triphosphate (ATP), N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 3-iodo-tyrosine, salsolinol and SCH-23390 were purchased from Sigma-Aldrich Chemicals Co. (St Louis, MO, USA). Risperidone, quetiapine and haloperidol were acquired from Nallin Farmácia e Manipulação Ltda (Itatiba-SP, Brazil). Dopamine-d3 hydrochloride, DL-noradrenaline-d6 hydrochloride and adrenaline-d6 hydrochloride were acquired from CDN Isotopes (Point Claire, Canada). Aluminium oxide was purchased from Dinamica Quimica Contemporanea Ltda (Indaiatuba-SP, Brazil). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$), potassium phosphate monobasic (KH_2PO_4), and glucose were bought from Merck KGaA (Darmstadt, Germany). Acetonitrile was obtained from J.T Baker (Phillipsburg, NJ, USA) and formic acid (HPLC grade) was purchased from Mallinckrodt (St. Louis, MO, USA).

Aortic ring preparations and isometric tension recordings

The tortoises were anesthetized with ketamine and propofol (40 mg/kg IM and 15 mg/kg IV, respectively) after sedation with midazolam (2 mg/kg; IM). The animals were euthanized by exsanguination. A segment of dorsal aorta was removed and immediately placed in oxygenated ($95\%O_2/5\%CO_2$) Krebs-Henseleit solution at 27°C. Subsequently, aortic rings (3 mm) were suspended vertically between two metal hooks in 10 ml organ baths containing Krebs-Henseleit solution (mM): NaCl (118), KCl (4.7), CaCl2 (2.5), MgSO4 (1.2), NaCO3 (25), KH2PO4 (1.2) and glucose (5.6), gassed with a mixture of 95% O2: 5% CO2 (pH 7.4) at 27°C, since it is the temperature often used for reptile tissue experiments (Stephens, 1984; Miller and Vanhoutte, 1986; Campos et al., 2019a,b). Isometric force was recorded using a PowerLab 400TM data acquisition system (Software Chart, version 7.0, AD Instrument, MA, USA). The tissues were allowed to equilibrate for 1 h before starting the experiments.

Concentration-response curves to dopamine

Dopamine-induced concentration-dependent contractions were performed in endothelium-intact aortic rings in the absence and in the presence of the NO synthase inhibitor L-NAME (100 µM) and the NO-sensitive inhibitor of the guanylyl cyclase ODQ (100 µM). In L-NAME-treated aortic rings, dopamine-induced concentration-dependent contractions were also performed in the presence of the D1-like receptor antagonist SCH-23390 (0.3, 1 and 3 µM) and the D2-like receptor antagonists (risperidone, quetiapine and haloperidol; 0.3, 1 and 3 µM each), as well as of the tyrosine hydroxylase inhibitors salsolinol (100 µM) and 3-Iodo-L-tyrosine (0.1 and 1 mM). Nonlinear regression analysis to determine the pEC50 was carried out using GraphPad Prism (GraphPad Software, version 6.0, San Diego, CA, USA) with the constraint that F=0. All concentration–response data were evaluated for a fit to a logistics function in the form: $E=E_{max}/([1+ (10c/10x)n]+F$, where E represents the increase in response contractile induced by the agonist, E_{max} is the effect agonist maximum, c is the logarithm of concentration of the agonist that produces 50% of E_{max}, x is the logarithm of the concentration of the drug; the exponential term, n, is a curve fitting parameter that defines the slope of the concentration–response line, and F is the response observed in the absence of added drug. The values of EC50 data represent the mean±s.e.m. Values of E_{max} were represented by mN.

Electrical-field stimulation-induced aorta contractions

The aortic rings were submitted to EFS at 60 V for 30 s, at 16 Hz in square-wave pulses, 0.3 ms pulse width and 0.1 ms delay, using a Grass S88 stimulator (Astro-Medical, Industrial Park, RI, USA). Electrical-field

Biology Open (2021) 10, bio057042. doi:10.1242/bio.057042

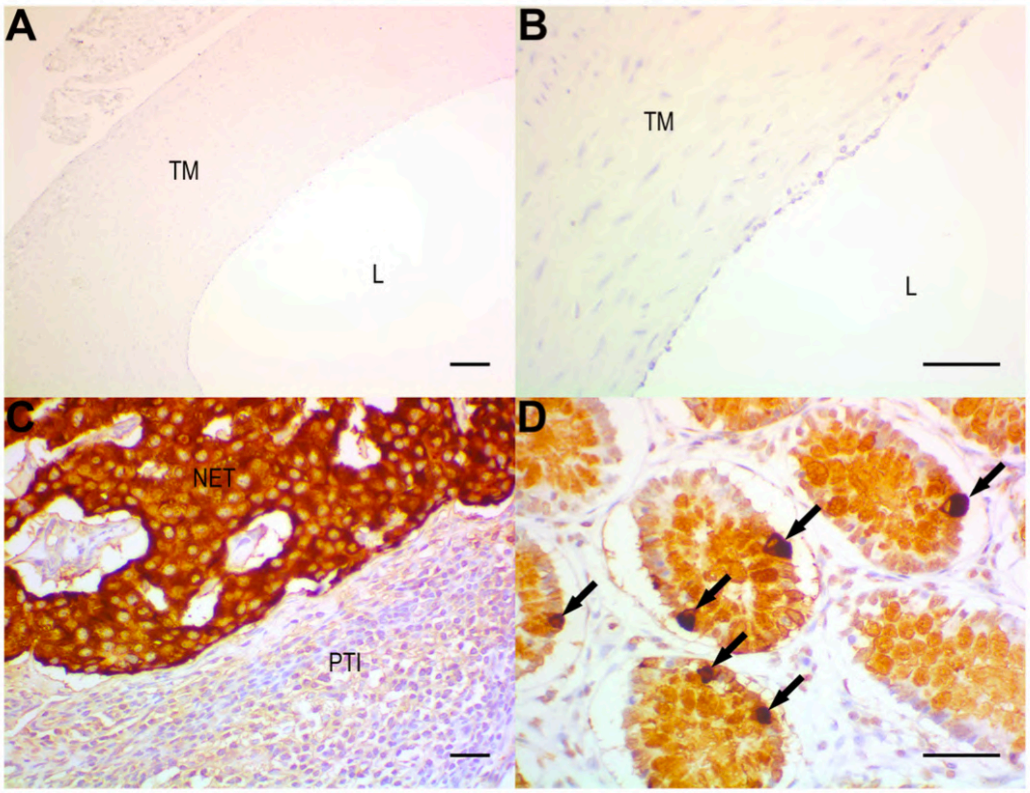


Fig. 8. Chromogranin A detection by immunohistochemistry. (A) lack of positivity for chromogranin A (CgA) in Chelonoidis aortic smooth muscle cells of the tunica media (TM) and in endothelial cells lining the lumen (L), low-power field (100X, original magnification); (B) same as in previous photomicrograph, at high-power field (400X). (C) Strong and diffuse positivity for CgA in a neuroendocrine tumor (NET) of the appendix, serving as a positive control. (D) Strong positivity also seen in scattered chromaffin cells (arrows), in a normal intestinal mucosae specimen (another positive control tissue). Immunoperoxidase, scale bars: 100 µm in (A) and (C); 50 µm in (B) and (D). PTI, peritumoral inflammation lacking positivity for chromogranin A.

simulations were performed with and without L-NAME (100 µM), SCH-23390 (1 µM), risperidone (1 µM), quetiapine (1 µM), haloperidol (1 and 3 µM), salsolinol p(100 µM) and 3-Iodo-L-tyrosine (1 mM). Potassium chloride (KCl, 80 mM) was added at the beginning and at the end of the experimental protocols to ensure the tissue integrity after EFS.

LC-MS-MS analysis

Two aortic rings per animal (15 mm) from *Chelonoidis carbonaria*, one endothelium-intact and another endothelium-denuded aortic ring were suspended in 5 ml organ baths containing Krebs-Henseleit's solution and O_2/CO_2 mixture at 27°C. The removal of endothelial cells was done mechanically by gently rubbing the arteries with forceps.

The basal release of dopamine, noradrenaline and adrenaline in Henseleit's solution was measured by LC-MS-MS following a 30 min incubation period. The dopamine, noradrenaline and adrenaline concentrations in the Krebs-Henseleit solution were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The extraction procedure was similar to that described for extracting methyldopa from plasma (Oliveira et al., 2002). Briefly, 100 µl of the internal standards (dopamine-d3, noradrenaline-d6 and adrenaline-d6 at 100 ng/ml) were added to the Krebs' solution (2 ml) followed by 1.5 ml of deionized water. After vortexing for 10 s, 100 mg of Al_2O_3 was added and left for incubation for 20 min in an orbital agitator (Centrifuge 5810/5810 R). The tubes were then centrifuged at 2000 g for 4 min at 4°C and the supernatant discarded. The residue was washed four times with 2 ml of deionized water. After the final washing, 200 µl of a solution containing trifluoroacetic acid 0.1% in HCN/H2O (60/40 l; v/v) were added. After vortexing for 40 s, the Eppendorf tubes were centrifuged for 2000 ***g*** for 5 min and the supernatant transferred to the vials for injection. The samples were analyzed by liquid chromatography coupled to a triple quadrupole mass spectrometer, LCMS-8050 (Shimadzu, Kyoto, Japan). The separation of catecholamines was performed on a 100×4.6 mm Lichrospher RP-8 column (GL Sciences Inc., Tokyo, Japan) using acetonitrile/water (5/95, v/v) with 0.1% formic acid as mobile phase at a flow rate of 0.4 ml/min. The mass spectrometer operated in positive electrospray ionization mode (ES+) for catecholamine detection. The analyses were executed in selected Multiple Reaction Monitoring (MRM) detection mode. This method has been fully validated, and the results reported elsewhere (Britto-Junior et al., 2020c).

Data analysis

Data are expressed as mean±standard error of mean (s.e.m.) of the number of experiments. In the pharmacological experiments, the number of

experiments in expressed as x/y, where x represents the number of aortas (animal) and y the number of rings employed in the experiment. The contractions were quantified in milli-Newtons (mN). A P value <0.05 was considered significant. When paired contractions were used, for example, in the absence and in the presence of an antagonist/inhibitor (the first contraction being the control response), Student's paired t-test was employed for statistical analysis. When one ring was used as the control response, and another ring was incubated with an antagonist/inhibitor, Student's unpaired t-test was used. For E_{max} analysis and pEC50, unpaired Student's t-test was used.

Immunohistochemistry

Immunohistochemistry was performed manually. Briefly, 4-µm sections of formalin-fixed, paraffin-embedded samples of Chelonoidis aorta (n=4) were deparaffinized in xylene and rehydrated in a series of ethanol baths of increasing concentration. They were then incubated in citrate buffer at pH 6.0 in a steamer set for 40 min (at approximately 95°C). Following this, the sections were incubated for 2hs at room temperature (25°C) with a mouse monoclonal anti-chromogranin A antibody (clone DAK-A3, code M0869, 1:700, Dako/Agilent). Subsequently, these sections were incubated with the NovoLink Max Polymer Detection System (Novocastra/Leica Biosystems), following the manufacturer's instructions, and using diaminobenzidine (liquid DAB, DakoCytomation, Carpenteria, CA, USA) as a chromogen (which renders a brown precipitate at the antibody binding site). Finally, the sections were counter-stained with Harris' hematoxylin and cover-slipped in Entellan. Formalin-fixed, paraffin-embedded sections of a neuroendocrine tumor and a sample of normal intestinal (colonic) mucosae were used as positive controls for the presence of chromogranin A. All slides were examined using a trinocular Eclipse E200 microscope (Nikon, Tokyo, Japan) coupled to a 10MP CMOS digital camera (Amscope, USA).

Acknowledgements

J.B.-J. thanks CAPES for PhD fellowship. F.F.J. thanks FAPESP for PhD fellowship (2018/24971-9). E.A. and F.M. thank FAPESP (2017/15175-1). G.D.N. thanks CNPq (303839/2019-8). A.A.S. thanks FAPESP (2016/04731-8).

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.B.-J, R.A.M., E.A., G.D.N.; Methodology: J.B.-J, F.F.J., R.C., D.H.A.P., G.M.F.M., V.B.d.S., A.A.S., F.Z.M., R.A.M., E.A., G.D.N.; Software: J.B.-J, G.D.N.; Validation: J.B.-J, G.D.N.; Formal analysis: J.B.-J, G.D.N.; Investigation: J.B.-J., G.D.N.; Resources: J.B.-J, G.D.N.; Data curation: J.B.-J,G.M.F.M, G.D.N.; Writing - original draft: J.B.-J, G.D.N.; Writing - review & editing: J.B.-J, G.D.N.; Visualization: J.B.-J, G.D.N.; Supervision: E.A, F.Z.M., G.D.N.; Project administration:G.D.N.; Funding acquisition: E.A, F.Z.M., G.D.N.

Funding

The work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo [2017/15175-1, 2018/24971-9, 2016/04731-8], Conselho Nacional de Desenvolvimento Científico e Tecnológico [2019-8].

References

Adams, D. J. and Hill, M. A. (2004). Potassium channels and membrane potential in the modulation of intracellular calcium in vascular endothelial cells. *J. Cardiovasc. Electrophysiol.* **15**, 598-610. doi:10.1046/j.1540-8167.2004.03277.x

Agrawal, D. K., Triggle, C. R. and Daniel, E. E. (1984). Pharmacological characterization of the postsynaptic alpha adrenoceptors in vascular smooth muscle from canine and rat mesenteric vascular beds. *J. Pharmacol. Exp. Ther.* **229**, 831-838.

Ahlquist, R. P. (1948). A study of the adrenotropic receptors. *Am. J. Physiol.* **153**, 586-600. doi:10.1152/ajplegacy.1948.153.3.586

Amenta, F. and Ricci, A. (1990). Autoradiographic localization of dopamine DA-1 receptors in the rat renal vasculature using [3H]-SCH 23390 as a ligand. *J. Auton. Pharmacol.* **10**, 373-383. doi:10.1111/j.1474-8673.1990.tb00037.x

Amenta, F., Collier, W. L. and Ricci, A. (1990). Autoradiographic localization of vascular dopamine receptors. *Am. J. Hypertens.* **3**, 34S-36S. doi:10.1093/ajh/3.6.34S

Awad, M., Czer, L. S. C., Hou, M., Golshani, S. S., Goltche, M., De Robertis, M., Kittleson, M., Patel, J., Azarbal, B., Kransdorf, E. et al. (2016). Early denervation and later reinnervation of the heart following cardiac transplantation: a review. *J. Am. Heart Assoc.* **5**, e004070. doi:10.1161/JAHA.116.004070

Banner, N. R., Williams, T. D., Patel, N., Chalmers, J., Lightman, S. L. and Yacoub, M. H. (1990). Altered cardiovascular and neurohumoral responses to head-up tilt after heart-lung transplantation. *Circulation* **82**, 863-871. doi:10.1161/01.CIR.82.3.863

Bevan, J. A. (1962). Some characteristics of the isolated sympathetic nerve-pulmonary artery preparation of the rabbit. *J. Pharmacol. Exp.Ther.* **137**, 213-218.

Billard, W., Ruperto, V., Crosby, G., Iorio, L. C. and Barnett, A. (1984). Characterization of the binding of 3H-SCH 23390, a selective D-1 receptor antagonist ligand, in rat striatum. *Life Sci.* **35**, 1885-1893. doi:10.1016/0024-3205(84)90540-X

Britto-Júnior, J., Jacintho, F. F., Figueiredo Murari, G. M., Campos, R., Moreno, R. A., Antunes, E., Mónica, F. Z. and De Nucci, G. (2020a). Electrical field stimulation induces endothelium-dependent contraction of human umbilical cord vessels. *Life Sci.* **243**, 117257. doi:10.1016/j.lfs.2020.117257

Britto-Júnior, J., Pinheiro, D. H. A., Justo, A. F. O., Figueiredo Murari, G. M., Campos, R., Mariano, F. V., de Souza, V. B., Schenka, A. A., Mónica, F. Z., Antunes, E. et al. (2020b). Endothelium-derived dopamine modulates EFS-induced contractions of human umbilical vessels. *Pharmacol. Res. Perspect.* **8**, e00612. doi:10.1002/prp2.612

Britto-Junior, J., Antunes, N. J., Campos, R., Sucupira, M., Mendes, G. D., Fernandes, F., Moraes, M. O., Moraes, M. E. A. and De Nucci, G. (2020c). Determination of dopamine, noradrenaline and adrenaline in Krebs-Henseleit's solution by liquid chromatography coupled tandem mass spectrometry. Measurement of basal release from *Chelonoidis carbonaria* aortae *in vitro*. *Biomed. Chromatogr.* **34**, e4978. doi:10.1002/bmc.4978

Bruzzone, P., D'Andrea, V., Motta, C. and Cavallotti, C. (2002). Occurrence of dopaminergic (D2) receptors within the rabbit pulmonary circulation. *Pulm. Pharmacol. Ther.* **15**:393-398. doi:10.1006/pupt.2002.0388

Burt, D. R., Creese, I. and Snyder, S. H. (1976). Properties of [3H]haloperidol and [3H]dopamine binding associated with dopamine receptors in calf brain membranes. *Mol. Pharmacol.* **12**, 800-812.

Campos, R., Justo, A. F. O., Mónica, F. Z., Cogo, J. C., Moreno, R. A., de Souza, V. B., Schenka, A. A. and De Nucci, G. (2018a). Electrical field-induced contractions on *Crotalus durissus terrificus* and *Bothrops jararaca* aortae are caused by endothelium-derived catecholamine. *PLoS ONE* **13**, e0203573. doi:10.1371/journal.pone.0203573

Campos, R., Mónica, F. Z., Justo, A. F. O., Cogo, J. C., de Toledo Oliveira, E. , Moreno, R. A., Antunes, E. and De Nucci, G. (2018b). Electrical field stimulation-induced contractions on *Pantherophis guttatus* corpora cavernosa and aortae. *PLoS ONE* **13**, e0196123. doi:10.1371/journal.pone.0196123

Campos, R., Claudino, M. A., De Oliveira, M. G., Franco-Penteado, C. F., Del Grossi Ferraz Carvalho, F., Zaminelli, T., Antunes, E. and De Nucci, G. (2019a). Amiloride relaxes rat corpus cavernosum relaxation *in vitro* and increases intracavernous pressure *in vivo*. *J. Sex. Med.* **16**, 500-511. doi:10.1016/j.jsxm.2019.01.315

Campos, R., Justo, A. F. O., Jacintho, F. F., Mónica, F. Z., Rojas-Moscoso, J. A., Moreno, R. A., Napolitano, M., Cogo, J. C. and De Nucci, G. (2019b). Pharmacological and transcriptomic characterization of the nitric oxide pathway in aortic rings isolated from the tortoise *Chelonoidis carbonaria*. *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.* **222**, 82-89. doi:10.1016/j.cbpc.2019.04.015

Campos, R., Jacintho, F. F., Britto-Júnior, J., Mónica, F. Z., Justo, A. F. O., Pupo, A. S., Moreno, R. A., de Souza, V. B., Schenka, A. A., Antunes, E. et al. (2020). Endothelium modulates electrical field stimulation-induced contractions of *Chelonoidis carbonaria* aortic rings. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **233**, 108763. doi:10.1016/j.cbpc.2020.108763

Chiou, G. C. and Chen, Y. J. (1992). Effects of dopamine agonist, bromocriptine, and some dopamine antagonists on ocular blood flow. *J. Ocul. Pharmacol.* **8**, 285-294. doi:10.1089/jop.1992.8.285

Dail, W. G., McGuffee, L., Minorsky, N. and Little, S. (1987). Responses of smooth muscle strips from penile erectile tissue to drugs and transmural nerve stimulation. *J. Auton. Pharmacol.* **7**, 287-294. doi:10.1111/j.1474-8673.1987.tb00157.x

De Oliveira, J. F., Teixeira, C. E., Arantes, E. C., De Nucci, G. and Antunes, E. (2003). Relaxation of rabbit corpus cavernosum by selective activators of voltage-gated sodium channels: role of nitric oxide-cyclic guanosine monophosphate pathway. *Urology* **62**, 581-588. doi:10.1016/S0090-4295(03)00462-X

De Oliveira, M. G., Alexandre, E. C., Bonilla-Becerra, S. M., Bertollotto, G. M., Justo, A. F. O., Mónica, F. Z. and Antunes, E. (2019). Autonomic dysregulation at multiple sites is implicated in age-associated underactive bladder in female mice. *Neurourol. Urodyn.* **38**, 1212-1221. doi:10.1002/nau.23990

Doxey, J. C., Lane, A. C., Roach, A. G. and Virdce, N. K. (1984). Comparison of the alpha-adrenoceptor antagonist profiles of idazoxan (RX 781094), yohimbine, rauwolscine and corynanthine. *Naunyn. Schmiedeberg's. Arch. Pharmacol.* **325**, 136-144. doi:10.1007/BF00506193

Farrell, A. P., Franklin, C. E., Arthur, P. G., Thorarensen, H. and Cousins, K. L. (1994). Mechanical performance of an in situ perfused heart from the turtle *Chrysemys scripta* during normoxia and anoxia at 5C and 15C. *J. Exp. Biol.* **191**, 207-229.

Félétou, M. (2011). The Endothelium, Part II: EDHF-Mediated Responses "The Classical Pathway". In *Colloquium Series on Integrated Systems Physiology: From Molecule to Function. Morgan & Claypool Life Sciences*. 3, 4, 1-306.
Furchgott, R. F. and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**, 373-376. doi:10.1038/288373a0
Goodwill, A. G., Dick, G. M., Kiel, A. M. and Tune, J. D. (2017). Regulation of coronary blood flow. *Compr. Physiol.* **7**, 321-382. doi:10.1002/cphy.c160016
Guyton, A. C. (1981). The relationship of cardiac output and arterial pressure control. *Circulation* **64**, 1079-1088. doi:10.1161/01.CIR.64.6.1079
Hicks, J. M., Farrell, A. P. (2000). The cardiovascular responses of the red-eared slider (Trachemys scripta) acclimated to either 22 or 5 degrees CI Effects of anoxic exposure on in vivo cardiac performance. *Journal of Experimental Biology*. **203**, 3765-3774.
Ignarro, L. J., Bush, P. A., Buga, G. M., Wood, K. S., Fukuto, J. M. and Rajfer, J. (1990). Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem. Biophys. Res. Commun.* **170**, 843-850. doi:10.1016/0006-291X(90)92168-Y
Jamieson, S. W., Stinson, E. B., Oyer, P. E., Baldwin, J. C. and Shumway, N. E. (1984). Operative technique for heart-lung transplantation. *J. Thorac. Cardiovasc. Surg.* **87**, 930-935. doi:10.1016/S0022-5223(19)38424-7
Joyce, W. and Wang, T. (2020). What determines systemic blood flow in vertebrates? *J. Exp. Biol.* **223**, jeb215335. doi:10.1242/jeb.215335
Kadowitz, P. J., Knight, D. S., Hibbs, R. G., Ellison, J. P., Joiner, P. D., Brody, M. J. and Hyman, A. L. (1976). Influence of 5- and 6-hydroxydopamine on adrenergic transmission and nerve terminal morphology in the canine pulmonary vascular bed. *Circ. Res.* **39**, 191-199. doi:10.1161/01.RES.39.2.191
Knottenbelt, D. C., Patterson-Kane, J. C. and Snalune, K. L. (2015). Endocrine and neuroendocrine tumors. *Clinical Equine Oncol.* **24**, 376-392. doi:10.1016/B978-0-7020-4266-9.00024-6
Kyriakopoulos, G., Mavroeidi, V., Chatzellis, E., Kaltsas, G. A. and Alexandraki, K. I. (2018). Histopathological, immunohistochemical, genetic and molecular markers of neuroendocrine neoplasms. *Ann. Transl. Med.* **6**, 252. doi:10.21037/atm.2018.06.27
La Perle, K. M. D. and Dintzis, S. M. (2018). *Endocrine System. Comparative Anatomy and Histology (Second Edition), A Mouse, Rat, and Human Atlas*. pp. 251-273. Academic Press. doi:10.1016/B978-0-12-802900-8.00015-4
Matsuyama, T., Shiosaka, S., Wanaka, A., Yoneda, S., Kimura, K., Hayakawa, T., Emson, P. C. and Tohyama, M. (1985). Fine structure of peptidergic and catecholaminergic nerve fibers in the anterior cerebral artery and their interrelationship: an immunoelectron microscopic study. *J. Comp. Neurol.* **235**, 268-276. doi:10.1002/cne.902350209
Miller, V. M. and Vanhoutte, P. M. (1986). Endothelium-dependent responses in isolated blood vessels of lower vertebrates. *Blood Vessels* **23**, 225-235. doi:10.1159/000158643
Moncada, S., Gryglewski, R., Bunting, S. and Vane, J. R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature* **263**, 663-665. doi:10.1038/263663a0
Narahashi, T., Moore, J. W. and Scott, W. R. (1964). Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. Gen. Physiol.* **47**, 965-974. doi:10.1085/jgp.47.5.965
Nilsson, S. (2010). On the autonomic nervous and chromaffin control systems of lungfish. *Aust. Zool.* **35**, 363-368. doi:10.7882/AZ.2010.024
Oliveira, C. H., Barrientos-Astigarraga, R. E., Sucupira, M., Graudenz, G. S., Muscará, M. N. and De Nucci, G. (2002). Quantification of methyldopa in human plasma by high-performance liquid chromatography–electrospray tandem mass spectrometry: Application to a bioequivalence study. *J. Chromatogr. B* **768**, 341-348. doi:10.1016/S1570-0232(01)00612-2
Paterson, G. (1965). The response to transmural stimulation of isolated arterial strips and its modification by drugs. *J. Pharm. Pharmacol.* **17**, 341-349. doi:10.1111/j.2042-7158.1965.tb07680.x
Ross, J., Jr., Linhart, J. W. and Brauwald, E. (1965). Effects of changing heart rate in man by electrical stimulation of the right atrium. studies at rest, during exercise, and with isoproterenol. *Circulation* **32**, 549-558. doi:10.1161/01.cir.32.4.549
Scheuermann, D. W. (1993). Comparative morphology, cytochemistry and innervation of chromaffin tissue in vertebrates. *J. Anat.* **183**, 327-342.
Stecyk, J. A. W., Overgaard, J., Farrell, A. P. and Wang, T. (2004). α-Adrenergic regulation of systemic peripheral resistance and blood flow distribution in the turtle Trachemys scripta during anoxic submergence at 5°C and 21°C. *J. Exp. Biol.* **207**, 269-283. doi:10.1242/jeb.00744
Stephens, G. A. (1984). Angiotensin and norepinephrine effects on isolated vascular strips from a reptile. *Gen. Comp. Endocrinol.* **54**, 175-180. doi:10.1016/0016-6480(84)90170-9
Wasser, J. S. and Jackson, D. C. (1991). Effects of anoxia and graded acidosis on the levels of circulating catecholamines in turtles. *Respir. Physiol.* **84**, 363-377. doi:10.1016/0034-5687(91)90130-B
Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**, 411-415. doi:10.1038/332411a0

Anexo 4

Artigo 6 – 6-Nitrodopamine is released by human umbilical cord vessels and modulates vascular reactivity

Autores: Britto-Júnior J, Coelho-Silva WC, Murari GF, Serpellone Nash CE, Mónica FZ, Antunes E, De Nucci G.

Revista: Life Sciences

Situação: Aceito a publicação em 16 de fevereiro de 2021. Publicado on-line em 10 de março de 2021

Life Sciences 276 (2021) 119425

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

6-Nitrodopamine is released by human umbilical cord vessels and modulates vascular reactivity

José Britto-Júnior [a,*], Weverton C. Coelho-Silva [a], Guilherme Figueiredo Murari [a], Charles Elliot Serpellone Nash [a], Fabíola Z. Mónica [a], Edson Antunes [a], Gilberto De Nucci [a,b,c]

[a] Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), São Paulo, Campinas, Brazil
[b] Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo (USP), São Paulo, Brazil
[c] Faculty of Medical Sciences, Universidade do Brasil, Fernandópolis, São Paulo, Brazil

ARTICLE INFO

Keywords:
Human umbilical artery
Human umbilical vein
Nitrocatecholamines
Endothelium
Nitric oxide
Sumanirole
Haloperidol
Fenoldopam

ABSTRACT

Aims: Human umbilical cord vessels (HUCV) release dopamine and nitric oxide (NO). This study aims to verify whether HUCV release nitrocatecholamines such as 6-nitrodopamine (6-ND).
Main methods: Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used to identify 6-ND release from HUCV rings incubated in Krebs-Henseleit's solution. Vascular reactivity of HUCV rings was tested (with and without endothelium integrity) by suspension of the rings in an organ bath under isometric tension and application of 6-ND and other known mediators.
Key findings: LC-MS/MS revealed a basal release of 6-ND from endothelium intact from both human umbilical artery (HUA) and vein (HUV). The endothelium intact release was inhibited by the pre-treatment with NO synthesis inhibitor L-NAME (100 μM). In contrast to dopamine, noradrenaline and adrenaline, 6-ND did not contract HUCV, even in presence of L-NAME or ODQ. 6-ND (10 μM) produced a rightward shift of the concentration-response curves to dopamine (pA2: 5.96 in HUA and 5.72 in HUV). Contractions induced by noradrenaline and adrenaline were not affected by pre-incubation with 6-ND (10 μM).
In U-46619 (10 nM) pre-contracted endothelium intact tissues, 6-ND and the dopamine D_2-receptor antagonist haloperidol induced concentration-dependent relaxations of HUA and HUV. Incubation with the dopamine D_1-receptor antagonist SCH-23390 (10 nM) abolished relaxation induced by fenoldopam but did not affect those induced by 6-ND.
Significance: 6-ND is released by HUCV and acts as a selective dopamine D_2-receptor antagonist in this tissue. This represents a novel mechanism by which NO may modulate vascular reactivity independently of cGMP production.

1. Introduction

Human umbilical cord vessels (HUCV) exhibit endothelium-dependent basal release of dopamine, as detected by liquid chromatography coupled to mass spectrometry (LC-MS/MS) [1]. Endothelium-derived dopamine modulates vascular reactivity in human umbilical artery (HUA) and vein (HUV), since the dopamine D_2-like receptor antagonist haloperidol inhibits electrical-field stimulation (EFS)-induced contractions of this tissue [1]. Chelonoidis carbonaria aortic rings also release endothelium-dependent catecholamines, and the dopamine D_2-like receptor antagonists such as haloperidol, quetiapine and risperidone modulate the spasmogenic activity induced by EFS [2].

Nitrocatecholamines such as nitronoradrenaline and nitroadrenaline have been extracted from rat brain and quantified by LC-peroxyoxalate chemiluminescence reaction detection [3], and 6-nitronoradrenaline was found to inhibit noradrenaline transport in rat synaptosomes [4]. Endothelium-dependent release of 6-nitrodopamine (6-ND) has been identified by LC-MS/MS from Chelonoidis carbonaria aortic rings [2]. Considering that HUCV liberates both dopamine and nitric oxide (NO), and the latter has been shown to be able to nitrate dopamine *in vitro* generating 6-ND [5], we have investigated whether 6-ND could be biologically synthetized in human tissue. In this study, a basal release of 6-ND from HUCV was identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Furthermore, our results

* Corresponding author.
E-mail address: josebrittojr@gmail.com (J. Britto-Júnior).

https://doi.org/10.1016/j.lfs.2021.119425
Received 16 February 2021; Received in revised form 10 March 2021; Accepted 15 March 2021
Available online 26 March 2021
0024-3205/© 2021 Elsevier Inc. All rights reserved.

indicate that 6-ND modulates vascular reactivity by acting as a highly selective dopamine D2-receptor antagonist.

2. Methods

2.1. Study participants

Participants over the age of eighteen, undergoing the natural or cesarean delivery from Maternity Hospital (Campinas, SP) were invited to take part in the study. The women were normotensive, and did not have preeclampsia, pregestational or gestational diabetes mellitus and none was on regular medication. The Informed Consent Form was obtained from those who agreed to participate. Umbilical cords from 96 volunteers aged 18–42 years. The investigation followed the principles outlined in the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Institute of Biomedical Sciences of the University of São Paulo – ICB/USP (protocol number 3.165.417).

2.2. Reagents

Dopamine, noradrenaline, adrenaline, serotonin (5-HT), ascorbic acid, SCH-23390, adenosine triphosphate (ATP), N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and 1H-[1,2,4]-oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) were acquired from Sigma-Aldrich Chemicals Co. (St Louis, Missouri, USA). Sumanirole and U-46619 were obtained from Tocris (Bristol, UK). 6-nitrodopamine and 6-nitrodopamine-d4 were purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Fenoldopam was obtained from Cayman Chemical Co (Michigan, USA). Haloperidol and were provided by Vamsi Labs Ltd. (Maharashtra, India). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$), potassium phosphate monobasic (KH_2PO_4) and glucose were acquired from Merck KGaA (Darmstadt, Germany). Acetonitrile and methanol were bought from J.T. Baker (Phillipsburg, NJ, USA) and formic acid from Mallinckrodt (St Louis, MO, USA).

2.3. Preparation of human umbilical artery (HUA) and vein (HUV) for extraction and quantification of 6-ND by LC MS-MS analysis

A segment of the umbilical cord (10–20 cm) from the insertion point in the placenta and 5 cm from the umbilicus was removed by the obstetrician and placed in a container with Krebs-Henseleit's solution (KHS). The Wharton's jelly was removed and HUA and HUV were dissected. Two HUA and two HUV rings (15 mm each) per subject with intact endothelium and another two with endothelium-denuded were suspended in 5 mL organ bath containing KHS (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5.6 mM glucose) containing ascorbic acid (3 mM) continuously gassed with a mixture of 95%O_2:5% CO_2 (pH 7.4) at 37°C for 30 min. The removal of endothelial cells was done mechanically by gently rubbing the vessels with forceps. Isolated HUA and HUV were incubated in the absence and in the presence of the NO synthesis inhibitor N^{ω}-Nitro-L-arginine (L-NAME, 100 μM). Then, an aliquot of 2 mL of the supernatant was transferred to a tube and stored at −20 °C until analysis [1].

The extraction and quantification of 6-ND in KHS was carried out according as described elsewhere [6]. Briefly, 6-ND was extracted from 1 mL of KHS by solid phase extraction (SPE). Calibrators and quality controls (QCs) prepared in blank KHS and KHS samples obtained from HUA and HUV rings for 30 min were spiked with 50 μL of the internal standard (IS) solution (6-ND-d4, 100 ng/mL). Extraction cartridges were conditioned with 1 mL of methanol and then equilibrated with 2 mL of deionized water. The samples were transferred to the extraction cartridges and washed three times with deionized water. The samples were eluted with 0.9 mL methanol/deionized water (90/10, v/v) plus 0.1% formic acid and followed by evaporation under N_2 flow at 50 °C. The dry residues were dissolved with 100 μL acetonitrile/deionized water (50/50, v/v) plus 0.1% formic acid, transferred to vials and submitted to chromatographic analysis.

The separation of 6-ND was performed on a 150 mm × 3.0 mm Shimpack GIST-HP C_{18} column, 3-μm particle size (Shimadzu, Duisburg, Germany) held at 65 °C. A 75% of mobile phase A consisting of deionized water with 0.1% formic acid (v/v) and 25% of mobile phase B consisting of acetonitrile/deionized water (90/10, v/v) plus 0.1% formic acid at a flow rate of 350 μL/min in an isocratic mode were used. The detection of 6-ND and IS was carried out by a LCMS-8060 triple quadrupole mass spectrometer (MS/MS) (Shimadzu, Kyoto, Japan), operating in positive ionization mode. The analyses were performed in the multiple reaction monitoring (MRM) mode. The protonated ions $[M + H]^+$ and their respective ion products were monitored in the 199.10 > 181.95 for 6-ND and 203.10 > 186.00 for 6-ND-d4.

2.4. Preparation of HUA and HUV for the in vitro vascular reactivity assays

Human umbilical arteries and HUV were obtained as mentioned before. Briefly, rings (3-mm) of HUA and HUV were suspended vertically between two metal hooks in 10-mL organ baths containing KHS, continuously gassed with a mixture of 95%O2: 5% CO_2 (pH 7.4) at 37 °C. Tissues were allowed to equilibrate under a resting tension of 10 mN, and the isometric tension was registered using a PowerLab system (ADInstruments, Sydney, Austrália). Following a 90-min stabilization period, rings were pre-contracted with 5-hydroxytriptamine (5-HT, 1 μM). The integrity of the endothelium in both HUA and HUV was evaluated through ATP-induced relaxation (10 μM) [7].

Cumulative concentration-response curves to 6-ND were performed in endothelium-intact HUA and HUV rings, in the absence and presence of L-NAME (100 μM, 30 min) or the heme-site inhibitor of the soluble guanylyl cyclase ODQ (100 μM, 30 min). Additionally, in L-NAME (100 μM, 30 min) pre-treated vessels, cumulative concentration-response curves to dopamine, noradrenaline, adrenaline, sumanirole and the thromboxane A_2 (TXA_2) mimetic U-46619 were carried out in endothelium-intact HUA and HUV rings in the absence and presence of 6-ND (1-10 μM, 30 min). Nonlinear regression analysis to determine the pEC_{50} was carried out with the constraint that F = 0. All concentration–response data were evaluated for a fit to a logistics function in the form: $E = E_{max}/[1 + (10c / 10\times)n] + F$, where E represents the increase in response contractile induced by the agonist, E_{max} is the maximum effect possible caused by the agonist, c is the logarithm of concentration of the agonist that produces 50% of E_{max}, x is the logarithm of the concentration of the drug; the exponential term, n, is a curve fitting parameter that defines the slope of the concentration–response line, and F is the response observed in the absence of added drug. The pA_2 values were calculated from the intercept on the concentration axis and by application of the equation; pA_2 = log (antagonist concentration) − log (CR-1) − log (antagonist concentration) [8]. The values of EC_{50} data represent the mean ± standard error of the mean (SEM) of *n* experiments. Values of E_{max} were expressed in mN.

Contractions of HUA and HUV induced by electrical-field stimulation (EFS) were also evaluated. Briefly, HUA and HUV rings pre-treated with L-NAME (100 μM, for 30 min) in the absence or presence of 6-ND (10 μM, for 30 min) were submitted to EFS at 60 V for 30 s, at 8–16 Hz in square-wave pulses, 0.3 ms pulse width, and 0.1 ms delay, using a Grass S88 stimulator (Astro-Medical, Industrial Park, RI, USA).

In order to evaluate the relaxant effects of 6-ND, endothelium-intact or mechanical removal of endothelium in HUA and HUV rings were pre-contracted with U-46619 (10 nM). After a sustained contraction was obtained, cumulative concentration-response curves to 6-ND (0.1–300 nM), a selective D_2-receptor haloperidol (0.1–300 nM) a selective D_1-receptor fenoldopam (0.1–300 nM). The 6-ND and fenoldopam were performed in the presence or not of the dopamine D_1-like antagonist SCH-23390 (10 nM). The relaxation responses were expressed as percentage of the contractile response. Data from *in vitro* experiments were

analyzed as described below.

2.5. Statistical analysis

All results were expressed as the mean ± standard error of the mean (SEM) of *n* experiments. All data were analyzed using GraphPad Prism 6.0 Software. The number of experiments was expressed as *x/y*, where *x* represents the number of umbilical vessels and *y* the number of rings employed in the experiment. When one ring was used as the control response and another ring was incubated with an antagonist/inhibitor, Student's unpaired *t*-test was used for the analysis. In the EFS experiments, where the same ring was submitted to the stimulus before and after treatment, Student's paired t-test was employed. For all analysis, differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Determination of the 6-ND concentrations in HUA and HUV rings by LC-MS/MS

The calibration curve of 6-ND was linear for concentrations of 0.1–10 ng/mL, with a correlation coefficient higher than 0.99 (data not shown). The limit of quantification was 0.1 ng/mL; the accuracy (expressed as %) for the intra-batch ($n = 7$) and inter-batch ($n = 21$) runs were 103.7 and 103.67, respectively and the precision (expressed as % CV) for the intra-batch (n = 7) and inter-batch (n = 21) runs were 12.7 and 14.9, respectively. The basal release of 6-ND in HUA and HUV was significantly decreased ($p < 0.05$) by either endothelium removal (Fig. 1A,B) or pre-treatment with L-NAME (Fig. 1C,D), respectively.

3.2. Effect of 6-ND on the contractions induced by the dopamine, noradrenaline and adrenaline

The addition of 6-ND (0.001–300 μM) alone contracted neither HUA ($n = 5/10$) nor HUV (n = 5/10) in the absence or presence of L-NAME or ODQ (data not shown). However, In L-NAME pre-treated vessels, 6-ND (10 μM, 30 min) produced a significant rightward shift of the concentration-response curves to dopamine in both HUA (pEC_{50} 4.9 ± 0.1, 4.4 ± 0.2 and 4.2 ± 0.1; $n = 5/8$) and HUV (pEC_{50} 4.9 ± 0.10, 4.4 ± 0.2 and 4.2 ± 0.1; n = 5/8), for control 3 and 10 μM 6-ND, respectively (Fig. 2A,B). The pA_2 values for 6-ND were 5.96 ± 0.24 and 5.72 ± 0.13 for HUA (n = 5/8) and HUV (n = 5/8), respectively.

As opposed to dopamine, 6-ND (10 μM, 30 min) had no effect on noradrenaline-induced contractions of HUA (pEC_{50} 5.1 ± 0.2 and 5.37 ± 0.2; E_{max} 4.6 ± 0.4 and 4.9 ± 0.5 mN for control and 6-ND respectively; $n = 4/7$) or HUV (pEC_{50} 5.75 ± 0.1 and 5.70 ± 0.2; E_{max} 16.6 ± 1.4 and 15.8 ± 2.3 mN; for control and 6-ND respectively, $n = 4/7$; Fig. 2C,D).

The adrenaline-induced contractions were also unaffected by 6-ND (10 μM) in HUA (pEC_{50} 5.35 ± 0.23 and 5.62 ± 0.23; E_{max} 4.2 ± 0.7 and 3.7 ± 0.4 mN for control and 6-ND respectively; n = 4/7) or HUV (pEC_{50} 5.83 ± 0.22 and 6.18 ± 0.17; E_{max} 3.5 ± 0.7 and 3.4 ± 0.4 mN for control and 6-ND respectively, n = 4/7; Fig. 2E,F).

3.3. Effect of 6-ND on the sumanirole-induced contractions

The selective D_2-receptor agonist sumanirole caused a concentration-dependent contraction of HUA (Fig. 3A) and HUV (Fig. 3B) pre-treated with L-NAME.

In HUA preparation, pre-incubation with 6-ND at 1 μM caused a rightward shift of the concentration-response curve to sumanirole, whereas at 3 and 10 μM, 6-ND markedly reduced the contractions (E_{max}

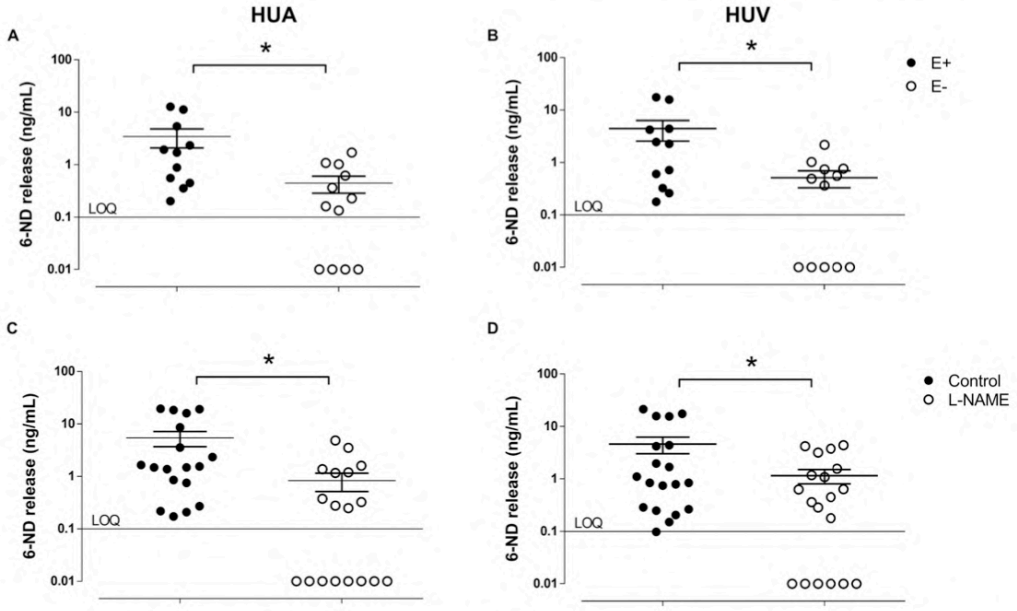


Fig. 1. Release of 6-ND by both human umbilical arteries (HUA) and human umbilical vein (HUV). The basal release of 6-ND was measured by LC-MS/MS following a 30 min-period incubation in Krebs-Henseleit's solution with endothelium-intact (E+) in HUA ($n = 11/11$; panel A) and HUV ($n = 9/9$; panel B). The mechanical removal of endothelium (E−) reduced the 6-ND release in both HUA ($n = 12/12$; panel A) and in HUV ($n = 10/10$; panel B) rings. Similar reduction was also observed in L-NAME (100 μM) treated both HUA (n = 10/18; panel C) and HUV (n = 10/19; panel D).

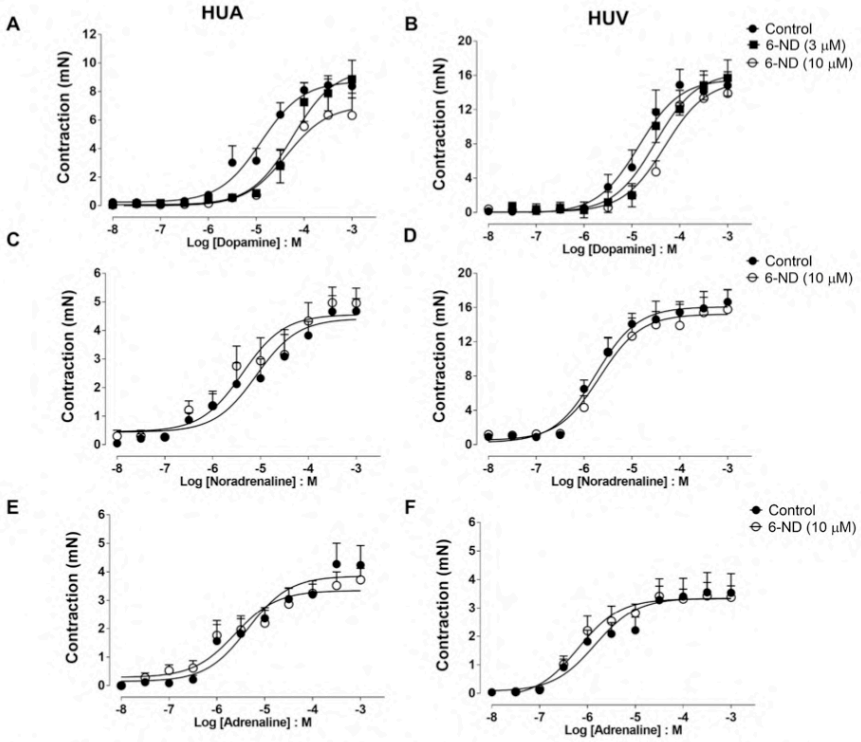


Fig. 2. Concentration-response curves (10 nM – 1 mM) to catecholamines in presence of L-NAME 100 μM in HUCV rings. Dopamine - [panel A: HUA ($n = 5/8$); panel B: HUV (n = 5/8)], noradrenaline - [panel C: HUA (n = 5/8); panel D: HUV (n = 5/8)] - adrenaline [panel E: HUA (n = 5/8); panel F: HUV (n = 5/8)] induced contractions in the presence and in the absence of 6-ND. 6-nitrodopamine (3 and 10 μM) caused a significant rightward shift to dopamine concentration-response curve. 6-ND (10 μM) had no effect on the contraction induced by noradrenaline and adrenaline.

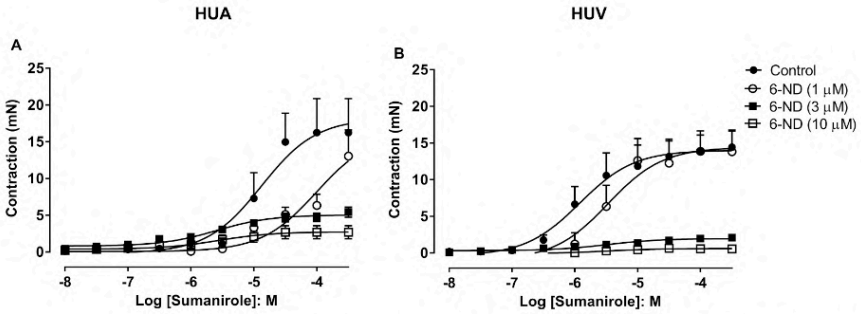


Fig. 3. Effect of 6-ND on Sumanirole (10 nM to 300 μM) induced contractions in HUA ($n = 4/8$; panel A) and HUV (n = 4/8; panel B). Pre-incubation with 6-ND 1 μM caused a rightward shift of the concentration-response curve, whereas at 3 and 10 μM 6-ND reduced the contractions in both HUA and HUV.

16.2 ± 2.1, 13.0 ± 3.6, 3.4 ± 0.7 and 2.7 ± 0.8 mN; $n = 4/8$) for control 1, 3 and 10 μM 6-ND, respectively (Fig. 3A). Similar results were observed in HUV preparations (Fig. 3B) where 6-ND produced a rightward shift at the lower concentration (1 μM), and nearly abolished the contractions at 3 and 10 μM (E_{max} 14.4 ± 2.3, 13.8 ± 2.7, 2.1 ± 0.1 and 0.5 ± 0.4 mN; n = 4/8; for control 1, 3 and 10 μM 6-ND).

3.4. *Effect of 6-ND in EFS-induced contractions*

In L-NAME-treated vessels, application of EFS at 8 and 16 Hz produced significant contractions of both HUA and HUV preparations (Fig. 4A, C). Pre-incubation with 6-ND (10 μM, 30 min) significantly reduced EFS-induced contractions of the HUA (4.8 ± 0.8 and 1.8 ± 0.5 mN for 8 Hz and 4.7 ± 0.8 and 2.7 ± 0.8 mN for 16 Hz; $p < 0.05$; $n = 5/8$; for control and 6-ND pre-treated vessels, respectively) similar in HUV (10.6 ± 1.8 and 3.8 ± 0.8 mN for 8 Hz and 12.1 ± 1.9 and 5.8 ± 0.9 mN

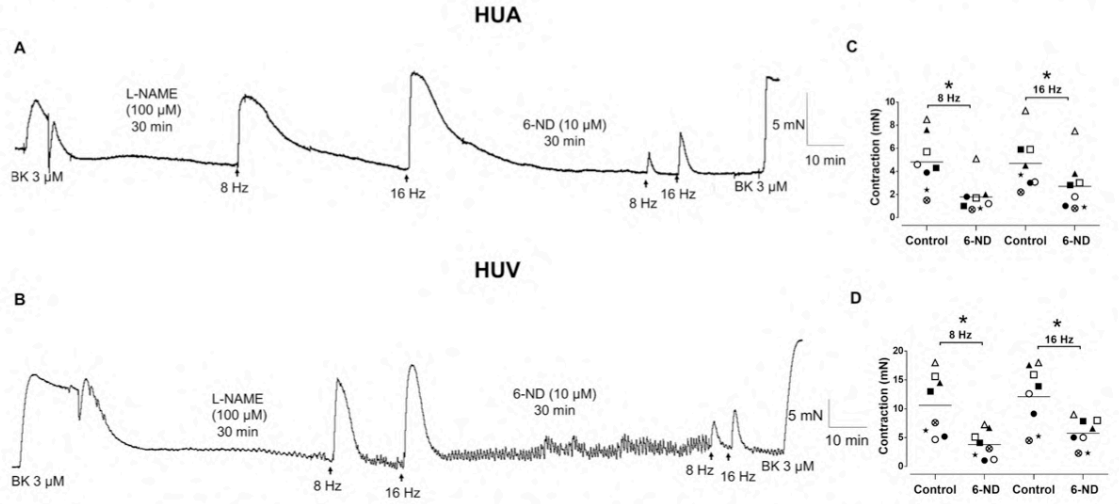


Fig. 4. Representative tracing of the treatment with 6-ND significant reduction in EFS-induced contractions in both HUA (panel A; control and 6-ND 10 μM) and HUV rings (panel B; control and 6-ND 10 μM). Scatter plot shows the individual values and mean ± SEM of the EFS-induced contractions in HUA (panel C; n = 5/8 for 8 Hz and 16 Hz) and HUV rings (panel D; n = 5/8 for 8 Hz and 16 Hz) in the absence and presence of 6-ND 10 μM ($P < 0.05$).

for 16 Hz; $p < 0.05$; n = 5/8; for control and 6-ND pre-treated vessels, respectively; Fig. 4B and D).

3.5. Relaxant effects of 6-ND in HUA and HUV

To evaluate the relaxation responses to 6-ND, tissues were initially pre-contracted with the TXA_2 mimetic U46619 (10 nM). Addition of 6-ND (0.1–300 nM) in the pre-contracted preparations produced concentration-dependent relaxations of both HUA (Fig. 5A; pEC_{50} 8.0 ± 0. 1; E_{max} 85.4 ± 5.9%; n = 5/9) and HUV (Fig. 5B; pEC_{50} 7.6 ± 0.1; E_{max} 62.1 ± 7.6%; n = 4/8), which were abolished by removal of the endothelium (Fig. 5A and B).

3.6. Relaxant effects of haloperidol and fenoldopam in HUA and HUV

To evaluate the relaxation responses to haloperidol (dopamine D_2-like receptor antagonist) and fenoldopam (dopamine D_1-receptor agonist), tissues were initially pre-contracted with the TXA_2 mimetic U46619 (10 nM). Haloperidol (0.1–300 nM) provoked concentration-dependent relaxations of both HUA (Fig. 5C) and HUV (Fig. 5D), which were abolished by endothelium removal.

Fenoldopam (0.1–300 nM) also induced concentration-dependent relaxations both HUA and HUV preparations, which were nearly abolished by pre-treatment with the dopamine D_1-receptor antagonist SCH-23390 (10 nM; Fig. 5E,F). In contrast to fenoldopam, SCH-23390 (10 nM) had no effect on 6-ND-induced HUA and HUV relaxations (Fig. 5G, H).

Incubation with 6-ND (10 μM) had no effect on the concentration-dependent contractions induced by U-46619 in both HUA (pEC_{50} 8.2 ± 0.2 and 8.4 ± 0.3 and E_{max} 9.8 ± 1.3 and 11.0 ± 2.6 mN; $n = 3/6$) and HUV (pEC_{50} 8.5 ± 0.2 and 8.6 ± 0.2 and E_{max} 14.5 ± 2.9 and 15.0 ± 2.9 mN; $n = 4/8$).

4. Discussion

The results presented here clearly demonstrate, for the first time in human vessels, that umbilical cord arteries and veins display a basal release of 6-ND. The release of 6-ND was inhibited by removal of the endothelium or by pre-treatment of the vessels with the NO synthesis inhibitor L-NAME, indicating that the synthesis of 6-ND is coupled to NO. Although the reduction was substantial (90%) in either case, the release of 6-ND was not abolished by either treatment. The biological half-life of 6-ND is unknown at the present moment, so it is possible that 6-ND could be stored in other cells once is formed by the endothelial cell. The expression of the dopamine transporter (DAT) is restricted mainly to dopaminergic neurons [9], however extraneuronal monoamine transporter has been found in smooth muscle cells [10]. Alternatively, the small amount of 6-ND detected after L-NAME incubation could be due to the lag-time for reaching full NO synthesis inhibition. It is interesting that 6-nitro-noradrenaline (6-NN) has been extracted from porcine and rat brains, and the amount extracted was reduced (50%) when the rats were pre-treated with L-NAME [3]. Nitric oxide is able to nitrate dopamine *in vitro* [4] which is consistent with our proposal that the formation of 6-ND is coupled to NO synthase activity. Whether the nitrosation/nitration of catecholamines *in vivo* is due to a chemical or enzymatic process is yet to be established.

Dopamine contracts human umbilical cord vessels (HUCV) due to interaction with D_2-like receptors [1]. Indeed, the dopamine D2-receptor agonist sumanirole [11] also induced contractions of HUA and HUV. Therefore, our findings that 6-ND antagonizes both dopamine- and sumanirole-induced HUCV contractions and did not affect either noradrenaline- or adrenaline-induced contractions indicate that 6-ND acts as a highly selective D_2-like receptor antagonist. Although the pA_2 values for 6-ND were on the lower range (5.7–5.9) when compared to classical D_2-like antagonists such as haloperidol (8.6) [12] and risperidone (8.3) [13], the observed selectivity is remarkable. The D_2-like antagonists do interact with adrenergic receptors [14], and the difference in potency is discrete (haloperidol, 1.4 and 4.7 nM and risperidone, 2.2 and 1.4 nM, for D_2 and α-1 adrenoceptor, respectively) [15]. The finding that 6-ND inhibited the contractions of HUCV induced by EFS further support the evidence that it is acting as an antagonist of D_2-like receptors in these vessels, since EFS-induced HUCV contractions are inhibited by haloperidol [1]. This is a novel mechanism of action for nitrocatecholamines in vascular tissue, and maybe restricted to 6-ND. Of interest, 6-nitronorepinephrine (6-NN) causes contraction of rat aortic rings, however it is 300 less potent than noradrenaline and that its

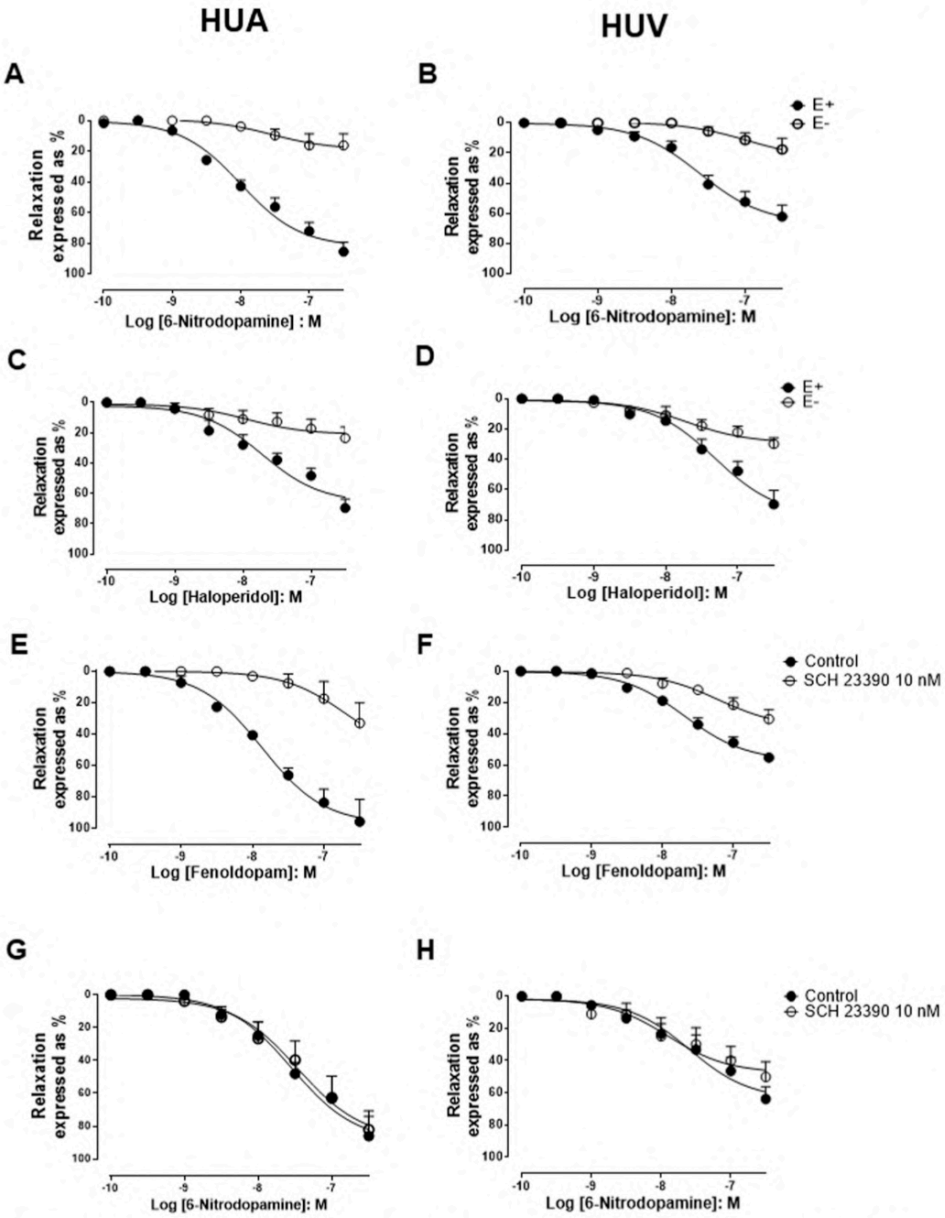


Fig. 5. In U-46619 pre-contracted (10 nM) HUCV 6-ND [panel A: HUA (n = 5/9); panel B: HUV (n = 4/8)] and Haloperidol [panel C: HUA (n = 5/10); panel D: HUV (n = 5/10)] caused concentration-dependent relaxation of both HUA and HUV. Which were abolished by mechanical removal of endothelium. Fenoldopam caused concentration dependent relaxation of both HUA and HUV which were abolished by SCH-23390 [panel E: HUA (*n* = 3/5); panel F: HUV (n = 3/5)]. SCH-23390 failed to affect the 6-ND-induced relaxation [panel G: HUA (n = 4/7); panel H: HUV (n = 3/5)].

contraction is abolished by the selective α1-adrenoceptor antagonist prazosin, indicating that 6-NN has a weak agonistic activity at α-1 adrenergic receptors [16]. It is worth mentioning that HUCV express functional α-1 adrenergic receptors, since the contractions induced by both noradrenaline in HUA rings [17] and adrenaline in HUV rings [18] are antagonized by the α-1 adrenergic receptor antagonist prazosin [19].

Another novel vascular action identified for 6-ND in our study was its ability to cause vasorelaxation in HUCV rings pre-contracted with the

thromboxane A_2 mimetic U46619 [20]. The relaxation was not due to interaction with the TXA_2 receptor [21] since 6-ND failed to affect the contractions induced by this agonist in HUCV. Although the D_1-receptor agonist fenoldopam [22] caused relaxations of both pre-contracted HUA and HUV, this mechanism of action is not involved in the 6-ND-induced relaxation, since it was not affected by the D_1-receptor antagonist SCH-23390 [12]. Our results indicate that the mechanism involved in 6-ND-induced relaxations is again antagonism of the D_2-like receptors present in the vasculature, for the following reasons: (i) it occurs in the presence of L-NAME, (ii) it is abolished by endothelium removal, (iii) it was not affected by SCH-23390 and (iv) the D_2-like antagonist haloperidol caused similar relaxation in both tissues. Indeed, D_1, D_2, D_3, D_4 and D_5 dopamine receptors are present in the peripheral vasculature [23–25].

The antagonism here described of the D_2-receptors by 6-ND has two major physiological consequences. First, it further supports the role of dopamine as an endogenous modulator of vascular reactivity *in vivo* [1]. It is interesting that schizophrenic women are at a lower risk of pre-eclampsia, although they have fewer antenatal care visits [26]. This could be due to the constant use of the D_2-receptor antagonists by these patients, since they are the main therapeutic agents for schizophrenia. A second point that needs addressing is the interpretation of the results obtained with the use of NO inhibitors, both *in vitro* and *in vivo*. Nitric oxide stimulates cGMP production [27], which causes vasorelaxation; the circulatory alterations caused by NO inhibition has been associated to the lack of vasodilatation due to decrease cGMP production. Our results may indicate that the culprit may not be the lack of NO, but the lack of 6-ND. This may explain the lack of improvement of short [28] or long-term nitrate therapy in patients who had acute myocardial infarction [29].

5. Conclusion

The endothelium-dependent release of 6-ND constitutes a novel mechanism by which NO may modulate vascular reactivity independently of cGMP production.

Declaration of competing interest

No conflict of interest.

Acknowledgements

JBJ & CESN thank CAPES for PhD fellowship. WCCS Thanks CNPq for master's fellowship. EA & FM thank FAPESP (2017/15175-1). GDN thanks CNPq (303839/2019-8) and FAEPEX (2170/20).

References

[1] J. Britto-Júnior, D.H.A. Pinheiro, A.F.O. Justo, G.M. Figueiredo-Murari, R. Campos, F.V. Mariano, V.B. de Souza, A.A Schenka, F.Z. Mónica, E. Antunes, G. De Nucci. Endothelium-derived dopamine modulates EFS-induced contractions of human umbilical vessels. Pharmacol. Res. Perspect.. (2020) Aug;8(4):e00612. doi:https://doi.org/10.1002/prp2.612.

[2] J. Britto-Júnior, F. Fernandes Jacintho, R. Campos, D.H.A. Pinheiro, G. M. Figueiredo Murari, V.B. de Souza, A.A. Schenka, F.Z. Mónica, R.A. Moreno, E. Antunes, G. De Nucci, The basal release of endothelium-derived catecholamines regulates the contractions of *Chelonoidis carbonaria* aorta caused by electrical-field stimulation, Biol Open. (2021) 057042, https://doi.org/10.1242/bio.057042.

[3] M. Tsunoda, E. Uchino, K. Imai, K. Hayakawa, T. Funatsu, Determination of nitrocatecholamines in rat brain using high-performance liquid chromatography-peroxyoxalate chemiluminescence reaction detection, J. Chromatogr. A 1164 (1–2) (2007) 162–166, https://doi.org/10.1016/j.chroma.2007.07.005, 14.

[4] F. Shintani, T. Kinoshita, S. Kanba, T. Ishikawa, E. Suzuki, N. Sasakawa, R. Kato, M. Asai, T. Nakaki, Bioactive 6-nitronorepinephrine identified in mammalian brain, J. Biol. Chem. 271 (23) (1996) 13561–13565, https://doi.org/10.1074/jbc.271.23.13561, 7. (PMID: 8662880).

[5] M. d'Ischia, C. Costantini, Nitric oxide-induced nitration of catecholamine neurotransmitters: a key to neuronal degeneration? Bioorg. Med. Chem. 3 (7) (1995) 923–927, https://doi.org/10.1016/0968-0896(95)00083-s (7582969).

[6] R. Campos, D.H. Pinheiro, J. Britto-Júnior, H.A. Castro, G. D. Mendes, O.M Moraes, M.E Moraes, R.A. Lopes-Martins, N.J. Antunes, G. De Nucci. Quantification of 6-nitrodopamine in Krebs-Henseleit's solution by liquid chromatography coupled to tandem mass spectrometry. Application in measurement of basal release from *Chelonoidis carbonaria* aortae in vitro (2021). J Chromatography, (in press).

[7] J. Britto-Júnior, F.F. Jacintho, G.M. Figueiredo-Murari, R. Campos, R.A. Moreno, E. Antunes, F.Z. Mónica, G. De Nucci, Electrical field stimulation induces endothelium-dependent contraction of human umbilical cord vessels, Life Sci. 243 (2020), 117257, https://doi.org/10.1016/j.lfs.2020.117257.

[8] O. Arunlakshana, H.O. Schild, Some quantitative uses of drug antagonists, Br. J. Pharmacol. Chemother. (1) (1959) 48–58, https://doi.org/10.1111/j.1476-5381.1959.tb00928.x.

[9] A.S. Kristensen, J Andersen, T.N Jørgensen, L. Sørensen, J. Eriksen, C.J. Loland, K. Strømgaard, U. Gether. SLC6 neurotransmitter transporters: structure, function, and regulation. Pharmacol. Rev. (2011);63(3):585–640. doi: https://doi.org/10.1124/pr.108.000869. Epub 2011 Jul 13. PMID: 21752877.

[10] G. Horvath, Z. Sutto, A. Torbati, G.E. Conner, M. Salathe, A. Wanner, Norepinephrine transport by the extraneuronal monoamine transporter in human bronchial arterial smooth muscle cells, Am J Physiol Lung Cell Mol Physiol 285 (4) (2003) L829–L837, https://doi.org/10.1152/ajplung.00054.2003.

[11] R.B. McCall, K.J. Lookingland, P.J. Bédard, R.M. Huff, Sumanirole, a highly dopamine D2-selective receptor agonist: in vitro and in vivo pharmacological characterization and efficacy in animal models of Parkinson's disease, J. Pharmacol. Exp. Ther. 314 (3) (2005) 1248–1256, https://doi.org/10.1124/jpet.105.084202.

[12] J.A. Bourne, SCH 23390: the first selective dopamine D1-like receptor antagonist, CNS Drug Rev. 7 (4) (2001) 399–414, https://doi.org/10.1111/j.1527-3458.2001.tb00207.x.

[13] Z. Nourian, T. Mow, D. Muftic, S. Burek, M.L. Pedersen, J. Matz, M.J. Mulvany, Orthostatic hypotensive effect of antipsychotic drugs in Wistar rats by in vivo and in vitro studies of alpha1-adrenoceptor function, Psychopharmacology 199 (1) (2008) 15–27, https://doi.org/10.1007/s00213-007-1064-9.

[14] G. Campiani, S. Butini, S. Gemma, V. Nacci, C. Fattorusso, B. Catalanotti, G. Giorgi, A. Cagnotto, M. Goegan, T. Mennini, P. Minetti, M.A.D. Cesare, N. Mastroianni, B. Scafetta, M.A. Galletti, M. Stasi, L. Castorina, L. Pacifici, O. Ghirardi, O. Tinti, P. Carminati, Pyrrolo[1,3]benzothiazepine-based atypical antipsychotic agents. Synthesis, structure-activity relationship, molecular modeling, and biological studies, J. Med. Chem. 45 (2) (2002) 344–359, https://doi.org/10.1021/jm010982y, 17.

[15] A.W. Schmidt, L.A. Lebel, H.R. Howard, S.H. Zorn, Ziprasidone: a novel antipsychotic agent with a unique human receptor binding profile, Eur. J. Pharmacol. 425 (3) (2001) 197–201, https://doi.org/10.1016/s0014-2999(01)01188-8, 17.

[16] T. Nakaki, T. Fujii, E. Suzuki, F. Shintani, Endothelium-independent and -dependent vasoactivity of 6-nitronorepinephrine, Eur. J. Pharmacol. 357 (2–3) (1998) 193–197, https://doi.org/10.1016/s0014-2999(98)00571-8, 18.

[17] G. Bodelsson, M. Stjernquist, Characterization of contractile adrenoceptors in the human umbilical artery, Eur. J. Pharmacol. 282 (1–3) (1995) 95–101, https://doi.org/10.1016/0014-2999(95)00277-r, 25.

[18] A.E. Errasti, M.P. Velo, R.M. Torres, S.P. Sardi, R.P. Rothlin, Characterization of alpha1-adrenoceptor subtypes mediating vasoconstriction in human umbilical vein, Br. J. Pharmacol. 126 (2) (1999) 437–442, https://doi.org/10.1038/sj.bjp.0702320.

[19] P. Greengrass, R. Bremner, Binding characteristics of 3H-prazosin to rat brain alpha-adrenergic receptors, Eur. J. Pharmacol. 55 (3) (1979) 323–326, 1.

[20] G.L. Bundy, The synthesis of prostaglandin endoperoxide analogs, Tetrahedron Lett. 16 (24) (1975) 1957–1960, https://doi.org/10.1016/S0040-4039(00)72333-1.

[21] Y. Kobayashi, A. Ricci, I. Rossodivita, F. Amenta, Autoradiographic localization of dopamine D2-like receptors in the rabbit pulmonary vascular tree, Naunyn Schmiedeberg's Arch. Pharmacol. 349 (6) (1994) 559–564, https://doi.org/10.1007/BF01258459.

[22] F. Amenta, Light microscope autoradiography of peripheral dopamine receptor subtypes, Clin. Exp. Hypertens. 19 (1–2) (1997) 27–41, https://doi.org/10.3109/10641969709080802.

[23] F. Amenta, P. Barili, E. Bronzetti, L. Felici, F. Mignini, A. Ricci, Localization of dopamine receptor subtypes in systemic arteries, Clin. Exp. Hypertens. 22 (3) (2000) 277–288, https://doi.org/10.1081/ceh-100100077.

[24] B.E. Bennedsen, P.B. Mortensen, A.V. Olesen, T.B. Henriksen, M. Frydenberg, Obstetric complications in women with schizophrenia, Schizophr. Res. 47 (2–3) (2001) 167–175, https://doi.org/10.1016/s0920-9964(99)00234-0, 1.

[25] A.J. Nichols, R.R. Ruffolo, D.P. Brooks, The pharmacology of fenoldopam, Am. J. Hypertens. 3 (6 Pt 2) (1990) 116S–119S, https://doi.org/10.1093/ajh/3.6.116s. Jun.

[26] M. Feelisch, Biotransformation to nitric oxide of organic nitrates in comparison to other nitrovasodilators, Eur. Heart J. 14 (1993) 123–132.

[27] GISSI-3, Effects of lisinopril and transdermal glyceryl trinitrate singly and together on 6-week mortality and ventricular function after acute myocardial infarction. Gruppo Italiano per lo studio della Sopravvivenza nell'infarto Miocardico, Lancet 343 (8906) (1994) 1115–1122, 7.

[28] ISIS-4, A randomised factorial trial assessing early oral captopril, oral mononitrate, and intravenous magnesium sulphate in 58,050 patients with suspected acute myocardial infarction. ISIS-4 (fourth international study of infarct survival) collaborative group, Lancet 345 (8951) (1995) 669–685, 18.

[29] S. Kojima, K. Matsui, T. Sakamoto, M. Ishihara, K. Kimura, S. Miyazaki, M. Yamagishi, C. Tei, H. Hiraoka, M. Sonoda, K. Tsuchihashi, N. Shimoyama, T. Honda, Y. Ogata, H. Ogawa, Japanese Acute Coronary Syndrome Study (JACSS) Investigators, Long-term nitrate therapy after acute myocardial infarction does not

improve or aggravate prognosis, Circ. J. 71 (3) (2007) 301–307, https://doi.org/10.1253/circj.71.301.

Anexo 5

Artigo 7 – Quantification of 6-nitrodopamine in Krebs-Henseleit's solution by LC-MS/MS for the assessment of its basal release from Chelonoidis carbonaria aortae *in vitro.*

Autores: Campos R, Pinheiro DHA, Britto-Júnior J, Castro HA, Mendes GD, Moraes MO, Moraes MEA, Lopes-Martins RÁB, Antunes NJ, De Nucci G.

Revista: Journal of Chromatography B

Situação: Aceito a publicação em 24 de setembro de 2020. Publicado on-line em 22 de março de 2021

Journal of Chromatography B 1173 (2021) 122668

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Quantification of 6-nitrodopamine in Krebs-Henseleit's solution by LC-MS/MS for the assessment of its basal release from *Chelonoidis carbonaria* aortae *in vitro*

Rafael Campos [a,b,1], David Halen Araújo Pinheiro [c,1], José Britto-Júnior [d], Heleson Alves de Castro [f], Gustavo Duarte Mendes [f,g], Manoel Odorico Moraes [b], Maria Elisabete A. Moraes [b], Rodrigo Álvaro Brandão Lopes-Martins [d,e], Natalícia J. Antunes [d,*], Gilberto De Nucci [b,f,g]

[a] Superior Institute of Biomedical Sciences, Ceará State University (UECE), Fortaleza, CE, Brazil
[b] Clinical Pharmacology Unit, Drug Research and Development Center, Federal University of Ceará (UFC), Fortaleza, CE, Brazil
[c] Institute of Research and Development, University of Vale do Paraíba, São José dos Campos, SP, Brazil
[d] Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, SP, Brazil
[e] Laboratório de Biofotônica e Terapêutica Experimental em Saúde e Estética, Programa de Pós-graduação em Bioengenharia, Universidade Brasil, Itaquera, SP, Brazil
[f] Faculty of Medicine, São Leopoldo Mandic, Campinas, SP, Brazil
[g] Department of Pharmacology, Faculty of Medicine, Metropolitan University of Santos, Santos, SP, Brazil

ARTICLE INFO

Keywords:
6-nitrodopamine
LC-MS/MS
Solid phase extraction
Tortoise
Endothelium

ABSTRACT

In this study, the development and validation of a method for quantification of 6-nitrodopamine in Krebs-Henseleit's solution by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with positive ion electrospray ionization is described. Aortic rings taken from tortoise were either denuded or left with endothelium intact (15 mm, N = 6) and were incubated for 30 min in 5 mL Krebs-Henseleit's solution in an organ bath. Solid phase extraction (SPE) was performed for aliquots of 1 mL of the supernatant. The separation of 6-nitrodopamine was obtained on a 150 mm × 3 mm Shim-pack GIST-HP C18 column, using 75% of mobile phase A consisted of deionized water with 0.1% formic acid (*v/v*) and 25% of mobile phase B consisted of acetonitrile/deionized water (50/50, *v/v*) + 0.1% formic acid at a flow rate of 350 μL/min in an isocratic mode. The method was linear over the concentration range of 0.1–20 ng/mL. The method was sensitive, precise and accurate for the assessment of the basal release of 6-nitrodopamine from *Chelonoidis carbonaria* aortae *in vitro*. The mean ± SEM concentrations of 6-nitrodopamine released from endothelium-intact and endothelium-denuded aortae were 0.44 ± 0.06 ng/mL and 0.18 ± 0.05 ng/mL, respectively. These results indicate that tortoise's aortae display a basal endothelium-derived 6-nitrodopamine release.

1. Introduction

6-Nitrodopamine is a member of 6-nitrocatecholamines family and a putative product of the nitric oxide (NO)-dependent nitration of dopamine [1,2]. *In vitro*, 6-nitrodopamine inhibits neuronal nitric oxide synthase (nNOS) activity [2]. Tortoise *Chelonoidis carbonaria* aortic endothelium releases mediator(s) capable of modulating electrical field stimulation (EFS) induced spasmogenic activity [3], similarly shown in human umbilical cord vessels (HUCV) [4]. *Chelonoidis carbonaria* aortic rings released dopamine, noradrenaline and adrenaline *in vitro* [5]. In humans, both umbilical arteries (HUA) and human umbilical vein vessels (HUV) also present a basal release of endothelium-derived dopamine [6]. Within the central nervous system (CNS), dopamine is a key neurotransmitter as well as being an important modulator of blood pressure, sodium balance, and renal and adrenal function and thus relevant to the pathophysiology of hypertension [7]. Endothelium-

* Corresponding author at: State University of Campinas (UNICAMP), Faculty of Medical Sciences, Department of Pharmacology, Alexander Fleming St, 105, 13083-881, Campinas, SP, Brazil.
E-mail address: nataliciaja@gmail.com (N.J. Antunes).
[1] R.C. and D.H.A.P. contributed equally to this work.

https://doi.org/10.1016/j.jchromb.2021.122668
Received 24 September 2020; Received in revised form 9 March 2021; Accepted 11 March 2021
Available online 22 March 2021
1570-0232/© 2021 Elsevier B.V. All rights reserved.

derived dopamine presents itself an important role in vascular reactivity [6]. These findings raised the question about the possibility of dopamine-derived products such as 6-nitrodopamine could also be released by the endothelium of blood vessels.

To date, no method of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to measure 6-nitrodopamine in biological samples has been described in the literature. This study shows for the first time the development and validation of a specific and sensitive method to quantify 6-nitrodopamine in Krebs-Henseleit's solution by LC-MS/MS. The method was applied to measure the basal release of 6-nitrodopamine from *Chelonoidis carbonaria* aortae *in vitro*.

2. Material and methods

2.1. Animals

Tortoises (*Chelonoidis carbonaria;* N = 6) of either sexes (2.0–7.0 kg weight) were obtained from the Parque Ecológico do Tietê (São Paulo, SP, Brazil) and its use was allowed by the Brazilian Institute for Environment (Sisbio; number 20910). The experimental protocols were approved by the Institutional Animal Care and Use Committee (CEUA/UNICAMP: 5265-1/2019) and were performed according to the ARRIVE guidelines.

2.2. Material

6-Nitrodopamine and 6-nitrodopamine-d4 (internal standard - IS) were obtained from Toronto Research Chemicals (TRC, North York, Canada). Ascorbic acid and trifluoroacetic acid were obtained from Sigma-Aldrich Chemicals Co. (Missouri, USA). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$), potassium phosphate monobasic (KH_2PO_4) and glucose were acquired from Merck KGaA (Darmstadt, Germany). Acetonitrile and methanol were supplied by J.T Baker (Phillipsburg, NJ, USA). Ultrapure water was prepared in-house using the Synergy UV® purification system (Millipore, Molsheim, France).

2.3. Calibrators and quality controls preparation

Stock solutions of 6-nitrodopamine and the IS 6-nitrodopamine-d4 were made in acetonitrile/ deionized water (20/80, *v/v*) at concentrations of 100 µg/mL for both analyte and IS. The work solution of 6-nitrodopamine was diluted in acetonitrile/deionized water (20/80, *v/v*) to generate the work solutions for calibration standards at concentrations of 1, 2, 5, 10, 20, 50, 100 and 200 ng/mL. Calibration curves were prepared in duplicate by spiking 1 mL of blank Krebs-Henseleit's solution with 100 µL of each standard solution to obtain the concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng/mL. Krebs-Henseleit's solution consisted of 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaCO_3$, 1.2 mM KH_2PO_4, 5.6 mM glucose and 3 mM ascorbic acid. The IS stock solution was diluted to 1 µg/mL in acetonitrile/ deionized water (20/80, *v/v*).

The quality control (QC) samples were prepared by diluting work solutions to obtain the concentrations of 0.3 (low), 1.5 (medium 1), 9 (medium 2) and 15 (high) ng/mL of 6-nitrodopamine in blank Krebs-Henseleit́s solution.

2.4. Aortic rings preparation

Sedation of tortoises was carried out with 2 mg/kg of midazolam (intramuscular), and anesthesia was made with 40 mg/kg of ketamine (intramuscular) and 15 mg/kg of propofol (intravenous). Then, the animals were euthanized by exsanguination. The dorsal aortae was removed, segmented into rings and immediately placed in Krebs-Henseleit's solution at 27 °C. Aortic rings were either left with the endothelium intact or rubbed gently with forceps to denude the endothelium. Then, two aortic rings per animal (15 mm), one endothelium-intact and another endothelium-denuded aortic ring were suspended in organ baths with 5 mL of Krebs-Henseleit's solution gassed with a mixture of 95% O_2:5% CO_2 (pH 7.4) at 27 °C. This temperature is commonly used for tissue experiments in reptiles [3,8]. After 30 min incubation, 2 mL aliquots of the supernatant were collected and stored at −20 °C.

2.5. Sample preparation

Extraction of 6-nitrodopamine from 1 mL Krebs-Henseleit's solution was performed by solid phase extraction (SPE). Calibrators, QCs and samples of of tortoise's aortic rings incubations were spiked with 50 µL of IS solution (100 ng/mL nitrodopamine-d4). Strata™-X 33 µm Polymeric Reversed Phase SPE cartridges, 30 mg/mL (Phenomenex, Torrance, CA, USA) were conditioned with 1 mL of methanol and then, equilibrated with 2 mL of deionized water. The samples were transferred to the extraction cartridges and washed three times with deionized water. The samples were eluted with 0.9 mL methanol/deionized water (90/10, *v/v*) + 0.1% formic acid followed by evaporation under N_2 flow at 50 °C. The dry residues were dissolved with 100 µL acetonitrile/ deionized water (50/50, *v/v*) + 0.1% formic acid and transferred to vials for analysis.

2.6. 6-Nitrodopamine quantification

The LC-MS/MS used was a Nexera HPLC system consisted of an autoinjector model SIL-30AC, a LC-30AD binary pump and a CTO-20AC column oven coupled to a LCMS-8060 triple quadrupole mass spectrometer (MS/MS) (Shimadzu, Kyoto, Japan).

6-Nitrodopamine was separated on a 150 mm × 3 mm Shim-pack GIST-HP C18 column, 3 µm particle size (Shimadzu, Duisburg, Germany), held at 65 °C and using 75% of mobile phase A consisting of deionized water with 0.1% formic acid (*v/v*) and 25% of mobile phase B consisting of acetonitrile/deionized water (50/50, *v/v*) + 0.1% formic acid at a flow rate of 350 µL/min in an isocratic mode. The auto-sampler temperature was kept at 8 °C.

The detection of 6-nitrodopamine and IS was carry out by a MS/MS system in positive ionization mode. The ion spray voltage was maintained at 4 kV, desolvation temperature at 250 °C, nebulizer gas flow at 3 L/min, heat block temperature at 400 °C, drying gas flow at 10 L/min. The cone voltage was 5 V and collision energy was 15 V for both 6-nitrodopamine and 6-nitrodopamine-d4. The analyses were performed in the multiple reaction monitoring (MRM) mode. The protonated ions $[M + H]^+$ and their respective ion products were monitored in the 199.1 > 181.95 for 6-nitrodopamine and 203.1 > 186 for 6-nitrodopamine-d4.

2.7. Method validation

The validation of the method for quantification of 6-nitrodopamine was performed following the Food and Drug Administration (FDA) guideline [9].

2.7.1. Linearity and limit of quantification

Method linearity was evaluated by constructing calibration curves from eight calibrator standards in the range of 0.1–20 ng/mL in duplicate. The peak area ratios of 6-nitrodopamine:IS were plotted against the nominal concentration of 6-nitrodopamine. A weighted (1/x) linear regression analysis was performed to determine the slope of the calibration lines, intercept, and correlation coefficient (r) for assessment of the linearity. The acceptance criteria for r was a value ≥ 0.98.

The limit of quantification (LLoQ) was evaluated by analyzing 7 replicates as the lowest concentration of 6-nitrodopamine on the calibration curve with a precision ≤ 20%, determined by the coefficient of variation (CV %), and accuracy within 80%–120%, calculated as the percentage relative error (% RE).

2.7.2. Selectivity

Selectivity was evaluated using 6 replicates of blank Krebs-Henseleit's solution samples. The samples were extracted, and the chromatograms compared to the LLoQ samples.

2.7.3. Carryover

In order to evaluate carryover, extracted blank Krebs-Henseleit's solution samples, were injected directly after the injection of the highest calibrator standard (20 ng/mL 6-nitrodopamine) sample. Thus, the peak area of blank sample was compared with the LLoQ. Carryover was considered to be acceptable if the peak area of 6-nitrodopamine in the blank analyzed after highest calibrator is ≤ 20% of LLoQ and ≤ 5% of IS.

2.7.4. Precision and accuracy

The intra- and inter-batch precision and accuracy studies was evaluated by analyzing seven replicates of LLoQ and QC samples (0.3, 1.5, 9 and 15 ng/mL) in the same analytical run and in three consecutive days, respectively. Intra and inter-batch precision (% CV) should be ≤ 15% for QCs and ≤ 20% for LLoQ, and accuracy (% RE) within the range of 85–115 % for QCs and 80–120% for LLoQ.

2.7.5. Matrix effect and recovery

Evaluation of matrix effect was done by comparing the peak area of 6-nitrodopamine (0.3 and 15 ng/mL) and IS prepared in mobile phase, with the peak areas from standard solutions added to blank Krebs-Henseleit's solution extracts (N = 3). A matrix factor normalized by IS (MFN) was calculated for each sample as follows: MFN = (peak area of 6-nitrodopamine in matrix/IS peak area in matrix)/(peak area of 6-nitrodopamine in the absence of matrix /peak area of IS in the absence of matrix). The CV must be ≤ 15%.

Recovery was evaluated by direct comparison of the peak area of 6-nitrodopamine extracts at concentrations of 0.3 and 15 ng/mL, in triplicate, with peak area obtained from standard solutions (0.3 and 15 ng/mL) added to blank Krebs-Henseleit's solution extracts.

2.7.6. Stability

The stability of the method was evaluated by submitting 3 replicates of low and high QCs (0.3 and 15 ng/mL) to 6 h short-term at room temperature, 3 freeze-thaw (−20 to 25 °C) cycles and 53 h auto-sampler (8 °C) stability tests. The results were compared with those obtained by the analysis of freshly prepared QCs.

2.8. Data analysis

Data are expressed as mean ± standard error of the mean (SEM) of the number of experiments (N = 6). Unpaired Student's *t* test was used and a *p*-value < 0.05 was considered as significant.

3. Results and discussion

This study presents a novelty method for quantification of 6-nitrodopamine in Krebs-Henseleit's solution samples after incubation of tortoise aortic rings, using LC-MS/MS and SPE.

3.1. LC-MS/MS analysis and sample preparation

The mass spectra for 6-nitrodopamine and IS are presented in Fig. 1. The total run time was 3.5 min and the retention time was 1.78 ± 0.3 for both 6-nitrodopamine and 6-nitrodopamine-d4 (Fig. 2).

This method was developed based on a previous study from our group [5]. However, in this study, Strata™-X cartridges were used as sorbent for sample preparation instead adding aluminum oxide (Al_2O_3) directly into the samples. Using these cartridges for extraction of 6-nitrodopamine resulted in mean recoveries of 95.49% and 92.16% for low and high QC samples, respectively. When extracted with Al_2O_3, the recovery was much lower (29.7% and 17.46% for low and high QC samples, respectively). The introduction of a nitro group, an electron withdrawing substituent, in the aromatic catechol ring decreases both the protonation and Al (III) complex formation constants of the substituted ligands because of its inductive and resonance effects, which strongly diminish the basicity of the two phenolic groups in catechol [10]. The nitro group also decreases the covalent component leading to lower binding affinity [11].

Strata™-X cartridges were also tested for catecholamines extraction and the mean recoveries were 94.15% and 93.64% for low and high QC samples of dopamine, 98.97% and 95.03% for low and high QC samples of adrenaline, and 100.02% and 92.4% for low and high QC samples of noradrenaline. These results show that Strata™-X cartridges can be used for catecholamine sample preparation.

There are also some differences in the chromatographic conditions between the methods. Catecholamines were separated on a 100 × 4.6 mm Lichrospher RP-8 column using as mobile phase acetonitrile/water (90/10, v/v) + 2.5 mM ammonium hydroxide at a flow rate of 1.3 mL/min in the previous study. In this work, 6-nitrodopamine was separated on a 150 mm × 3 mm Shim-pack GIST-HP C18 column, 3 µm particle size, using 75% of mobile phase A consisting of deionized water with

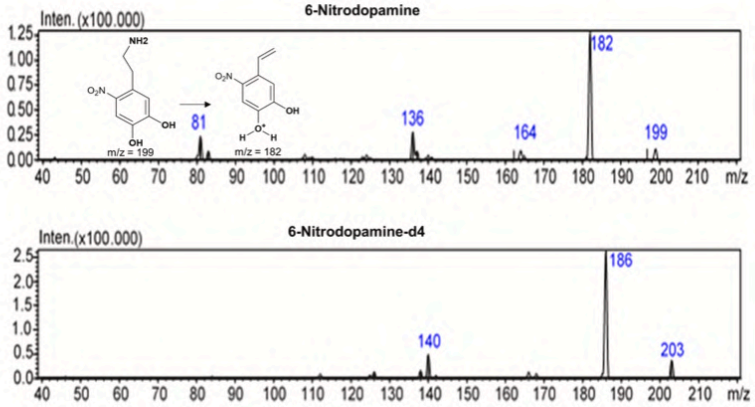


Fig. 1. Product-ion spectra of the protonated molecular ion of 6-nitrodopamine (m/z = 199) and 6-nitrodopamine-d4 (m/z = 203) acquired in positive-mode electrospray ionization.

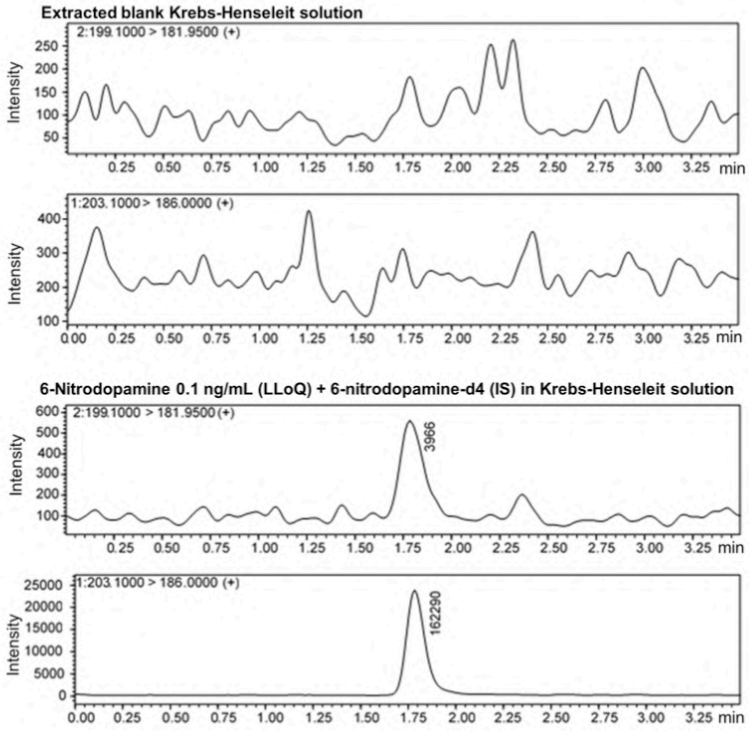


Fig. 2. Chromatograms obtained in the analysis of 6-nitrodopamine. Blank Krebs-Henseleit's solution and blank Krebs-Henseleit's solution spiked with 0.1 ng/mL 6-nitrodopamine and IS.

0.1% formic acid (v/v) and 25% of mobile phase B consisting of acetonitrile/deionized water (50/50, v/v) + 0.1% formic acid at a flow rate of 350 µL/min. The chromatographic conditions used in the present study generated a retention time of 1.78 min, while the previous one resulted in resulted in retention time of 4.75 min. As done for catecholamines, 3 mM ascorbic acid was added to Krebs-Henseleit's solution to prevent 6-nitrodopamine degradation [5,12].

3.2. Validation

3.2.1. Linearity

Fig. 3 shows the correlation obtained with different concentrations of standard 6-nitrodopamine (concentration–response curve). The method was linear in the concentrations range of 0.1–20 ng/mL. The r was ˃ 0.99.

3.2.2. Selectivity

The method was selective since the absence of endogenous interferences in the 6-nitrodopamine quantification was observed.

3.2.3. Carryover

No carryover was observed, no peaks in the blank samples analyzed immediately after ULQ sample were observed. No significant interference was found on the retention time of 6-nitrodopamine and 6-nitrodopamine-d4.

3.2.4. Precision and accuracy

Intra- and inter-batch precision (CV %) and accuracy (% RE) were < 14.9% and between 93.2 and 103.7 %, respectively (Table 1), guaranteeing the results were reproducible and repetitive.

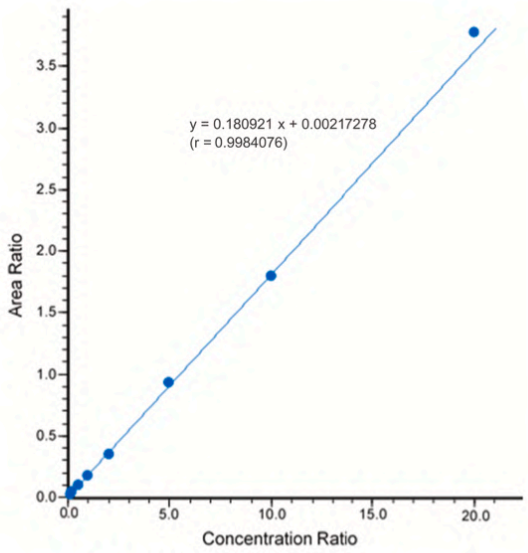


Fig. 3. Calibration curve of 6-nitrodopamine.

3.2.5. Matrix effect

No significant matrix effect on ionization of 6-nitrodopamine and IS was observed (Table 2).

Table 1
Precision and accuracy data from 6-nitrodopamine in Krebs-Henseleit's solution.

Parameter	6-Nitrodopamine (ng/mL)				
	LLoQ(0.1)	QC(0.3)	QC (1.5)	QC (9)	QC (15)
Intra-batch					
Mean (n = 7)	0.104	0.3	1.4	8. 8	14.6
Precision (% CV)	12.7	5.8	4.8	2.4	4.3
Accuracy (%)	103.7	94.3	94.7	97.5	97.2
Inter-batch					
Mean (n = 21)	0.104	0.3	1.4	8.9	14.8
Precision (% CV)	14.9	6.3	4.4	2.6	4.2
Accuracy (%)	103.6	93.2	93.8	98.5	98.3

CV % = [(SD/M) × 100]; Accuracy % = (E − T) × 100; CV, coefficient of variation; M, mean; SD, standard deviation of M; E, experimentally determined concentration; T, theoretical concentration; LLoQ, lower limit of quantification; QC, quality control.

Table 2
Matrix effect for 6-nitrodopamine in Krebs-Henseleit's solution.

6-Nitrodopamine	MFN	CV (%)
0.3 ng/mL (n = 5)	1.08	9
15 ng/mL (n = 5)	1.04	13.3

MFN = matrix factor normalized by internal standard [(response of the analyte in matrix/internal standard response matrix)/(response of the analyte in the absence of matrix /response of the internal standard in the absence of matrix)]; CV %= coefficient of variation [(standard deviation MFN/mean MFN) × 100].

3.2.6. Stability

6-Nitrodopamine was stable in Krebs-Henseleit's solution after 6 h at room temperature, 3 freeze–thaw cycles, and after 53 h in the auto-injector at 8 °C (Table 3).

3.3. Method application

The developed and validated method was applied to the measurement of basal release of 6-nitrodopamine from *Chelonoidis carbonaria* aortae *in vitro*. Table 4 shows the concentrations of 6-nitrodopamine detected in aortic rings (15 mm) of 6 animals placed in Krebs-Henseleit's solution (5 mL) for 30 min, and the comparison between the amount released from endothelium-intact and endothelium-denuded aortae. The amounts were not corrected by either weight or length of the vessel. The removal of endothelium decreased the release of 6-nitrodopamine from aortae indicating that tortoise's aortae display a basal endothelium-derived 6-nitrodopamine release. This is the first evidence of release of 6-nitrodopamine from a biological tissue. 6-Nitrodopamine was previously extracted in rat brain by detecting a chemiluminescence reaction of peroxyoxalate-high performance liquid chromatography (POCL), but as reproducibility (<10%) was not reliable, it was not quantified [13].

4. Conclusion

The method developed and validated for quantification of 6-nitrodopamine by LC-MS/MS in Krebs-Henseleit solution was sensitive, precise and accurate enough for its application in the measurement of basal release from *Chelonoidis carbonaria* aortae *in vitro*. Tortoise's aortae display a basal endothelium-derived 6-nitrodopamine release.

Funding

This study was funded by Grant n. 88887.342238/2019-00 CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Grant n. 303839/2019-8 CNPq (National Council for Scientific and Technological Development) and Grant n. 2016/22506-1 FAPESP (São Paulo Research Foundation).

Table 3
Summary stability data of 6-nitrodopamine in Krebs-Henseleit's solution using LC-MS/MS.

Stability	Mean (ng/mL)	CV (%)	Accuracy (%)
Freshly prepared			
0.3 ng/mL	0.3	9.9	100
15 ng/mL	14.7	4.6	98
Short term (6 h)			
0.3 ng/mL	0.298	5.8	99.3
15 ng/mL	14.9	0.9	99.3
Freeze/thaw (3 cycles)			
0.3 ng/mL	0.31	8.9	103.3
15 ng/mL	14.7	3.6	98
Post-processing (53 h at 8 °C)			
0.3 ng/mL	0.3	6.9	100.3
15 ng/mL	15	3.1	100

CV % = coefficient of variation [(SD/mean) × 100]; Accuracy % = [(E/T) × 100]; E, experimentally determined concentration; T, theoretical concentration; QC, quality control.

Table 4
Concentration of 6-nitrodopamine in Krebs-Henseleit's solution samples after incubation of *Chelonoidis carbonaria* aortic rings.

Sample number	6-Nitrodopamine (ng/mL)	
	E^+	E^-
1	0.25	0.15
2	0.7	0.3
3	0.36	0.08
4	0.41	0.34
5	0.55	0.1
6	0.38	0.1
Mean	0.44	0.18
Standard error of the mean	0.06	0.05
***p*-value**	0.0076	

E^+, Aortae endothelium-intact rings; E^-, Aortae endothelium-denuded rings.

CRediT authorship contribution statement

Rafael Campos: Investigation, Methodology, Writing - original draft. **David Halen Araújo Pinheiro:** Investigation, Methodology, Writing - original draft. **José Britto-Júnior:** Data curation, Formal analysis, Writing - original draft. **Heleson Alves Castro:** Visualization. **Gustavo Duarte Mendes:** Validation. **Manoel Odorico Moraes:** Conceptualization. **Maria Elisabete A. Moraes:** Conceptualization. **Rodrigo Álvaro Brandão Lopes Martins:** Supervision. **Natalícia J. Antunes:** Formal analysis, Writing - original draft, Validation, Writing - review & editing. **Gilberto De Nucci:** Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] A. Palumbo, A. Napolitano, P. Barone, M. D'Ischia, Nitrite- and peroxide-dependent oxidation pathways of dopamine: 6- nitrodopamine and 6-hydroxy-dopamine formation as potential contributory mechanisms of oxidative stress- and nitric oxide-induced neurotoxicity in neuronal degeneration, Chem. Res. Toxicol. 12 (1999) 1213–1222, https://doi.org/10.1021/tx990121g.
[2] A. Palumbo, G. Astarita, M. D'Ischia, Inhibition of neuronal nitric oxide synthase by 6-nitrocatecholamines, putative reaction products of nitric oxide with catecholamines under oxidative stress conditions, Biochem. J. 356 (2001) 105–110, https://doi.org/10.1042/0264-6021:3560105.
[3] R. Campos, F.F. Jacintho, J. Britto-Júnior, F.Z. Mónica, A.F.O. Justo, A.S. Pupo, R. A. Moreno, V.B. de Souza, A.A. Schenka, E. Antunes, G. De Nucci, Endothelium modulates electrical field stimulation-induced contractions of Chelonoidis carbonaria aortic rings, Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 233 (2020) 108763, https://doi.org/10.1016/j.cbpc.2020.108763.

[4] J. Britto-Júnior, F. Fernandes, G.M. Figueiredo, R. Campos, R. Agnaldo, E. Antunes, F.Z. Mónica, G. De Nucci, Electrical field stimulation induces endothelium-dependent contraction of human umbilical cord vessels, Life Sci. 243 (2020), 117257, https://doi.org/10.1016/j.lfs.2020.117257.

[5] J. Britto-Júnior, N.J. Antunes, R. Campos, M. Sucupira, G.D. Mendes, F. Fernandes, M.O. Moraes, M.E.A. Moraes, Determination of dopamine, noradrenaline and adrenaline in Krebs-Henseleit's solution by liquid chromatography coupled tandem mass spectrometry. Measurement of basal release from Chelonoidis carbonaria aortae in vitro, Biomed. Chromatogr. (2020) 1–10, https://doi.org/10.1002/bmc.4978.

[6] J. Britto-Júnior, D.H.A. Pinheiro, A.F.O. Justo, G.M. Figueiredo Murari, R. Campos, F.V. Mariano, V.B. Souza, A.A. Schenka, F.Z. Mónica, E. Antunes, G. De Nucci, Endothelium-derived dopamine modulates EFS-induced contractions of human umbilical vessels, Pharmacol. Res. Perspect. 8 (2020) 1–12, https://doi.org/10.1002/prp2.612.

[7] C. Zeng, H. Sanada, H. Watanabe, G.M. Eisner, R.A. Felder, P.A. Jose, H. Sanada, H. Watanabe, G.M. Eisner, R.A. Felder, P.A. Jose, Functional genomics of the dopaminergic system in hypertension, Physiol. Genom. 2197 (2004) 233–246, https://doi.org/10.1152/physi-olgenomics.00127.2004.

[8] R. Campos, A.F.O. Justo, F.F. Jacintho, F.Z. Mónica, J.A. Rojas-Moscoso, R. A. Moreno, M. Napolitano, J.C. Cogo, G. De Nucci, Pharmacological and transcriptomic characterization of the nitric oxide pathway in aortic rings isolated from the tortoise Chelonoidis carbonaria, Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 222 (2019) 82–89, https://doi.org/10.1016/j.cbpc.2019.04.015.

[9] FDA, Food and Drug Administration. Bioanalytical Method Validation Guidance, Food Drug Adm. 1043 (2018) 25. https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf (accessed April 22, 2020).

[10] V.M. Nurchi, T. Pivetta, J.I. Lachowicz, G. Crisponi, Effect of substituents on complex stability aimed at designing new iron(III) and aluminum(III) chelators, J. Inorg. Biochem. 103 (2009) 227–236, https://doi.org/10.1016/j.jinorgbio.2008.10.011.

[11] G. Dalla Torre, J.I. Mujika, E. Formoso, E. Matito, M.J. Ramos, X. Lopez, Tuning the affinity of catechols and salicylic acids towards Al(III): Characterization of Al-chelator interactions, Dalt. Trans. 47 (2018) 9592–9607, https://doi.org/10.1039/c8dt01341a.

[12] J.C. Van Oene, P. Sminia, A.H. Mulder, A.S. Horn, The purported dopamine agonist DPI inhibits [3H]noradrenaline release from rat cortical slices but not [3H] dopamine and [14C]acetylcholine release from rat striatal slices in-vitro, J. Pharm. Pharmacol. 35 (1983) 786–792, https://doi.org/10.1111/j.2042-7158.1983.tb02897.x.

[13] M. Tsunoda, E. Uchino, K. Imai, K. Hayakawa, T. Funatsu, Determination of nitrocatecholamines in rat brain using high-performance liquid chromatography-peroxyoxalate chemiluminescence reaction detection, J. Chromatogr. A. 1164 (2007) 162–166, https://doi.org/10.1016/j.chroma.2007.07.005.

Anexo 6

Artigo 8 – 6-Nitrodopamine is an endogenous mediator of rat isolated epididymal vas deferens contractions induced by electric-field stimulation.

Autores: Britto-Júnior J, Ximenes L, Ribeiro A, Fregonesi A, Campos R, Ricardo de Almeida Kiguti L, Mónica FZ, Antunes E, De Nucci G.

Revista: Journal of Chromatography B

Situação: Aceito a publicação em 29 de setembro de 2021. Publicado on-line em 01 de setembro de 2021

European Journal of Pharmacology 911 (2021) 174544

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

6-Nitrodopamine is an endogenous mediator of rat isolated epididymal vas deferens contractions induced by electric-field stimulation

José Britto-Júnior [a,*], Luiz Ximenes [a], André Ribeiro [a], Adriano Fregonesi [a], Rafael Campos [b,c], Luiz Ricardo de Almeida Kiguti [d], Fabíola Z. Mónica [a], Edson Antunes [a], Gilberto De Nucci [a,b,d,e]

[a] Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), São Paulo, Campinas, Brazil
[b] Clinical Pharmacology Unit, Drug Research and Development Center, Federal University of Ceará (UFC), Fortaleza, CE, Brazil
[c] Superior Institute of Biomedical Sciences, Ceará State University (UECE), Fortaleza, Brazil
[d] Metropolitan University of Santos (UNIMES), Santos, Brazil
[e] Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo (USP), São Paulo, Brazil

ARTICLE INFO

Keywords:
Dopamine
Nitric oxide
Ejaculation
Tricyclic antidepressants
Carbamazepine
Cyclobenzaprine

ABSTRACT

6-nitrodopamine (6-ND) is released from human umbilical cord vessels and modulates vascular reactivity by acting as a dopamine antagonist. Here we investigate whether 6-ND is released by the rat isolated vas deferens and its effect on this tissue. Dopamine, noradrenaline, adrenaline and 6-ND levels were quantified in rat isolated vas deferens by LC-MS-MS. Electric-field stimulation (EFS) and concentration-response curves to 6-ND, noradrenaline, dopamine and adrenaline were performed in the absence and in the presence (30 min) of L-NAME, SCH-23390, haloperidol, PG-01037, sonepiprazole, desipramine, clomipramine, amitriptyline, cyclobenzaprine, carbamazepine, maprotiline, paroxetine, oxcarbazepine and ketanserin in the rat isolated epididymal vas deferens (RIEVD).

Basal releases of 6-ND and noradrenaline were detected from the rat isolated vas deferens. 6-ND release was reduced by tissue incubation with L-NAME and from the vas deferens obtained from L-NAME-treated rats.

SCH-23390 caused leftward shifts on concentration-response curves to 6-ND without affecting dopamine- or EFS-induced RIEVD contractions. Haloperidol, PG-01037 and sonepiprazole caused significant rightward shifts on concentration-response curves to dopamine but had no effect on either the 6-ND or EFS-induced RIEVD contractions.

The tricyclic compounds desipramine, clomipramine, amitriptyline, cyclobenzaprine and carbamazepine induced rightward shifts on 6-ND concentration-response curve but did not reduce the noradrenaline, dopamine and adrenaline contractile responses. They also reduced the EFS-induced RIEVD contractions in control but not in tissues obtained from L-NAME-treated animals. Maprotiline, oxcarbazepine, paroxetine and ketanserin had no effect in either 6-ND or EFS-induced RIEVD contractions.

Thus, 6-ND modulates RIEVD contractility, and desipramine, clomipramine, amitriptyline, cyclobenzaprine and carbamazepine act as selective 6-ND receptor antagonists.

1. Introduction

The nitrocatecholamines nitronoradrenaline (NN) and nitroadrenaline have been extracted from rat brain (Tsunoda et al., 2007) and 6-nitronoradrenaline (6-NN) was detected in microdialysates of the rat spinal cord dorsal horn (Chiari et al., 2000). Noradrenaline transport in rat synaptosomes is inhibited by 6-NN (Shintani et al., 1996) and the intratechal administration of 6-NN induces analgesia due to release of noradrenaline (Chiari et al., 2000). These observations indicate that the nitrocatecholamines may act as neuronal mediators in the central nervous system.

Endothelium-dependent basal release of both dopamine (Britto-Júnior et al., 2020a) and 6-nitrodopamine (6-ND) was identified by tandem mass spectrometry in *Chelonoidis carbonaria* aorta (Campos et al., 2021). Human umbilical cord vessels (HUCV) also present a basal release of both dopamine (Britto-Júnior et al., 2020b) and 6-ND

* Corresponding author. University of Campinas (Unicamp), Faculty of Medical Sciences, Department of Pharmacology, Rua Tessália Vieira de Camargo, 126 - Cidade Universitária, 13083-887, Campinas, São Paulo, Brazil.
E-mail address: josebrittojr@gmail.com (J. Britto-Júnior).

https://doi.org/10.1016/j.ejphar.2021.174544
Received 7 September 2021; Received in revised form 22 September 2021; Accepted 29 September 2021
Available online 1 October 2021
0014-2999/© 2021 Elsevier B.V. All rights reserved.

(Britto-Júnior et al., 2021) and the synthesis of 6-ND was inhibited by incubation with the nitric oxide (NO) synthesis inhibitor L-NAME. Interestingly, 6-ND modulates HUCV reactivity through selective antagonism of dopamine D_2-like receptors, indicating that 6-ND may have modulatory role outside the central nervous system (Britto-Júnior et al., 2021) Electric field stimulation (EFS) causes a biphasic muscular contraction in the rat isolated vas deferens, which is characterized by an initial purinergic (ATP) component, followed by an adrenergic (noradrenaline) component (French and Scott, 1983). The purinergic element is more prominent in the prostatic portion whereas the adrenergic contraction is more predominant in the epididymal part (Anton, Duncan & McGrath, 1977). In this manuscript, the potential release of 6-ND from rat isolated vas deferens and the effect of this novel catecholamine in the vas deferens contractility have been investigated.

The results here reported show that rat isolated vas deferens presents a basal release of 6-ND in addition to noradrenaline, as detected by tandem mass spectrometry (LC-MS-MS). The dopamine receptor antagonist D_1 (SCH-23390; Hyttel, 1983) caused a left-shift on the concentration-response curve to 6-ND, but it had no effect on the contractions induced by dopamine or EFS in the rat isolated epididymal vas deferens (RIEVD). The D_2 like (haloperidol; Miranda et al., 1988), D_3 (PG-01037; Grundt et al., 2005) and D_4 (sonepiprazole; Merchant et al., 1996) receptor antagonists caused significant right-shifts on the concentration-response curves to dopamine, but they did not inhibit the 6-ND- or EFS-induced RIEVD contractions.

The tricyclic compounds desipramine, clomipramine, amitriptyline, cyclobenzaprine and carbamazepine caused selective rightward shifts on the concentration-response curves to 6-ND in the RIEVD, without inhibiting the contractile responses to dopamine, noradrenaline and adrenaline. The EFS-induced RIEVD contractions were significantly reduced by these tricyclic compounds, however they had no effect on the EFS-induced contractions of RIEVD obtained from animals chronically treated with L-NAME. Maprotiline, paroxetine, ketanserin and the tricyclic compound oxcarbazepine had no effect on either 6-ND or EFS-induced contractions.

These results indicate that 6-ND is an endogenous mediator of EFS-induced contractions of RIEVD, and that the selective antagonism of the 6-ND receptor by the tricyclic antidepressants may constitute a novel mechanism of action by which these drugs delay ejaculation in man (Hsu and Shen, 1995).

2. Methods

2.1. Animals

Adult male Wistar rats (280–320 g) were provided by the animal care of University of Campinas (UNICAMP) and University of Sao Paulo (USP). All experimental protocols were authorized by the Institutional Animal Care and Use Committee (CEUA/UNICAMP: 5557-1/2020) and followed the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al., 2020). Animals were housed in cages (n = 3 per cage) located in ventilated cage shelters with constant humidity of 55% ± 5% and temperature of 24 ± 1 °C under a 12-h light-dark cycle and received filtered water and standard food *ad libitum*.

2.2. Reagents

Dopamine, adrenaline, noradrenaline, desipramine, serotonin, SCH-23390, ascorbic acid, carbachol, atropine and N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) were acquired from Sigma-Aldrich Chemicals Co. (St Louis, Missouri, USA). Amitriptyline, clomipramine, cyclobenzaprine, haloperidol and paroxetine were obtained from Vamsi Labs Ltd. (Solapur, Maharashtra, India). Maprotiline was obtained from Beijing Merson Pharm (Daxing District, Beijing, China). Carbamazepine, oxcarbazepine, ketanserin and sonepiprazole were purchased from Cayman Chemical Co (Michigan, USA). PG-01037 was bought from Bio-Techne Corporation (Minneapolis, Minnesota, EUA).

Dopamine-d_3 hydrochloride, DL-noradrenaline-d_6 hydrochloride and adrenaline-d_6 hydrochloride were acquired from CDN Isotopes (Point Claire, Quebec, Canada). 6-nitrodopamine and 6-nitrodopamine-d_4 were purchased from Toronto Research Chemicals Inc (Toronto, Ontario, Canada). Aluminium oxide was purchased from Dinamica Quimica Contemporanea Ltda (Indaiatuba, São Paulo, Brazil). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$), potassium phosphate monobasic (KH_2PO_4) and glucose were acquired from Merck KGaA (Darmstadt, Germany). Acetonitrile and methanol were bought from J.T. Baker (Phillipsburg, NJ, USA) and formic acid from Mallinckrodt (St Louis, Missouri, USA).

2.3. L-NAME treatment

Rats were chronically treated with the nitric oxide synthase inhibitor L-NAME (20 mg/rat/day) in the drinking water for 4 weeks minimum (Ribeiro et al., 1992). Control animals received tap water alone.

2.4. Rat isolated vas deferens (RIVD) preparations

Euthanasia was performed by isoflurane overdose, in which animals were exposed to a concentration greater than 5% until 1 min after the breathing stops. Exsanguination was performed to confirm the euthanasia. The vas deferens was removed and immediately placed in Krebs-Henseleit's solution (KHS) of the following composition (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5.6 mM glucose). Epididymal portions of vas deferens were surgically dissected (length, 1.5 cm, each) for functional studies.

2.5. Basal release of catecholamines in RIVD

Two whole strips of RIVD from one rat were suspended in 5-mL organ bath containing KHS (pH 7.4) and ascorbic acid (3 mM) continuously gassed with a mixture of 95%O_2:5%CO_2 at 37 °C for 30 min. The RIVD whole strips were incubated in the absence and in the presence of L-NAME (100 μM, 30 min). Two aliquots of 2 mL of the supernatant were transferred to two tubes and stored at −20 °C until analysis (Britto et al., 2020b, 2021). The same procedure was employed to evaluate the basal release of catecholamines from RIEVD of animals chronically treated with L-NAME.

2.5.1. Extraction and quantification of 6-nitrodopamine (6-ND) by LC-MS/MS analysis

The extraction and quantification of 6-ND in KHS were performed as reported previously (Campos et al., 2021). Briefly, 6-ND was extracted from 1 mL of KHS by solid phase extraction (SPE). Calibrators and quality controls (QCs) prepared in blank KHS and the KHS samples obtained from RIVD strips for 30 min were spiked with 50 μL of the internal standard (IS) solution (6-ND-d_4, 100 ng/mL). Extraction cartridges were conditioned with 1 mL of methanol and then equilibrated with 2 mL of deionized water. The samples were transferred to the extraction cartridges and washed three times with deionized water. The samples were eluted with 0.9 mL methanol/deionized water (90/10, v/v) plus 0.1% formic acid and followed by evaporation under N_2 flow at 50 °C. The dry residues were dissolved with 100 μL acetonitrile/deionized water (50/50, v/v) plus 0.1% formic acid, transferred to vials and submitted to chromatographic analysis.

The separation of 6-ND was performed on a 150 mm × 3.0 mm Shimpack GIST-HP C_{18} column, 3-μm particle size (Shimadzu, Duisburg, Germany) held at 65 °C. A 75% of mobile phase A consisting of deionized water with 0.1% formic acid (v/v) and 25% of mobile phase B consisting of acetonitrile/deionized water (90/10, v/v) plus 0.1% formic acid at a flow rate of 350 μL/min in an isocratic mode were used. The detection of 6-ND and IS was carried out by a LC-MS-8060 triple

quadrupole mass spectrometer (MS/MS) (Shimadzu, Kyoto), operating in positive ionization mode. The analyses were performed in the multiple reaction monitoring (MRM) mode. The protonated ions $[M + H]^+$ and their respective ion products monitored were 199.10 > 181.95 and 203.10 > 186.00 for 6-ND and 6-ND-d4, respectively.

2.5.2. Extraction and quantification of dopamine, noradrenaline and adrenaline by LC-MS/MS analysis

The extraction and quantification of dopamine, noradrenaline and adrenaline in KHS with ascorbic acid (3 mM) were carried out as previously described (Britto et al., 2020c). Briefly, 100 μL of the internal standards (dopamine-d_3, noradrenaline-d_6 and adrenaline-d_6 at 100 ng/mL) were added to the KHS (2 mL) followed by 1.5 mL of deionized water. After vortexing for 10 s, 100 mg of Al_2O_3 was added and left for incubation for 20 min in an orbital agitator. The tubes were then centrifuged at 2000 g for 4 min at 4 °C and the supernatant discarded. The residue was washed four times with 2 mL of deionized water. After the final washing, 200 μL of a solution containing trifluoroacetic acid 0.1% in HCN/H2O (60/40; v/v) were added. After vortexing for 40 s, the Eppendorf tubes were centrifuged for 2000 g for 5 min and the supernatant transferred to the vials for injection. The samples were analyzed by liquid chromatography coupled to a triple quadrupole mass spectrometer, LC-MS-8050 (Shimadzu, Kyoto). The separation of catecholamines was performed on a 100 × 4.6 mm Lichrospher RP-8 column (GL Sciences Inc., Tokyo) using acetonitrile/water (5/95, v/v) with 0.1% formic acid as mobile phase at a flow rate of 0.4 mL/min. The mass spectrometer operated in positive electrospray ionization mode (ES^+) for catecholamine detection. The analyses were executed in selected Multiple Reaction Monitoring (MRM) detection mode. The protonated ions $[M + H]^+$ and their respective ion products monitored were 154.00 > 91.15, 170.10 > 107.10 and 184.20 > 107.00 for dopamine, noradrenaline and adrenaline, respectively and 157.00 > 93.00, 176.10 > 158.10 and 190.00 > 171.95 for dopamine-d3, noradrenaline-d6 and adrenaline-d6, respectively.

2.6. Preparation of rat isolated epididymal vas deferens (RIEVD) for the in vitro functional assays

The RIEVD was suspended vertically between metal hooks in 10-mL organ baths containing KHS (pH 7.4), continuously gassed with a mixture of 95%O_2: 5%CO_2 at 37 °C. Tissues were allowed to equilibrate under a resting tension of 10 mN, and the isometric tension was registered using a PowerLab system (ADInstruments, Sydney, Australia). Following a 45-min stabilization period, the RIEVD were contracted with a single concentration of noradrenaline (NA, 3 μM) to verify the tissue viability.

Cumulative concentration-response curves to 6-ND, dopamine, noradrenaline, adrenaline, carbachol and serotonin (5-HT) were performed in RIEVD strips, in the absence and presence of L-NAME (100 μM, 30 min), SCH-23390 (100 nM, 30 min), haloperidol (100 nM, 30 min), PG-01037 (10 nM, 30 min), sonepiprazole (100 nM, 30 min), desipramine (100, 30 min), clomipramine (100 nM, 30 min), amitriptyline (100, 30 min), cyclobenzaprine (100 nM, 30 min), carbamazepine (100 nM, 30 min), maprotiline (100 nM, 30 min), paroxetine (100 nM, 30 min), oxcarbazepine (100 nM, 30 min), ketanserin (10 nM, 30 min) and atropine (10 nM, 30 min). In another set of experiments the effect of desipramine, clomipramine, amitriptyline, cyclobenzaprine and carbamazepine on the 6-ND concentration-response curve were evaluated at 3 different concentrations (30, 100 and 300 nM).

Contractions of RIEVD induced by EFS were also evaluated. The RIEVD were submitted to EFS at 60 V for 20 s, at 2–16 Hz in square-wave pulses, 0.3 ms pulse width, and 0.1 ms delay, using a Grass S88 stimulator (Astro-Medical, Industrial Park, RI, USA). EFS-induced RIEVD contractions were evaluated in the absence and in the presence of L-NAME (100 μM, 30 min), SCH-23390 (100 nM, 30 min), haloperidol (100 nM, 30 min), PG-01037 (10 nM, 30 min), sonepiprazole (100 nM, 30 min), desipramine (100 nM, 30 min), clomipramine (100 nM, 30 min), amitriptyline (100 nM, 30 min),cyclobenzaprine (100 nM, 30 min), carbamazepine (100 nM, 30 min), maprotiline (100 nM, 30 min), paroxetine (100 nM, 30 min), oxcarbazepine (100 nM, 30 min), ketanserin (10 nM, 30 min) and atropine (10 nM, 30 min).

2.7. Data analysis

Nonlinear regression analysis to determine the pEC_{50} was carried out using GraphPad Prism (GraphPad Software, version 6.0, San Diego, California, USA) with the constraint that F = 0. All concentration–response data were evaluated for a fit to a logistics function in the form: $E = E_{max}/([1 + (10c/10x)n] + F$, where E represents the increase in response contractile induced by the agonist, E_{max} is the effect agonist maximum, c is the logarithm of concentration of the agonist that produces 50% of E_{max}, x is the logarithm of the concentration of the drug; the exponential term, n, is a curve fitting parameter that defines the slope of the concentration–response line, and F is the response observed in the absence of added drug. The results were expressed as mN. One strip was used as the control response and the other strip was incubated with an antagonist/inhibitor. Student's two-tail unpaired *t*-test was employed and the differences between groups. In addition, standard ANOVA, followed by the Newman–Keuls post-test, were used when more than two groups were involved, as for the experiments shown in Figs. 3–8. The distribution of the log values (pEC_{50}) was normal for each agonist, confirmed by the Shapiro-Wilk test (Motulsky, 2014). A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Levels of 6-ND, dopamine, noradrenaline and adrenaline in RIVD strips by LC-MS-MS

The calibration curve of 6-ND was linear for concentrations of 0.1–10 ng/mL, with a correlation coefficient higher than 0.99 (data not shown). The limit of quantification was 0.1 ng/mL. A basal release of 6-ND was detected in RIVD strips (Fig. 1A and B). Pre-treatment of the RIVD strips with L-NAME (100 μM) for 30 min caused a significant reduction of basal 6-ND release (p = 0.0021; Fig. 1A). A smaller, but significant reduction of 6-ND levels, was also observed in RIVD obtained from animals chronically treated with L-NAME (Fig. 1B).

The calibration curves for dopamine, noradrenaline and adrenaline were linear for concentrations of 0.1–10.0 ng/mL, with a correlation coefficient higher than 0.99 (data not shown). The limit of quantification was 0.1 ng/mL. A basal release of noradrenaline was detected (0.4 ± 0.2 ng/mL; n = 10), but it was not significantly affected by pre-treatment with L-NAME for 30 min (0.5 ± 0.3 ng/mL, n = 10; p = 0.6153). Dopamine and adrenaline concentrations in RIVD were below the limit of quantification (data not shown).

3.2. Effect of dopamine receptor antagonists on RIEVD contractions induced by dopamine, 6-ND and EFS

Dopamine (1nM-1 mM) and 6-ND (1 nM-1 mM; pEC50 4.64 ± 0.10 n = 16) produced concentration-dependent contractions and EFS (2–16 Hz) caused frequency-dependent contractions of RIEVD (Fig. 2). Incubation with the dopamine D_1 receptor antagonist SCH-23390 (100 nM) had no effect on dopamine concentration-curve (Fig. 2A) but caused a significant leftward shift on the concentration-response curves to 6-ND (p = 0.0349; Fig. 2B). The EFS-induced RIEVD contractions were not affected by prior incubation with SCH-23390 (100 nM; Fig. 2C).

Incubation with the dopamine D_2-like receptor antagonist haloperidol (100 nM) caused a significant rightward shift of the concentration-response curves to dopamine (p = 0.0017; Fig. 2D), but had no effect on the concentration-response curves to 6-ND (p = 0.4518; Fig. 2E). The

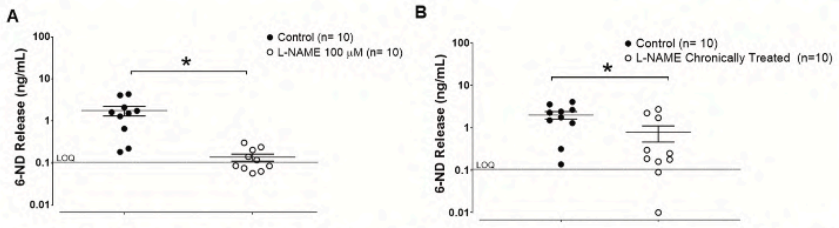


Fig. 1. Basal release of 6-nitrodopamine (6-ND) by rat isolated vas deferens (RIVD) as quantified by tandem mass spectrometry. Panel A shows the effect a 30 min-period incubation of the RIVD in Krebs-Henseleit's solution with or without L-NAME (100 μM). Panel B shows the basal release of 6-ND from RIVD obtained from control animals and from animals chronically treated with L-NAME. *P < 0.05 control vs L-NAME. n means the number of vas deferens strips.

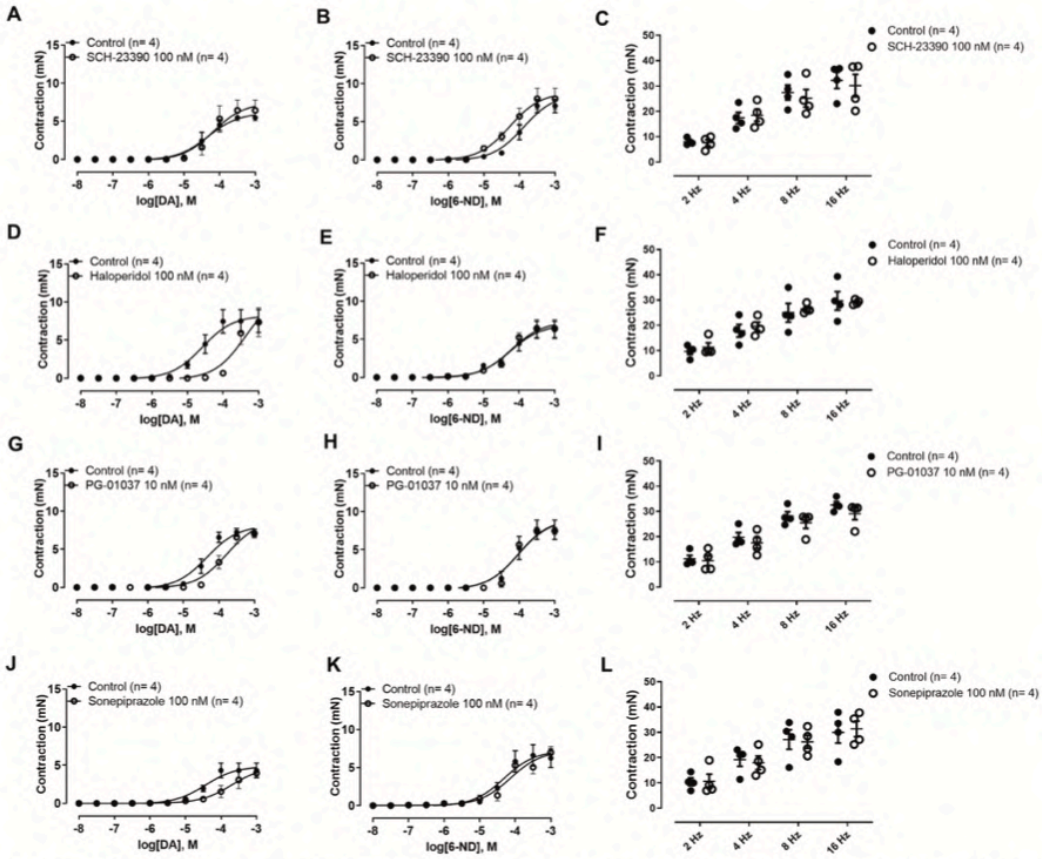


Fig. 2. Effects of dopaminergic receptor antagonists SCH-23390 (100 nM; panels A–C), haloperidol (100 nM; panels D–F), PG-01037 (10 nM; panels G–I) and sonepiprazole (100 nM; panels J–M) on the rat isolated epididymal vas deferens (RIEVD) contractions induced by dopamine, 6-nitrodopamine (6-ND) and electric-field stimulation (EFS). Note that haloperidol, PG-01037 and sonepiprazole (but not SCH-23390) causes leftward shifts on concentration-response curves to dopamine. None of these antagonists affected the 6-ND-induced contractions, except SCH-23390 that produced a leftward shift in the concentration-response curves. The EFS-induced contractions were not affected by any antagonist. Data are expressed as mean ± SEM. P < 0.05 compared with respective control values. n means the number of vas deferens strips.

EFS-induced RIEVD contractions were not affected by prior incubation with haloperidol (100 nM; Fig. 2F).

Incubation with the dopamine D_3 receptor antagonist PG-01037(10 nM) caused a significant rightward shift of the concentration-response curves to dopamine ($p = 0.0029$; Fig. 2G) but no had effect on the concentration-response curves to 6-ND ($p = 0.4977$; Fig. 2H). The EFS-

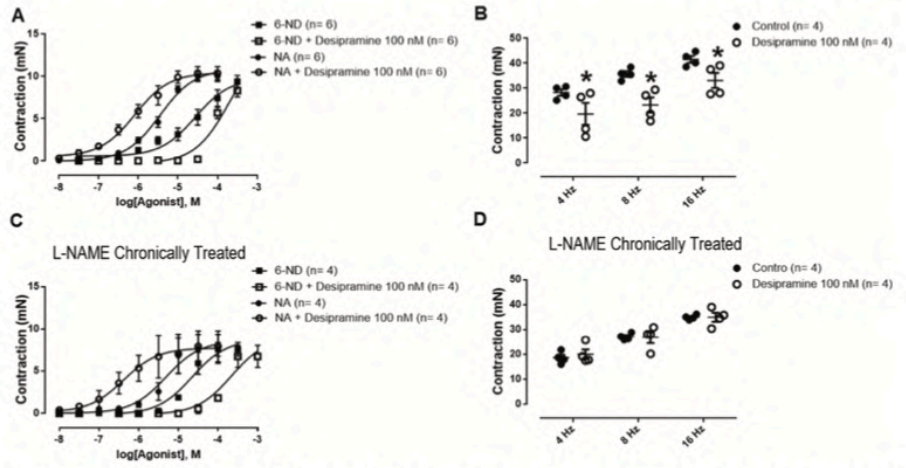


Fig. 3. Effect of desipramine (100 nM) in the rat isolated epididymal vas deferens (RIEVD) obtained from control (Panels A and B) and from L-NAME chronically treated rats (Panels C and D). Desipramine caused a significant rightward shift of the concentration-response curves to 6-ND and a significant leftward shift of the concentration-response curves to NA in RIEVD obtained from control (panel A) and from L-NAME chronically treated rats (Panel C). Electric-field stimulation (EFS) caused frequency-dependent contractions of the RIEVD obtained from control (Panel B) and from L-NAME chronically treated rats (Panel D). Desipramine (100 nM) caused a significant reduction in the contractions induced by EFS in RIEVD obtained from control animals (Panel B) but had no effect on those obtained from L-NAME chronically treated rats (Panel D). $P < 0.05$ compared with respective control values. n means the number of vas deferens strips.

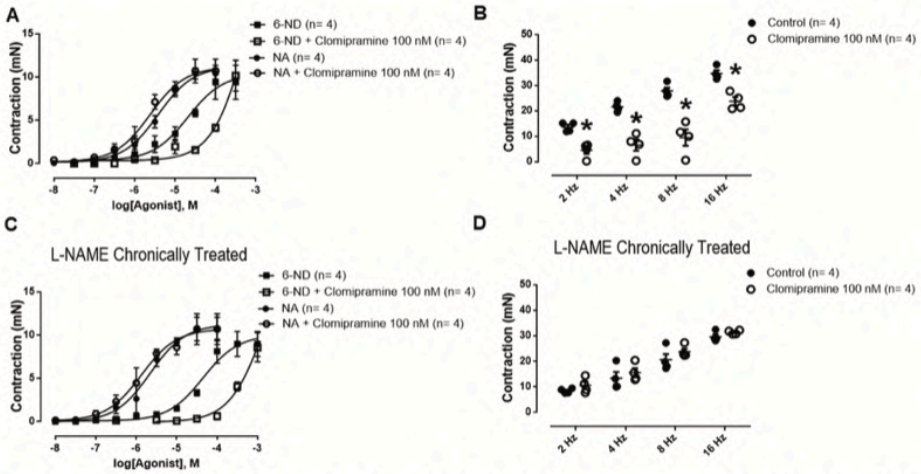


Fig. 4. Effect of clomipramine (100 nM) in rat isolated epididymal vas deferens (RIEVD) obtained from control (Panels A and B) and from L-NAME chronically treated rats (Panels C and D). Clomipramine caused a significant rightward shift of the concentration-response curves to 6-ND but it did not affect the concentration-response curves to NA in RIEVD obtained from control (panel A) and from L-NAME chronically treated rats (Panel C). Electric-field stimulation (EFS) caused frequency-dependent contractions of the RIEVD obtained from control (Panel B) and from L-NAME chronically treated rats (Panel D). Clomipramine (100 nM) caused a significant reduction in the contractions induced by EFS in RIEVD obtained from control animals (Panel B) but had no effect on those obtained from L-NAME chronically treated rats (Panel D). $P < 0.05$ compared with respective control values. n means the number of vas deferens strips.

induced RIEVD contractions were not affected by prior incubation with PG-01037 (10 nM; Fig. 2I).

Incubation with the dopamine D_4 receptor antagonist sonepiprazole (100 nM) caused a significant rightward shift of the concentration-response curves to dopamine ($p = 0.0121$; Fig. 2J) but had no effect on the concentration-response curves to 6-ND ($p = 0.2022$; Fig. 2L). The EFS-induced RIEVD contractions were not affected by prior incubation with sonepiprazole (100 nM; Fig. 2M).

3.3. Effect of desipramine on RIEVD contractions induced by catecholamines and EFS

Incubation with desipramine (100 nM) caused a significant rightward shift ($p = 0.0254$) on the concentration-response curves to 6-ND

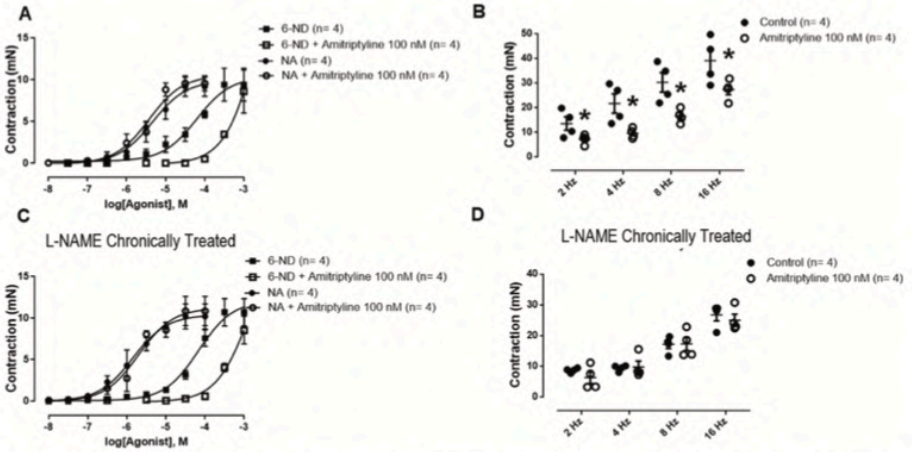


Fig. 5. Effect of amitriptyline (100 nM) in rat isolated epididymal vas deferens (RIEVD) obtained from control (Panels A and B) and from L-NAME chronically treated rats (Panels C and D). Amitriptyline caused a significant rightward shift of the concentration-response curves to 6-ND but it did not affect the concentration-response curves to NA in RIEVD obtained from control (panel A) and from L-NAME chronically treated rats (Panel C). Electric-field stimulation (EFS) caused frequency-dependent contractions of the RIEVD obtained from control (Panel B) and from L-NAME chronically treated rats (Panel D). Amitriptyline (100 nM) caused a significant reduction in the contractions induced by EFS in RIEVD obtained from control animals (Panel B) but had no effect on those obtained from L-NAME chronically treated rats (Panel D). $P < 0.05$ compared with respective control values. n means the number of vas deferens strips.

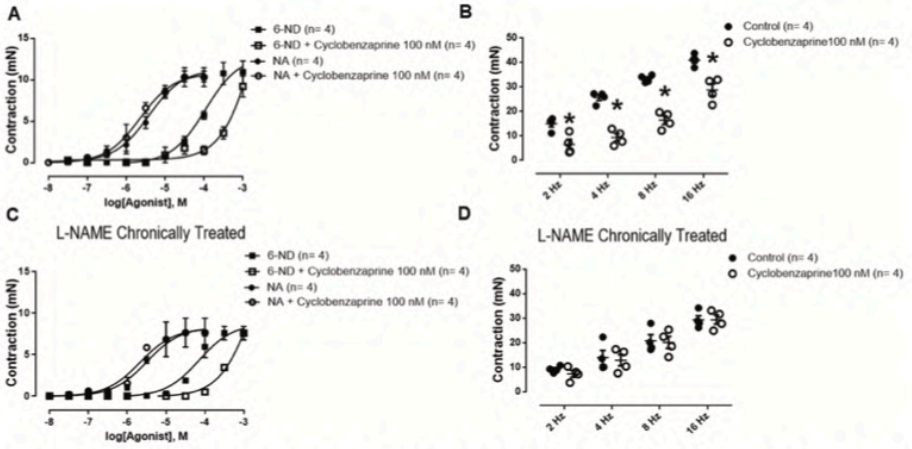


Fig. 6. Effect of cyclobenzaprine (100 nM) in rat isolated epididymal vas deferens (RIEVD) obtained from control (Panels A and B) and from L-NAME chronically treated rats (Panels C and D). Cyclobenzaprine caused a significant rightward shift of the concentration-response curves to 6-ND but it did not affect the concentration-response curves to NA in RIEVD obtained from control (panel A) and from L-NAME chronically treated rats (Panel C). Electric-field stimulation (EFS) caused frequency-dependent contractions of the RIEVD obtained from control (Panel B) and from L-NAME chronically treated rats (Panel D). Cyclobenzaprine (100 nM) caused a significant reduction in the contractions induced by EFS in RIEVD obtained from control animals (Panel B) but had no effect on those obtained from L-NAME chronically treated rats (Panel D). $P < 0.05$ compared with respective control values. n means the number of vas deferens strips.

(Fig. 3A) and a significant leftward shift (pEC_{50} 5.45 ± 0.08 and 6.08 ± 0.11 with and without desipramine, respectively) on the concentration-response curves to noradrenaline (Fig. 3A). The rightward shift on the 6-ND concentration-response curve was concentration-dependent (6.0 ± 0.7, 13.8 ± 1.7 and 30.5 ± 5.3 for 30, 100 and 300 nM, respectively, n = 4; p = 0.0013, Fig S01A). Electric-field stimulation caused frequency-dependent RIEVD contractions (Fig. 3B), which were all significantly reduced by prior incubation with desipramine (100 nM; Fig. 3B).

Similarly, in RIEVD obtained from chronic L-NAME treatment, desipramine (100 nM) also caused a significant rightward shift (p = 0.0001) on the concentration-response curves to 6-ND (Fig. 3C) and a significant leftward shift (p = 0.0109) on the concentration-response curves to noradrenaline (Fig. 3C). However, in chronic L-NAME-treated preparations, desipramine (100 nM) failed to significantly affect the EFS-induced RIEVD contractions in all frequencies tested (Fig. 3D).

Desipramine (100 nM) caused no significant shifts (p = 0.6289) on the concentration-response curve to dopamine (Table 1) but provoked a significant leftward shift (p = 0.0210) on concentration-response curve

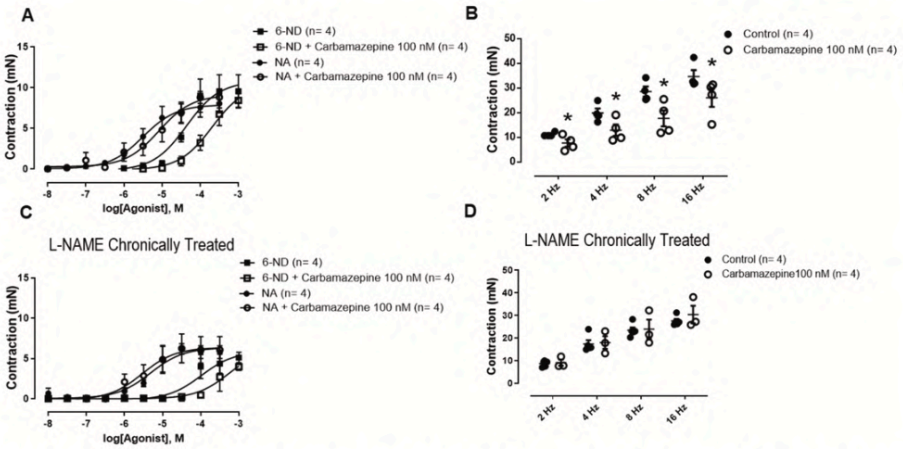


Fig. 7. Effect of carbamazepine (100 nM) in the rat isolated epididymal vas deferens (RIEVD) obtained from control (Panels A and B) and from L-NAME chronically treated rats (Panels C and D). Carbamazepine caused a significant rightward shift of the concentration-response curves to 6-ND but it did not affect the concentration-response curves to NA in RIEVD obtained from control (panel A) and from L-NAME chronically treated rats (Panel C). Electric-field stimulation (EFS) caused frequency-dependent contractions of the RIEVD obtained from control (Panel B) and from L-NAME chronically treated rats (Panel D). Carbamazepine (100 nM) caused a significant reduction in the contractions induced by EFS in RIEVD obtained from control animals (Panel B) but had no effect on those obtained from L-NAME chronically treated rats (Panel D). $P < 0.05$ compared with respective control values. n means the number of vas deferens strips.

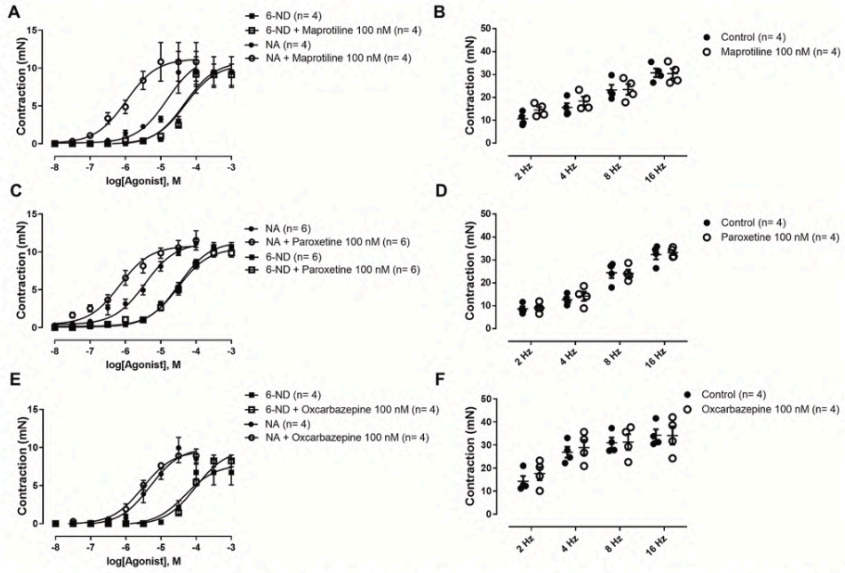


Fig. 8. Effect of maprotiline (100 nM, panel A and B), paroxetine (100 nM, panel C and D) and oxcarbazepine (100 nM, panel E and F) in the rat isolated epididymal vas deferens (RIEVD). Maprotiline caused significant leftward shift of the concentration-response curves to NA but it did not affect the concentration-response curves to 6-ND (panel A). The contractions of the RIEVD induced by EFS were not affected by maprotiline (100 nM; Panel B). Paroxetine caused significant leftward shift of the concentration-response curves to NA but it did not affect the concentration-response curves to 6-ND (panel C). The contractions of the RIEVD induced by EFS were not affected by paroxetine (100 nM; Panel D). Oxcarbazepine caused no significant shift of the concentration-response curves to NA and 6-ND (panel A). The contractions of the RIEVD induced by EFS were not affected by oxcarbazepine (100 nM; Panel F). $P < 0.05$ compared with respective control values. n means the number of vas deferens strips.

to adrenaline (Table 2). In RIEVD obtained from animals chronically treated with L-NAME, desipramine (100 nM) caused no significant shift ($p = 0.4702$) on the concentration-response curve to dopamine (Table 3) but maintained a significant leftward shift ($p = 0.0003$) on concentration-response curve to adrenaline (Table 4).

Desipramine had no effects on the Emax values of 6-ND (Fig. 3A,C),

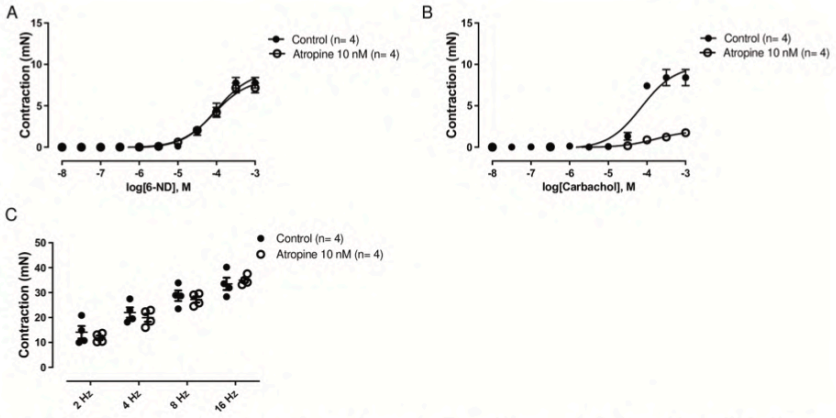


Fig. 9. Effect of atropine (10 nM) in the rat isolated epididymal vas deferens (RIEVD). Atropine no caused significant leftward shift of the concentration-response curves to ND (panel A) but it did not affect the concentration-response curves to carbachol (panel B). The contractions of the RIEVD induced by EFS were not affected by atropine (10 nM; Panel C). $P < 0.05$ compared with respective control values. n means the number of vas deferens strips.

Table 1
The potency (pEC_{50}) and maximum response (E_{max}) of the concentration-response curves to dopamine in rat isolated epididymal vas deferens.

Dopamine	pEC_{50} (log[M])	E_{max} (mN)	n
Control	4.93 ± 0.13	10.47 ± 1.34	6
Desipramine (100 nM)	4.83 ± 0.19	10.87 ± 1.34	6
Control	4.76 ± 0.25	11.90 ± 2.07	4
Clomipramine (100 nM)	4.82 ± 0.32	11.94 ± 2.42	4
Control	4.67 ± 0.30	8.50 ± 1.77	4
Amitriptyline (100 nM)	4.65 ± 0.28	7.94 ± 2.10	4
Control	4.60 ± 0.43	8.37 ± 1.26	4
Cyclobenzaprine (100 nM)	4.50 ± 0.45	8.46 ± 1.23	4
Control	4.37 ± 0.32	8.19 ± 1.10	4
Maprotiline (100 nM)	4.43 ± 0.20	8.16 ± 0.84	4
Control	4.44 ± 0.10	7.92 ± 0.92	4
Paroxetine (100 nM)	4.54 ± 0.15	6.58 ± 1.27	4
Control	4.47 ± 0.19	6.51 ± 1.60	4
Carbamazepine (100 nM)	4.48 ± 0.17	6.06 ± 1.21	4
Control	4.51 ± 0.14	8.15 ± 0.54	4
Oxcarbazepine (100 nM)	4.47 ± 0.19	8.98 ± 1.03	4

pEC50 is defined as the negative logarithm of the EC_{50}; Emax is the maximal effect at high drug; n means the number of vas deferens strips.

noradrenaline (Fig. 3A,C), dopamine (Tables 1 and 3) or adrenaline (Tables 2 and 4).

3.4. Effect of clomipramine on RIEVD contractions induced by catecholamines and EFS

Incubation with clomipramine (100 nM) caused a significant rightward shift ($p = 0.0001$) on the concentration-response curves to 6-ND without affecting the concentration-response curves to noradrenaline ($p = 0.2865$; Fig. 4A). The rightward shift on the 6-ND concentration-response curve was concentration-dependent (3.0 ± 1.4, 17.5 ± 5.7 and 19.5 ± 4.5 for 30, 100 and 300 nM, respectively, $n = 4$; $p = 0.0057$; Fig S01B). Electric-field stimulation caused frequency-dependent RIEVD contractions (Fig. 3B), which were significantly reduced by prior incubation with clomipramine in all frequencies tested (100 nM; Fig. 4B).

In RIEVD obtained from animals chronically treated with L-NAME, clomipramine (100 nM) caused significant rightward shift ($p = 0.0024$) on the concentration-response curves to 6-ND (Fig. 4C) but produced no significant shift ($p = 0.1146$) of the concentration-response curves to noradrenaline (Fig. 4C). In RIEVD obtained from animals chronically

Table 2
The potency (pEC_{50}) and maximum response (E_{max}) of the concentration-response curves to adrenaline on rat isolated epididymal vas deferens.

Adrenaline	pEC_{50} (log[M])	E_{max} (mN)	n
Control	4.43 ± 0.16	9.92 ± 1.60	4
Desipramine (100 nM)	5.07 ± 0.12*	9.58 ± 1.17	4
Control	4.58 ± 0.14	9.31 ± 0.83	4
Clomipramine (100 nM)	4.88 ± 0.24	8.02 ± 0.59	4
Control	4.76 ± 0.24	8.80 ± 1.32	4
Amitriptyline (100 nM)	5.06 ± 0.35	8.12 ± 1.15	4
Control	4.97 ± 0.30	7.56 ± 1.07	4
Cyclobenzaprine (100 nM)	4.98 ± 0.25	7.19 ± 1.10	4
Control	5.03 ± 0.29	8.19 ± 1.10	4
Maprotiline (100 nM)	5.71 ± 0.17**	8.16 ± 0.84	4
Control	4.76 ± 0.13	8.53 ± 0.71	4
Paroxetine (100 nM)	5.38 ± 0.10**	6.97 ± 0.80	4
Control	4.73 ± 0.11	6.86 ± 1.48	4
Carbamazepine (100 nM)	4.72 ± 0.06	5.80 ± 1.68	4
Control	4.95 ± 0.14	8.58 ± 0.42	4
Oxcarbazepine (100 nM)	4.73 ± 0.22	8.27 ± 0.42	4

pEC50 is defined as the negative logarithm of the EC_{50}; Emax is the maximal effect at high drug; n means the number of vas deferens strips.
*$P < 0.05$ compared with respective control values.
**$P < 0.01$ compared with respective control values.

Table 3
The potency (pEC_{50}) and maximum response (E_{max}) of the concentration-response curves to dopamine on isolated epididymal vas deferens of rats chronically treated with L-NAME.

Dopamine	pEC_{50} (log[M])	E_{max} (mN)	n
Chronic L-NAME	4.41 ± 0.16	6.75 ± 1.12	4
Desipramine (100 nM)	4.40 ± 0.10	8.32 ± 0.89	4
Chronic L-NAME	4.57 ± 0.17	7.67 ± 1.87	4
Clomipramine (100 nM)	4.26 ± 0.07	8.10 ± 0.70	4
Chronic L-NAME	4.78 ± 0.33	7.99 ± 1.61	4
Amitriptyline (100 nM)	4.62 ± 0.18	8.86 ± 0.90	4
Chronic L-NAME	4.69 ± 0.20	5.77 ± 1.01	4
Cyclobenzaprine (100 nM)	4.63 ± 0.20	5.16 ± 0.20	4
Chronic L-NAME	4.71 ± 0.22	6.32 ± 0.77	4
Carbamazepine (100 nM)	4.51 ± 0.14	6.37 ± 0.68	4

pEC50 is defined as the negative logarithm of the EC_{50}; Emax is the maximal effect at high drug; n means the number of vas deferens strips.

Table 4
The potency (pEC_{50}) and maximum response (E_{max}) of the concentration-response curves to adrenaline on isolated epididymal vas deferens of rats chronically treated with L-NAME.

Adrenaline	pEC_{50} (log[M])	E_{max} (mN)	n
Chronic L-NAME	5.48 ± 0.15	6.53 ± 0.80	4
Desipramine (100 nM)	6.63 ± 0.26**	6.44 ± 1.55	4
Chronic L-NAME	5.49 ± 0.21	6.53 ± 0.80	4
Clomipramine (100 nM)	5.18 ± 0.20	7.46 ± 1.33	4
Chronic L-NAME	5.26 ± 0.36	6.06 ± 0.98	4
Amitriptyline (100 nM)	5.07 ± 0.40	5.46 ± 1.21	4
Chronic L-NAME	5.41 ± 0.24	7.99 ± 1.61	4
Cyclobenzaprine (100 nM)	4.69 ± 0.35	8.86 ± 0.90	4
Chronic L-NAME	5.56 ± 0.33	7.63 ± 0.62	4
Carbamazepine (100 nM)	5.54 ± 0.13	7.38 ± 0.29	4

pEC50 is defined as the negative logarithm of the EC_{50}; Emax is the maximal effect at high drug; n means the number of vas deferens strips.
**P < 0.01 compared with respective control values.

treated with L-NAME, desipramine (100 nM) failed to significantly affect the EFS-induced contractions at any frequency (Fig. 4D).

Clomipramine (100 nM) caused no significant shifts on the concentration-response curves to dopamine (p = 0.8961; Table 1) and adrenaline (p = 0.3353; Table 2). In RIEVD obtained from animals chronically treated with L-NAME, clomipramine also failed to affect the concentration-response curves to dopamine (p = 0.1692; Table 3) and adrenaline (p = 0.3311; Table 4).

Clomipramine had no effects on the Emax values of 6-ND, noradrenaline (Fig. 4A and C), dopamine (Tables 1 and 3) or adrenaline (Tables 2 and 4).

3.5. Effect of amitriptyline on RIEVD contractions induced by catecholamines and EFS

Incubation with amitriptyline (100 nM) caused a significant rightward shift (p = 0.0151) on the concentration-response curves to 6-ND without significantly affecting the concentration-response curves to noradrenaline (p = 0.4190; Fig. 5A). The rightward shift on the 6-ND concentration-response curve was concentration-dependent (4.2 ± 3.0, 21.1 ± 4.8 and 29.1 ± 0.1 for 30, 100 and 300 nM, respectively, n = 4; p = 0.0001; Fig S01C). EFS-induced RIEVD contractions were significantly reduced by prior incubation with amitriptyline in all frequencies tested (100 nM; Fig. 5B).

In RIEVD obtained from animals chronically treated with L-NAME, amitriptyline (100 nM) caused a significant rightward shift (p = 0.0022) on the concentration-response curves to 6-ND (Fig. 5C), but no changes on the concentration-response curves to noradrenaline were observed (p = 0.9952; Fig. 5C). In RIEVD obtained from animals chronically treated with L-NAME, amitriptyline (100 nM) failed to significantly affect the EFS-induced contractions (Fig. 5D).

Amitriptyline (100 nM) did not significantly affect the concentration-response curves to dopamine (p = 0.9548; Table 1) or adrenaline (p = 0.5122; Table 2). The chronic treatment with L-NAME also failed to change the effects of amitriptyline on the concentration-response curves to dopamine (p = 0.3462; Table 3) and adrenaline (p = 0.3648; Table 4).

Amitriptyline had no effect on the Emax of 6-ND, noradrenaline (Fig. 5A and C), dopamine (Tables 1 and 3) or adrenaline (Tables 2 and 4).

3.6. Effect of cyclobenzaprine on RIEVD contractions induced by catecholamines and EFS

Incubation with cyclobenzaprine (100 nM) caused a significant rightward shift (p = 0.0205) on the concentration-response curves to 6-ND (Fig. 6A), without affecting the responses to noradrenaline (p = 0.3740; Fig. 6A). The rightward shift on the 6-ND concentration-response curve was concentration-dependent (2.4 ± 3.6, 13.3 ± 3.3 and 28.1 ± 7.0 for 30, 100 and 300 nM, respectively, n = 4; p = 0.0006 for 100 and 300 nM only; Fig S01D). The EFS-induced RIEVD contractions were significantly reduced by cyclobenzaprine in all frequencies tested (Fig. 6B).

In RIEVD obtained from animals chronically treated with L-NAME, cyclobenzaprine (100 nM) caused a significant rightward shift (p = 0.0002) on the concentration-response curves to 6-ND (Fig. 6C) but no changes on the responses to noradrenaline were observed (p = 0.23843; Fig. 6C). In RIEVD obtained from animals chronically treated with L-NAME, cyclobenzaprine failed to significantly affect the EFS-induced contractions at any frequency (Fig. 6D).

Cyclobenzaprine (100 nM) caused no significant shifts on the concentration-response curve to dopamine (p = 0.4849; Table 1) and adrenaline (p = 0.2060; Table 2). In the chronic L-NAME group, cyclobenzaprine affected neither the concentration-response curves to dopamine (p = 0.4186; Table 3) nor to adrenaline (p = 0.0754; Table 4).

Cyclobenzaprine had no effect on the Emax of 6-ND, noradrenaline (Fig. 6A and C), dopamine (Tables 1 and 3) or adrenaline (Tables 2 and 4).

3.7. Effect of carbamazepine on RIEVD contractions induced by catecholamines and EFS

Incubation with carbamazepine (100 nM) caused a significant rightward shift (p = 0.0071) on the concentration-response curves to 6-ND (Fig. 7A), without affecting (p = 0.0878) the concentration-response curves to noradrenaline (Fig. 7A). The rightward shift on the 6-ND concentration-response curve was concentration-dependent (7.7 ± 1.6 and 8.3 ± 3.2 for 30 and 100 nM, respectively, n = 4; p = 0.0212). For 300 nM, the shift was not significant (2.2 ± 1.8) due to the major E_{max} reduction *observed* (8.71 ± 1.16 and 3.73 ± 1.12 mN, control and 300 nM, respectively; n = 4; p = 0.0109; Fig S01E). Electric-field stimulation caused frequency-dependent contractions of the RIEVD (Fig. 7B), which were all significantly reduced by prior incubation with carbamazepine (100 nM; Fig. 7B).

In RIEVD obtained from animals chronically treated with L-NAME, carbamazepine (100 nM) caused a significant rightward shift (p = 0.0223) on the concentration-response curves to 6-ND (Fig. 7C) but no changes on the responses to noradrenaline were observed (p = 0.4956; Fig. 7C). In RIEVD obtained from animals chronically treated with L-NAME, carbamazepine (100 nM) failed to significantly affect the EFS-induced contractions at any frequency (Fig. 7D).

Carbamazepine (100 nM) caused no significant shifts on the concentration-response curve to dopamine (p = 0.4948; Table 1) and adrenaline (p = 0.4736; Table 2). In the chronic L-NAME group, carbamazepine affected neither the concentration-response curves to dopamine (p = 0.4510; Table 3) nor to adrenaline (p = 0.9784; Table 4).

Carbamazepine (100 nM) had no effect on the E_{max} of 6-ND, noradrenaline (Fig. 7A and C), dopamine (Tables 1 and 3) or adrenaline (Tables 2 and 4).

3.8. Effect of maprotiline, paroxetine and oxcarbazepine on RIEVD contractions induced by catecholamines and EFS

Incubation with maprotiline (100 nM) caused no significant rightward shift (p = 0.4129) on the concentration-response curves to 6-ND (Fig. 8A) but produced a significant leftward shift on the concentration-response curves to noradrenaline (p = 0.0378; Fig. 8A). The EFS-induced RIEVD contractions were not affected by maprotiline in all frequencies tested (Fig. 8B).

Paroxetine (100 nM) caused no significant shift (p = 0.8830) on the concentration-response curves to 6-ND (Fig. 8C) but produced a significant leftward shift (p = 0.0002) on the concentration-response curves to noradrenaline (Fig. 8C). The EFS-induced RIEVD contractions were not affected by prior incubation with paroxetine (Fig. 8D).

Incubation with oxcarbazepine (100 nM) caused no significant shifts on the concentration-response curves to 6-ND ($p = 0.2017$; Fig. 8E) and noradrenaline ($p = 0.0680$; Fig. 8E), The EFS-induced RIEVD contractions were not affected by prior incubation with oxcarbazepine (Fig. 8F).

These compounds had no effect on dopamine concentration-response curves (Table 1). Maprotiline and paroxetine, but not oxcarbazepine, caused a significant leftward shift on the concentration-response curves to adrenaline (Table 2).

3.9. Effect of ketanserin on RIEVD contractions induced by 6-ND, noradrenaline and 5-HT

Incubation with the 5-HT2A antagonist ketanserin (10 nM) caused no significant shifts on the concentration-response curves to 6-ND ($pEC_{50} = 4.35 \pm 0.12$ and 4.35 ± 0.14; control and treated, respectively; $n = 4$, $p = 0.4484$) and noradrenaline ($pEC_{50} = 5.03 \pm 0.20$ and 4.99 ± 0.09; control and treated, respectively; $n = 4$, $p = 0.8538$), but caused a significant right shift ($p = 0.0002$) on the concentration-response curves to 5-HT ($pEC_{50} = 4.70 \pm 0.12$ and 3.12 ± 0.02; control and treated respectively; $n = 4$).

Ketanserin (10 nM) had no effect on the E_{max} of 6-ND (9.94 ± 1.38 and 9.54 ± 2.04 mN; control and treated respectively; $n = 4$), noradrenaline (6.70 ± 1.86 and 7.90 ± 0.75 mN; control and treated, respectively; $n = 4$) and 5-HT (4.72 ± 0.75 and 4.14 ± 0.88 mN; control and treated, respectively; $n = 4$).

The EFS-induced RIEVD contractions were not affected by prior incubation with ketanserin (Table 5).

3.10. Effect of atropine on RIEVD contractions induced by carbachol, 6-ND and EFS

Incubation with the muscarinic antagonist receptor atropine (10 nM) caused no significant shifts on the concentration-response curves to 6-ND (Fig. 9 A; $p = 0.4060$) but abolish the concentration-response curves to carbachol Fig. 9B; $p = 0.0003$).

The EFS-induced RIEVD contractions were not affected by prior incubation with atropine (Fig. 9 C).

3.11. Effects of addition of L-NAME on RIEVD contractions induced by catecholamines and EFS

Incubation of RIEVD with L-NAME in vitro (100 μM) did not significantly change the concentration-response curves to dopamine ($p = 0.9349$), adrenaline ($p = 0.2277$), noradrenaline ($p = 0.9349$) and 6-ND ($p = 0.438$) at the level of both pEC50 and E_{max} values (Tables 3 and 6). Similarly, incubation with L-NAME (100 μM) caused no effect on the EFS-induced RIEVD contractions (Table 7).

4. Discussion

Our results clearly demonstrate for the first time that RIVD presents a basal release of 6-ND, as characterized by LC-MS-MS. The basal release of 6-ND was inhibited (92.3%) by incubation with L-NAME, indicating that the synthesis of 6-ND is coupled to NO synthesis, as observed in the human umbilical cord vessels. However, in RIEVD strips obtained from animals chronically treated with L-NAME, the inhibition of basal 6-ND release was less pronounced (60.7%). A similar degree of inhibition by chronic L-NAME treatment (50%) was reported for 6-nitronoradrenaline extracted from rat brain (Tsunoda et al., 2007). Despite the in vitro L-NAME incubation produces >90% inhibition of basal release of 6-ND, it had no effect on the EFS-induced RIVD contractions; in contrast, the inhibition of basal 6-ND release by chronic L-NAME treatment was lower (60%), but that was associated with a significant decrease of the EFS-induced contractions (Ventura and Burnstock, 1997). One possible explanation for this apparent paradox is that 6-ND may be stored in vesicles as it happens with noradrenaline and would be released upon EFS.

The contractile responses for 6-ND were observed in 1–10 μM range whereas the levels detected were under 50 nM. This apparent discrepancy could be explained by the difference between the local concentration (where the mediator is released) and the concentration needed to be reached in the organ bath to give the same concentration. This is illustrated in the rat isolated perfused mesenteric vascular bed, where exogenous NO at doses that produced smaller responses within the mesenteric bed than endogenously generated NO survived to the assay tissues whereas the endogenous generated NO did not (Warner et al., 1989). In addition, the amounts of 6-ND and noradrenaline released are in the same range as those of noradrenaline following extraction and quantification by HPLC coupled to electrochemical detection (Bell et al., 1984; Celuch and Sloley, 1988).

Dopamine and 6-ND presented basically the same pEC_{50} values in the RIEVD (4.73 and 4.69, respectively), which were less potent than noradrenaline (5.59) and adrenaline (5.48). Interestingly, the selective α_1-adrenoceptor antagonist prazosin inhibits the noradrenaline- and dopamine-induced RIEVD contractions, indicating that these catecholamines activate the same population of α_1-adrenergic receptors (Leedham and Pennefather, 1982). However, it is unlikely that 6-ND is acting on α_1-adrenergic receptors since in contrast to dopamine, noradrenaline and adrenaline, 6-ND does not cause contractions of HUCV (Britto-Júnior et al., 2021). The α_1-adrenoceptor antagonist blocks the contractions induced by noradrenaline in the human umbilical artery (Bodelsson and Stjernquist, 1995) and by adrenaline in the human umbilical vein (Errasti et al., 1999), establishing the presence of functional α_1-adrenoceptors in HUCV.

Table 5
Effect of ketanserin (10 nM) on isolated epididymal vas deferens contractions induced by electric-field stimulation (EFS) of control rats in the presence of L-NAME (100 μM).

Frequency	Control (mN)	L-NAME (mN)	n
2 Hz	10.67 ± 1.39	12.64 ± 2.43	4
4 Hz	15.60 ± 1.88	16.89 ± 2.81	4
8 Hz	23.33 ± 2.22	25.03 ± 1.83	4
16 Hz	30.76 ± 1.89	31.75 ± 1.96	4

Table 6
The potency (pEC_{50}) and maximum response (E_{max}) of the concentration-response curves to dopamine, noradrenaline, adrenaline and 6-nitrodopamine in rat isolated epididymal vas deferens in the presence of L-NAME (100 μM).

Agonist	pEC_{50} (log[M])	E_{max} (mN)	n
Control + Dopamine	5.22 ± 0.30	8.87 ± 1.61	5
L-NAME + Dopamine	5.54 ± 013	8.87 ± 1.18	5
Control + Noradrenaline	5.78 ± 0.13	9.32 ± 0.43	5
L-NAME + Noradrenaline	5.76 ± 0.18	10.68 ± 1.37	5
Control + Adrenaline	5.28 ± 0.13	10.15 ± 0.45	5
L-NAME + Adrenaline	5.01 ± 0.16	12.04 ± 1.54	5
Control + 6-Nitrodopamine	5.04 ± 0.19	10.71 ± 0.74	5
L-NAME + 6-Nitrodopamine	4.96 ± 0.08	10.18 ± 0.78	5

pEC50 is defined as the negative logarithm of the EC_{50}; Emax is the maximal effect at high drug; n means the number of vas deferens strips.

Table 7
Effect of L-NAME (100 μM) on electric-field stimulation (EFS)-induced contractions of rat isolated epididymal vas deferens from control rats in the presence of L-NAME (100 μM).

Frequency	Control (mN)	L-NAME (mN)	n
2 Hz	12.72 ± 1.59	13.17 ± 1.44	4
4 Hz	21.50 ± 2.68	21.04 ± 0.78	4
8 Hz	27.62 ± 2.26	26.62 ± 1.26	4
16 Hz	35.06 ± 2.12	32.31 ± 1.34	4

Could 6-ND be acting on dopaminergic receptors? Dopamine D_1 receptors have been identified in the sympathetic nerve endings of guinea-pig vas deferens (Furukawa and Morishita, 1997). Activation of dopamine D_1 receptors stimulates adenylate cyclase to increase the intracellular cAMP levels (Sibley and Monsma Jr., 1992). Interestingly, the D_1 receptor agonist fenoldopam (Zeng et al., 2004) causes relaxation of both human umbilical arteries and veins (Britto-Junior et al., 2021). The dopamine D1 antagonist SCH-23390 did not affect the contractions of the RIEVD induced by 6-ND. It rather caused a significant leftward shift on the concentration-response curve to 6-ND, indicating the presence of functional dopamine D_1 receptors in the rat vas deferens.

The dopamine D_2-like antagonist haloperidol antagonized the dopamine-induced contraction of the rat vas deferens, although at the concentration employed (250 nM), it certainly blocks the post-synaptic α_1 adrenergic receptor (K_i 7.6 nM; Arnt and Skarsfeldt, 1998). Indeed, the use of N-0923, a selective dopamine D_2 receptor agonist, demonstrated the presence of these receptors only in the mouse but not in rat vas deferens (Martin et al., 1993). However, the finding that haloperidol (100 nM) caused a significant right shift in the concentration-response curve to dopamine, without affecting the responses to 6-ND, demonstrates that 6-ND is not acting at this dopaminergic (or adrenergic) receptor.

To date, there is no report on the presence of dopamine D_3 receptors in vas deferens. The right shift caused by the D3 receptor antagonist PG-01037 on the concentration-response curve to dopamine suggests the presence of this receptor in the rat vas deferens. However, since it did not affect the responses to 6-ND, demonstrating that this dopaminergic receptor is not involved in the contraction induced by 6-ND in this tissue.

Guinea-pig vas deferens express functional dopamine D_4 receptors (Morishita and Katsuragi, 1999). Indeed, the D_4 receptor antagonist sonepiprazole did evoke a significant rightward shift in the concentration-response curve to dopamine, indicating the existence of functional dopamine D_4 receptors in the RIEVD. However, at the same concentration, it demonstrate did not affect the concentration-response curve to 6-ND, indicating that 6-ND is not activating this receptor. In addition, the lack of effect of all above mentioned dopamine antagonists on the EFS-induced contractions suggests that dopamine does not modulate this phenomenon. Indeed, no basal release of dopamine was detected from the rat vas deferens.

The tricyclic antidepressants can block α1-adrenergic receptors (Feighner, 1999; Nojimoto et al., 2010). Amitriptyline (Sánchez et al., 1999) and clomipramine (Millan et al., 2001) block α1-adrenergic receptor in rat cortex at 61 and 15.5 nM, respectively, whereas desipramine (Cusack et al., 1994) blocks human α1-adrenergic receptor at 100 nM. However, the finding that these tricyclic compounds caused rightward shifts only in the concentration-response curves to 6-ND indicates that they are not acting as α1 receptor antagonists. Indeed, the tetracyclic compound maprotiline inhibits human brain α1 adrenergic receptor at 90 nM (Richelson and Pfenning, 1984) but had no effect on either concentration-response curves to 6-ND or EFS-induced RIEVD contractions. These results suggest that mechanism by which the tricyclic antidepressants antagonizes 6-ND is not by interaction with the α1-adrenergic receptors.

The common mechanism of action of all tricyclic antidepressants are blockade of histaminergic H1 receptors (Gillman, 2007). It is unlikely that 6-ND is acting on these receptors in the RIEVD, since histamine has a depressor effect on EFS-induced vas deferens contractions (Vohra, 1979). Another mechanism of action of tricyclic antidepressants involves inhibition of serotonin (SERT) and noradrenaline (NET) transporters, favouring the serotonin and noradrenaline re-uptake (Gillman, 2007). For instance, amitriptyline inhibits human cloned SERT and NET at 67 nM and 63 nM, respectively (Vaishnavi et al., 2004), desipramine inhibits rat brain SERT (Dutta et al., 2019) and NET (Muth et al., 1986) at 106 and 0.15 nM, respectively, and clomipramine inhibits rat cortex SERT (Cheetham et al., 1993) and human NET (Tatsumi et al., 1997) at 5 and 38 nM, respectively. The centrally acting muscle relaxant cyclobenzaprine also inhibits human SERT and NET at 108 and 36 nM, respectively (Mestres et al., 2011). Paroxetine also inhibits rat cortex SERT (Kung et al., 1995) and rat NET (Sánchez et al., 1999) at 0.5 and 81 nM, respectively, but it had no effect on the concentration-response curves to 6-ND or EFS-induced RIEVD contractions, indicating that SERT and NET blockade cannot be responsible for the effects observed with the drugs above mentioned.

Serotonin also causes contractions of the rat vas deferens, primarily by activation of post-synaptic 5-HT receptors located in the epididymal half (Hay and Wadsworth, 1982; Karasawa et al., 1985). Ketanserin blocks 5-HT_{2A} (0.88 nM; Woutersc et al., 1986), 5-HT_{1A} (0.5 nM; Janowsky et al., 2014) and 5-HT_{1B} (1.9 μM; Markstein et al., 1986) receptors. Amitriptyline, clomipramine and desipramine also block 5-HT2A receptors (Gillman, 2007). However, the finding that ketanserin had no effect on the concentration-response curve to 6-ND exclude that the antagonism observed with the tricyclic antidepressants involves serotonin receptors. Acetylcholine is released by RIEVD (Knoll et al., 1972), however atropine failed to affect the contractions induced by nerve stimulation (Burnstock and Holman, 1964) or by 6-ND, indicating the lack of involvement of cholinergic receptors.

Thus, we propose that the tricyclic antidepressants cyclobenzaprine and carbamazepine are acting as antagonists of the 6-ND receptor. This is further supported by the lack of effect of these drugs on the EFS-induced contractions of RIEVD strips obtained from animals chronically treated with L-NAME. What do we know about this novel 6-ND receptor in the vas deferens? The results demonstrate that the 6-ND receptor is blocked by tricyclic structures such as dibenzoazepines (desipramine, clomipramine and carbamazepine) and dibenzocycloheptenes (amitriptyline and cyclobenzaprine), but not by tetracyclic compounds such as maprotiline or modified dibenzoazepines like oxcarbazepine. The inhibition was observed at concentrations in the lower range compared to the tricyclic antidepressant C_{max} (Aldeman et al., 1997; Filser et al., 1988; Kuss and Jungkrunz, 1988) or hundred times lower when compared to the therapeutic plasma levels of carbamazepine and oxcarbazepine (Patsalos et al., 2008).

What is the physiological role of 6-ND in the vas deferens? Pharmacology identifies physiological roles of mediators through the actions of antagonists/inhibitors. As discussed above, the tricyclic antidepressants cyclobenzaprine and carbamazepine antagonize 6-ND in the vas deferens. Tricyclic antidepressants can cause delayed ejaculation (Rothmore, 2020). This adverse reaction is also observed with cyclobenzaprine (Kraus et al., 2015) and carbamazepine (Leris et al., 1997). Clomipramine is used as treatment of premature ejaculation in man (Waldinger, 2018). In a randomized, double-blind clinical trial, using on-demand fixed dose of either clomipramine (25 mg) or paroxetine (20 mg) in 30 men with lifelong premature ejaculation (Waldinger et al., 2004), clomipramine led to a clinical relevant ejaculation delay whereas paroxetine had no clinical relevant effect. During ejaculation, sperm moves through the epididymis to the vas deferens (Wu and De Cicco, 2020). The finding that 6-ND is released in substantially greater amounts than noradrenaline (20-fold approximately) generates the exciting possibility of its involvement in the ejaculatory process.

5. Conclusion

6-nitrodopamine is an endogenous modulator of the rat vas deferens contractility. The antagonism of the 6-ND receptor by tricyclic compounds reveals a novel mechanism of action of tricyclic antidepressants.

CRediT authorship contribution statement

José Britto-Júnior: Conceptualization, Data curation, Investigation, Methodology, Writing – review & editing, Writing – original draft. **Luiz Ximenes:** Methodology. **André Ribeiro:** Methodology. **Adriano Fregonesi:** Methodology. **Rafael Campos:** Methodology. **Luiz Ricardo de**

Almeida Kiguti: Methodology. **Fabíola Z. Mónica:** Methodology, Supervision. **Edson Antunes:** Funding acquisition, Methodology, Supervision, Visualization, Writing – review & editing, Writing – original draft. **Gilberto De Nucci:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare no competing or financial interests.

Acknowledgment

JBJ and LX thank CAPES for PhD fellowship (001). EA & FM thank FAPESP (2017/15175-1). GDN thanks FAPESP (2019/16805-4), FAEPEX (2469/21) and CNPq (303839/2019-8).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.174544.

References

Alderman, J., Preskorn, S.H., Greenblatt, D.J., Harrison, W., Penenberg, D., Allison, J., Chung, M., 1997. Desipramine pharmacokinetics when coadministered with paroxetine or sertraline in extensive metabolizers. J. Clin. Psychopharmacol. (4), 284–291.

Anton, P.G., Duncan, M.E., McGrath, J.C., 1997. An analysis of the anatomical basis for the mechanical response to motor nerve stimulation of the rat vas deferens. J. Physiol. 273 (1), 23–43. https://doi.org/10.1113/jphysiol.1977.sp012079.

Arnt, J., Skarsfeldt, T., 1998. Do novel antipsychotics have similar pharmacological characteristics? A review of the evidence. Neuropsychopharmacology 18 (2), 63–101. https://doi.org/10.1016/S0893-133X(97)00112-7.

Bell, C., Gillespie, J.S., Macrae, I.M., 1984. Release of noradrenaline and dopamine by nerve stimulation in the Guinea-pig and rat vas deferens. Br. J. Pharmacol. Mar. 81 (3), 563–569. https://doi.org/10.1111/j.1476-5381.1984.tb10110.x.

Bodelsson, G., Stjernquist, M., 1995. Characterization of contractile adrenoceptors in the human umbilical artery. Eur. J. Pharmacol. 25 (282), 95–101. https://doi.org/10.1016/0014-2999(95)00277-r (1-3).

Britto-Júnior, J., Jacintho, F.F., Figueiredo Murari, G.M., Campos, R., Moreno, R.A., Antunes, E., Mónica, F.Z., De Nucci, G., 2020a. Electrical field stimulation induces endothelium-dependent contraction of human umbilical cord vessels. Life Sci. 15 (243), 117257. https://doi.org/10.1016/j.lfs.2020.117257.

Britto-Júnior, J., Pinheiro, D.H.A., Justo, A.F.O., Figueiredo Murari, G.M., Campos, R., Mariano, F.V., de Souza, V.B., Schenka, A.A., Mónica, F.Z., Antunes, E., De Nucci, G., 2020b. Endothelium-derived dopamine modulates EFS-induced contractions of human umbilical vessels. Pharmacol. Res. Perspect. 8 (4), e00612 https://doi.org/10.1002/prp2.612.

Britto-Júnior, J., Antunes, N.J., Campos, R., Sucupira, M., Mendes, G.D., Fernandes, F., Moraes, M.O., Moraes, M.E.A., De Nucci, G., 2020c. Determination of dopamine, noradrenaline, and adrenaline in Krebs-Henseleit solution by liquid chromatography coupled with tandem mass spectrometry and measurement of their basal release from Chelonoidis carbonaria aortae in vitro. Biomed. Chromatogr. 35 (2), e4978 https://doi.org/10.1002/bmc.4978. Epub 2020 Sep 13. PMID: 32866321.

Britto-Júnior, J., Coelho-Silva, W.C., Murari, G.F., Serpellone Nash, C.E., Mónica, F.Z., Antunes, E., De Nucci, G., 2021. 6-Nitrodopamine is released by human umbilical cord vessels and modulates vascular reactivity. Life Sci. 26 (276), 119425. https://doi.org/10.1016/j.lfs.2021.119425.

Burnstock, G., Holman, M.E., 1964. An electrophysiological investigation of the actions of some autonomic blocking drugs on transmission in the guinea-pig vas deferens. Br. J. Pharmacol. Chemother. 23 (3), 600–612. https://doi.org/10.1111/j.1476-5381.1964.tb01613.x.

Campos, R., Pinheiro, D.H.A., Britto-Júnior, J., de Castro, H.A., Mendes, G.D., Moraes, M. O., Moraes, M.E.A., Lopes-Martins, R.Á.B., Antunes, N.J., De Nucci, G., 2021. Quantification of 6-nitrodopamine in Krebs-Henseleit's solution by LC-MS/MS for the assessment of its basal release from Chelonoidis carbonaria aortae in vitro. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 22 (1173), 122668. https://doi.org/10.1016/j.jchromb.2021.122668.

Celuch, S.M., Sloley, B.D., 1988. Regional distribution of dopamine, 5-hydroxytryptamine, and noradrenaline in the rat vas deferens. Neurochem. Res. 13 (10), 967–972. https://doi.org/10.1007/BF00970770.

Cheetham, S.C., Viggers, J.A., Slater, N.A., Heal, D.J., Buckett, W.R., 1993. [3H] paroxetine binding in rat frontal cortex strongly correlates with [3H]5-HT uptake: effect of administration of various antidepressant treatments. Neuropharmacology 32 (8), 737–743. https://doi.org/10.1016/0028-3908(93)90181-2.

Chiari, A., Li, X.H., Xu, Z., Pan, H.L., Eisenach, J.C., 2000. Formation of 6-nitro-norepinephrine from nitric oxide and norepinephrine in the spinal cord and its role in spinal analgesia. Neuroscience 101 (1), 189–196. https://doi.org/10.1016/s0306-4522(00)00328-6.

Cusack, B., Nelson, A., Richelson, E., 1994. Binding of antidepressants to human brain receptors: focus on newer generation compounds. Psychopharmacology (Berl) 114 (4), 559–565. https://doi.org/10.1007/BF02244985.

Dutta, A.K., Santra, S., Harutyunyan, A., Das, B., Lisieski, M.J., Xu, L., Antonio, T., Reith, M.E.A., Perrine, S.A., 2019 Nov 5. D-578, an orally active triple monoamine reuptake inhibitor, displays antidepressant and anti-PTSD like effects in rats. Eur. J. Pharmacol. 862, 172632. https://doi.org/10.1016/j.ejphar.2019.172632. Epub 2019 Aug 29. PMID: 31473161.

Errasti, A.E., Velo, M.P., Torres, R.M., Sardi, S.P., Rothlin, R.P., 1999. Characterization of alpha1-adrenoceptor subtypes mediating vasoconstriction in human umbilical vein. Br. J. Pharmacol. 126 (2), 437–442. https://doi.org/10.1038/sj.bjp.0702320.

Feighner, J.P., 1999. Mechanism of action of antidepressant medications. J. Clin. Psychiatr. 60 (Suppl. 4), 4–11 discussion 12-3.

Filser, J.G., Kaumeier, S., Brand, T., Schanz, H., Terlinden, R., Müller, W.E., 1988. Pharmacokinetics of amitriptyline and amitriptylinoxide after intravenous or oral administration in humans. Pharmacopsychiatry 21 (6), 381–383.

French, A.M., Scott, N.C., 1983. Feedback inhibition of responses of rat vas deferens to twin pulse stimulation. Eur. J. Pharmacol. 21 https://doi.org/10.1016/0014-2999(83)90187-5, 86(3-4):379-83.

Furukawa, T., Morishita, H., 1997. Existence of dopamine D1 receptor on the sympathetic nerve endings in the Guinea-pig vas deferens. Eur. J. Pharmacol. 11, 229–234. https://doi.org/10.1016/s0014-2999(97)83049-x, 328(2-3).

Gillman, P.K., 2007. Tricyclic antidepressant pharmacology and therapeutic drug interactions updated. Br. J. Pharmacol. 151 (6), 737–748. https://doi.org/10.1038/sj.bjp.0707253.30.

Grundt, P., Carlson, E.E., Cao, J., Bennett, C.J., McElveen, E., Taylor, M., Luedtke, R.R., Newman, A.H., 2005. Novel heterocyclic trans olefin analogues of N-{4-[4-(2,3-dichlorophenyl)piperazin-1-yl]butyl}arylcarboxamides as selective probes with high affinity for the dopamine D3 receptor. J. Med. Chem. 10, 839–848. https://doi.org/10.1021/jm049465g, 48(3).

Hay, D.W., Wadsworth, R.M., 1982. The contractile effects of 5-hydroxytryptamine on the rat isolated vas deferens. Br. J. Pharmacol. 77 (4), 605–613. https://doi.org/10.1111/j.1476-5381.1982.tb09338.x.

Hsu, J.H., Shen, W.W., 1995. Male sexual side effects associated with antidepressants: a descriptive clinical study of 32 patients. Int. J. Psychiatr. Med. 25 (2), 191–201. https://doi.org/10.2190/1DHU-Y7L7-9GKG-V7WV.

Hyttel, J., 1983. SCH 23390 - the first selective dopamine D-1 antagonist. Eur. J. Pharmacol. 15, 153–154. https://doi.org/10.1016/0014-2999(83)90381-3, 91(1).

Janowsky, A., Eshleman, A.J., Johnson, R.A., Wolfrum, K.M., Hinrichs, D.J., Yang, J., Zabriskie, T.M., Smilkstein, M.J., Riscoe, M.K., 2014 Jul. Mefloquine and psychotomimetics share neurotransmitter receptor and transporter interactions in vitro. Psychopharmacology (Berl) 231 (14), 2771–2783. https://doi.org/10.1007/s00213-014-3446-0. Epub 2014 Feb 2. PMID: 24488404; PMCID: PMC4097020.

Karasawa, A., Kubo, K., Shuto, K., Nakamizo, N., 1985. Interaction of 5-hydroxytryptamine and ketanserin in rat vas deferens subjected to low frequency field stimulation. Jpn. J. Pharmacol. 37 (3), 285–291. https://doi.org/10.1254/jjp.37.285.

Knoll, J., Somogyi, G.T., Illés, P., Vizi, E.S., 1972. Acetylcholine release from isolated vas deferens of the rat. Naunyn Schmiedebergs Arch. Pharmacol. 274 (2), 198–202. https://doi.org/10.1007/BF00501855.

Kraus, M.B., Wie, C.S., Gorlin, A.W., Wisenbaugh, E.S., Rosenfeld, D.M., 2015. Painful ejaculation with cyclobenzaprine: a case report and literature review. Sex. Med. 19 (4), 343–345. https://doi.org/10.1002/sm2.93, 3.

Kung, M.P., Essman, W.D., Frederick, D., Meegalla, S., Goodman, M., Mu, M., Lucki, I., Kung, H.F., 1995 Aug. IPT: a novel iodinated ligand for the CNS dopamine transporter. Synapse 20 (4), 316–324. https://doi.org/10.1002/syn.890200405. PMID: 7482291.

Kuss, H.J., Jungkunz, G., 1986. Nonlinear pharmacokinetics of chlorimipramine after infusion and oral administration in patients. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 10 (6), 739–748.

Leedham, J.A., Pennefather, J.N., 1982 Oct. Dopamine acts at the same receptors as noradrenaline in the rat isolated vas deferens. Br. J. Pharmacol. 77 (2), 293–299. https://doi.org/10.1111/j.1476-5381.1982.tb09298.x. PMID: 6291687; PMCID: PMC2044591.

Leris, A.C., Stephens, J., Hines, J.E., McNicholas, T.A., 1997 Mar. Carbamazepine-related ejaculatory failure. Br. J. Urol. 79 (3), 485. https://doi.org/10.1046/j.1464-410x.1997.11336.x. PMID: 9117241.

Markstein, R., Hoyer, D., Engel, G., 1986 Aug. 5-HT1A-receptors mediate stimulation of adenylate cyclase in rat hippocampus. Naunyn-Schmiedeberg's Arch. Pharmacol. 333 (4), 335–341. https://doi.org/10.1007/BF00500006. PMID: 2945992.

Martin, P.L., Kelly, M., Cusack, N.J., 1993. (-)-2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin (N-0923), a selective D2 dopamine receptor agonist demonstrates the presence of D2 dopamine receptors in the mouse vas deferens but not in the rat vas deferens. J. Pharmacol. Exp. Therapeut. 267 (3), 1342–1348.

Merchant, K.M., Gill, G.S., Harris, D.W., Huff, R.M., Eaton, M.J., Lookingland, K., Lutzke, B.S., Mccall, R.B., Piercey, M.F., Schreur, P.J., Sethy, V.H., Smith, M.W., Svensson, K.A., Tang, A.H., Vonvoigtlander, P.F., Tenbrink, R.E., 1996. Pharmacological characterization of U-101387, a dopamine D4 receptor selective antagonist. J. Pharmacol. Exp. Therapeut. 279 (3), 1392–1403.

Mestres, J., Seifert, S.A., Oprea, T.I., 2011 Nov. Linking pharmacology to clinical reports: cyclobenzaprine and its possible association with serotonin syndrome. Clin. Pharmacol. Ther. 90 (5), 662–665. https://doi.org/10.1038/clpt.2011.177. Epub 2011 Oct 5. PMID: 21975349; PMCID: PMC3809033.

Millan, M.J., Dekeyne, A., Papp, M., La Rochelle, C.D., MacSweeny, C., Peglion, J.L., Brocco, M., 2001 Aug. S33005, a novel ligand at both serotonin and norepinephrine transporters: II. Behavioral profile in comparison with venlafaxine, reboxetine, citalopram, and clomipramine. J. Pharmacol. Exp. Therapeut. 298 (2), 581–591. PMID: 11454919.

Miranda, H.F., Wolstenholme, W.W., Moreu, G.M., Santiago, P.A., 1988. Effects of haloperidol on neurotransmitter activity in the rat vas deferens. Gen. Pharmacol. 19 (1), 123–127. https://doi.org/10.1016/0306-3623(88)90017-1.

Morishita, H., Katsuragi, T., 1999. Existence and pharmacological properties of dopamine D4 receptors in Guinea pig vas deferens. Eur. J. Pharmacol. 18 (2), 255–261. https://doi.org/10.1016/s0014-2999(99)00312-x, 374.

Motulsky, H., 2014. Normality tests. In: Intuitive Biostatistics: A Nonmathematical Guide to Statistical Thinking. Oxford University Press, New York, NY, pp. 203–208.

Muth, E.A., Haskins, J.T., Moyer, J.A., Husbands, G.E., Nielsen, S.T., Sigg, E.B., 1986 Dec 15. Antidepressant biochemical profile of the novel bicyclic compound Wy-45,030, an ethyl cyclohexanol derivative. Biochem. Pharmacol. 35 (24), 4493–4497. https://doi.org/10.1016/0006-2952(86)90769-0. PMID: 3790168.

Nojimoto, F.D., Mueller, A., Hebeler-Barbosa, F., Akinaga, J., Lima, V., Kiguti, L.R., Pupo, A.S., 2010 Jul-Aug. The tricyclic antidepressants amitriptyline, nortriptyline and imipramine are weak antagonists of human and rat alpha1B-adrenoceptors. Neuropharmacology 59 (1–2), 49–57. https://doi.org/10.1016/j.neuropharm.2010.03.015. Epub 2010 Apr 2. PMID: 20363235.

Patsalos, P.N., Berry, D.J., Bourgeois, B.F., Cloyd, J.C., Glauser, T.A., Johannessen, S.I., Leppik, I.E., Tomson, T., Perucca, E., 2008. Antiepileptic drugs–best practice guidelines for therapeutic drug monitoring: a position paper by the subcommission on therapeutic drug monitoring, ILAE Commission on Therapeutic Strategies. Epilepsia 49 (7), 1239–1276.

Percie du Sert, N., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W.J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Hurst, V., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E.J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E. S., Silberberg, S.D., Steckler, T., Würbel, H., 2020. Reporting animal research: explanation and elaboration for the ARRIVE guidelines 2.0. PLoS Biol. 14, e3000411 https://doi.org/10.1371/journal.pbio.3000411, 18(7).

Ribeiro, M.O., Antunes, E., de Nucci, G., Lovisolo, S.M., Zatz, R., 1992 Sep. Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. Hypertension 20 (3), 298–303. https://doi.org/10.1161/01.hyp.20.3.298. PMID: 1516948.

Richelson, E., Pfenning, M., 1984. Blockade by antidepressants and related compounds of biogenic amine uptake into rat brain synaptosomes: most antidepressants selectively block norepinephrine uptake. Eur. J. Pharmacol. 17 (104) https://doi.org/10.1016/0014-2999(84)90403-5 (3-4):277-86.

Rothmore, J., 2020. Antidepressant-induced sexual dysfunction. Med. J. Aust. 212 (7), 329–334. https://doi.org/10.5694/mja2.50522.

Sánchez, C., Hyttel, J., 1999. Comparison of the effects of antidepressants and their metabolites on reuptake of biogenic amines and on receptor binding. Cell. Mol. Neurobiol. 19 (4), 467–489. https://doi.org/10.1023/a:1006986824213. PMID: 10379421.

Shintani, F., Kinoshita, T., Kanba, S., Ishikawa, T., Suzuki, E., Sasakawa, N., Kato, R., Asai, M., Nakaki, T., 1996. Bioactive 6-nitronorepinephrine identified in mammalian brain. J. Biol. Chem. 7 (271) https://doi.org/10.1074/jbc.271.23.13561 (23): 13561-5.

Sibley, D.R., Monsma Jr., F.J., 1992. Molecular biology of dopamine receptors. Trends Pharmacol. Sci. 13 (2), 61–69. https://doi.org/10.1016/0165-6147(92)90025-2.

Tatsumi, M., Groshan, K., Blakely, R.D., Richelson, E., 1997. Pharmacological profile of antidepressants and related compounds at human monoamine transporters. Eur. J. Pharmacol. 11 (340) https://doi.org/10.1016/s0014-2999(97)01393-9 (2-3):249-58.

Tsunoda, M., Uchino, E., Imai, K., Hayakawa, K., Funatsu, T., 2007. Determination of nitrocatecholamines in rat brain using high-performance liquid chromatography-peroxyoxalate chemiluminescence reaction detection. J. Chromatogr. A 1164 (1–2), 162–166. https://doi.org/10.1016/j.chroma.2007.07.005.

Vaishnavi, S.N., Nemeroff, C.B., Plott, S.J., Rao, S.G., Kranzler, J., Owens, M.J., 2004. Milnacipran: a comparative analysis of human monoamine uptake and transporter binding affinity. Biol. Psychiatr. 1 (3), 320–322. https://doi.org/10.1016/j.biopsych.2003.07.006, 55.

Ventura, S., Burnstock, G., 1997. Evidence against nitrergic neuromodulation in the rat vas deferens. Eur. J. Pharmacol. 3 (1), 87–93. https://doi.org/10.1016/s0014-2999(97)01172-2, 334.

Vohra, M.M., 1979. A postsynaptic inhibitory histamine H2-receptor in the mouse isolated vas deferens. Agents Actions 9 (4), 319–326. https://doi.org/10.1007/BF01970655.

Waldinger, M.D., 2018. Drug treatment options for premature ejaculation. Expet Opin. Pharmacother. 19 (10), 1077–1085. https://doi.org/10.1080/14656566.2018.1494725.

Waldinger, M.D., Zwinderman, A.H., Olivier, B., 2004. On-demand treatment of premature ejaculation with clomipramine and paroxetine: a randomized, double-blind fixed-dose study with stopwatch assessment. Eur. Urol. 46 (4), 510–515. https://doi.org/10.1016/j.eururo.2004.05.005 discussion 516.

Warner, T.D., de Nucci, G., Vane, J.R., 1989. Comparison of the survival of endothelium-derived relaxing factor and nitric oxide within the isolated perfused mesenteric arterial bed of the rat. Br. J. Pharmacol. 97 (3), 777–782. https://doi.org/10.1111/j.1476-5381.1989.tb12016.x.

Wouters, W., van Hertrooy, J.J., Krekels, M.D., Laduron, P.M., 1986. Reconstitution of solubilized serotonin-S2 receptors into phospholipid vesicles. J. Recept. Res. 6 (3–4), 271–282. https://doi.org/10.3109/10799898609074814.

Wu, E.H., De Cicco, F.L., 2021. Anatomy, abdomen and pelvis, male genitourinary tract. 2020 Sep 15. In: StatPearls [Internet]. StatPearls Publishing, Treasure Island (FL.

Zeng, C., Wang, D., Yang, Z., Wang, Z., Asico, L.D., Wilcox, C.S., Eisner, G.M., Welch, W. J., Felder, R.A., Jose, P.A., 2004. Dopamine D1 receptor augmentation of D3 receptor action in rat aortic or mesenteric vascular smooth muscles. Hypertension 43 (3), 673–679. https://doi.org/10.1161/01.HYP.0000118958.27649.6f.

Anexo 7

Artigo 9 – Alpha1-adrenergic antagonists block 6-nitrodopamine contractions on the rat isolated epididymal vas deferens.

Autores: Britto-Júnior J, Ribeiro A, Ximenes L, Lima AT, Jacintho FF, Fregonesi A, Mónica FZ, Antunes E, De Nucci G

Revista: European Journal of Pharmacology

Situação: Aceito a publicação em 20 de dezembro de 2021. Publicado on-line em 22 de dezembro de 2021

European Journal of Pharmacology 915 (2022) 174716

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Alpha1-adrenergic antagonists block 6-nitrodopamine contractions on the rat isolated epididymal vas deferens

José Britto-Júnior [a,*], André Ribeiro [a], Luiz Ximenes [a], Antonio Tiago Lima [a], Felipe Fernandes Jacintho [a], Adriano Fregonesi [a], Fabíola Z. Mónica [a], Edson Antunes [a], Gilberto De Nucci [a,b,c,d]

[a] Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, SP, Brazil
[b] Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo (USP), São Paulo, SP, Brazil
[c] Department of Pharmacology, Faculty of Medicine, São Leopoldo Mandic, Campinas, SP, Brazil
[d] Brazil University, Fernandópolis, SP, Brazil

ARTICLE INFO

Keywords:
Premature ejaculation
Nitrocatecholamines
EFS
L-NAME
Idazoxan

ABSTRACT

6-nitrodopamine (6-ND) is released from rat isolated vas deferens and modulates electrical-field stimulation (EFS) contractions of the rat isolated epididymal vas deferens (RIEVD) via a specific receptor which is blocked by tricyclic antidepressants. Here, the effects of selective α_1-adrenergic receptor antagonists on RIEVD contractions induced by 6-ND, dopamine, noradrenaline, adrenaline and EFS were investigated. Doxazosin and tamsulosin (3–10 nM) caused significant rightward shifts of the concentration-response curve to 6-ND, but had no effect on dopamine-, noradrenaline- and adrenaline-induced contractions. Alfuzosin (10 nM) produced rightward shifts on concentration-response curves to all catecholamines. Silodosin (10 nM) and terazosin (100 nM) displaced to the right the noradrenaline, dopamine and adrenaline curves, but higher concentrations of both antagonists (100 and 300 nM, respectively) were required to displace the 6-ND curves. The EFS-induced contractions were significantly inhibited only at the concentrations that the α_1-adrenergic receptor antagonists caused rightward shifts on the 6-ND concentration-response curves. The inhibition of EFS-induced contractions by doxazosin (10 nM), tamsulosin (10 nM), alfuzosin (10 nM), silodosin (100 nM) and terazosin (300 nM), were not observed in RIEVD obtained from animals chronically treated with L-NAME. This work demonstrates that α_1-adrenoceptor antagonists act as 6-ND receptor antagonists in RIEVD, opening the possibility that many actions previously attributed to noradrenaline could be due to 6-ND antagonism. In addition, blockade of the 6-ND receptors by both tricyclic antidepressants and α_1-adrenergic receptor antagonists may represent the common mechanism of action responsible for their therapeutic use in the treatment of premature ejaculation.

1. Introduction

Ejaculation is used as a synonym for the external ejection of semen and comprises two phases, namely, emission, which is the ejection into the prostatic urethra of spermatozoa mixed with fluids secreted by the accessory sexual glands through epididymis, ductus deferens, seminal vesicles, and prostate smooth muscles; and expulsion, that is defined as the release of the semen from the urethra by involuntary contractions of the striated perineal muscles (Puppo and Puppo, 2016). Rat vas deferens exhibits basal release of both 6-nitrodopamine (6-ND) and noradrenaline, as identified by tandem mass spectrometry (Britto-Júnior et al., 2021a; Ximenes et al., 2021). 6-Nitrodopamine causes rat isolated epididymal vas deferens (RIEVD) contractions, which is blocked by the tricyclic antidepressants desipramine, clomipramine and amitriptyline, the skeletal muscle relaxant cyclobenzaprine and the anti-epileptic drug carbamazepine (Britto-Júnior et al., 2021a). Many of the antidepressant drugs are associated with male sexual adverse reactions such as anorgasmia or delayed ejaculation that can lead to non-compliance treatment (Monteiro et al., 1987; Segraves, 1989; Segraves, 2007; Segraves and Balon, 2014). Accordingly, tricyclic antidepressants are often used in the treatment of premature ejaculation (Giuliano and Hellstrom, 2008; Giuliano and Clèment, 2012). Since the mechanism(s) by which

* Corresponding author. University of Campinas (Unicamp), Department of Pharmacology, Faculty of Medical Sciences, Rua Tessália Vieira de Camargo, 126 - Cidade Universitária, 13083-887, Campinas, São Paulo, Brazil.
E-mail address: josebrittojr@gmail.com (J. Britto-Júnior).

https://doi.org/10.1016/j.ejphar.2021.174716
Received 25 October 2021; Received in revised form 25 November 2021; Accepted 20 December 2021
Available online 22 December 2021
0014-2999/© 2021 Elsevier B.V. All rights reserved.

the tricyclic antidepressants delay the ejaculation is(are) not clear, the antagonism of 6-ND action on the vas deferens could be an important peripheral mechanism of action of these drugs (Britto-Júnior et al., 2021a).

Alpha$_1$-adrenoceptor antagonists are now commonly used in the treatment of the symptoms of lower urinary tract obstruction (Andersson et al., 1997). Prazosin (U'Prichard et al., 1978; Docherty, 2019), terazosin (Frishman et al., 1988; O'Leary, 2001), alfuzosin (Ramsay et al., 1988) and doxazosin (Elliott et al., 1982; Wilt and MacDonald, 2006) are classified as non-subtype selective α_1-adrenergic antagonists (Martin, 2010) whereas tamsulosin (Lepor et al., 1988; Dunn et al., 2002) and silodosin (Yamagishi et al., 1966; Keating, 2015) are considered highly selective α_{1A}-adrenergic antagonists (Roehrborn, 2009). However, the use of this class of drugs in the treatment of lower urinary tract symptoms (LUTS) has been linked to ejaculatory abnormalities such as retrograde ejaculation, reduced ejaculate volume and absence of ejaculate in 4–11% of patients (Höfner et al., 1999; Djavan et al., 2004). These adverse reactions are apparently not dependent on the receptor subtype selectivity since the incidence of abnormal ejaculation was similar in patients receiving either tamsulosin or alfuzosin (Hellstrom and Sikka, 2006). Interestingly, terazosin has been successfully used for the treatment of premature ejaculation (Başar et al., 2005).

Here it was investigated the effect of several α_1-adrenoceptor antagonists on the contractile activity of 6-ND and other catecholamines (dopamine, noradrenaline and adrenaline) on the rat isolated epididymal vas deferens (RIEVD). In addition, it was also assessed the action of these α_1-adrenoceptor antagonists on the electric-field stimulation (EFS)-induced RIEVD contractions. Because the release of 6-ND is coupled to nitric oxide (NO) and that is inhibited by the NO synthesis inhibitor N$^{\omega}$-L-nitro-L-arginine methyl ester (L-NAME; Britto- et al., 2021b), we also investigated the effects α_1-adrenoceptor antagonists on the contractions of RIEVD obtained from control and animals chronically treated with the nitric oxide inhibitor N$^{\omega}$-L-nitro-L-arginine methyl ester (L-NAME).

2. Methods

2.1. Animals

Adult male Wistar rats (280–320 g) were provided by the animal care of University of Campinas (UNICAMP; Campinas, São Paulo, Brazil) and Animais de Laboratorio Criação e Com. LTDA (ANILAB; Paulinia, São Paulo, Brazil). All experimental protocols were authorized by the Ethics Committee in Animal Use of UNICAMP (CEUA/UNICAMP, protocols numbers 5746-1/2020 and 5831-1/2021) and followed the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al., 2020). Animals were housed in cages (three per cage) located in ventilated cage shelters with constant humidity of 55% ± 5% and temperature of 24 ± 1 °C under a 12-h light-dark cycle. Animals received filtered water and standard food *ad libitum*.

2.2. Reagents

Dopamine, adrenaline, prazosin, idazoxan and N$^{\omega}$-Nitro-L-arginine methyl ester hydrochloride (L-NAME) were obtained from Sigma-Aldrich Chemicals Co. (St Louis, Missouri, USA). Tamsulosin was obtained from Swati Spentose Pvt Ltd (Vapi, Gujarat, India). Doxazosin was obtained from Nifty Labs Pvt Ltd (Hyderabad, Telangana, India). Alfuzosin, silodosin, noradrenaline and terazosin were purchased from Cayman Chemical Co (Michigan, USA). 6-nitrodopamine was bought from Toronto Research Chemicals Inc (Toronto, Ontario, Canada). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$), potassium phosphate monobasic (KH_2PO_4) and glucose were acquired from Merck KGaA (Darmstadt, Germany).

2.3. L-NAME treatment

Rats were chronically treated with the nitric oxide synthase inhibitor L-NAME (20 mg/rat/day) in the drinking water for 4 weeks minimum (Ribeiro et al., 1992). Control animals received tap water alone.

2.4. Rat isolated vas deferens isolation and preparation

Euthanasia was performed by isoflurane overdose, in which animals were exposed to a concentration greater than 5% until 1 min after the breathing stops. Exsanguination was performed to confirm the euthanasia. The vas deferens was removed and immediately placed in Krebs-Henseleit's solution (KHS) of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 5.6 mM glucose. Epididymal portions of vas deferens were surgically dissected (length, 1.5 cm each) for functional studies. The RIEVD strips were suspended vertically between metal hooks in 10-mL organ baths containing KHS (pH 7.4), continuously gassed with a mixture of 95%O_2: 5%CO_2 at 37 °C. Tissues were allowed to equilibrate under a resting tension of 10 mN, and the isometric tension was registered using a PowerLab system (ADInstruments, Sydney, Australia). Following a 45-min stabilization period, the RIEVD strips were initially contracted with a single concentration of noradrenaline (NA, 10 μM) to verify the tissue viability.

2.5. In vitro functional assays in RIEVD preparations

Cumulative concentration-response curves to 6-ND were performed in RIEVD strips in the absence and presence of doxazosin (1, 3 and 10 nM, 30 min), tamsulosin (0.1, 1 and 10 nM, 30 min), alfuzosin (1, 3 and 10 nM, 30 min), silodosin (10, 30 and 100 nM, 30 min), terazosin 0.1, 0.3 and 1 μM, 30 min), prazosin (1, 3 and10 nM, 30 min) or idazoxan (1 μM, 30 min). In separate RIEVD preparations, cumulative concentration-response curves to dopamine, noradrenaline and adrenaline were performed in the absence and presence of doxazosin (10 nM, 30 min), tamsulosin (10 nM, 30 min), alfuzosin (10 nM, 30 min), silodosin (10 nM, 30 min), terazosin (100 nM, 30 min), prazosin (10 nM, 30 min) or idazoxan (1 μM, 30 min).

2.6. Electric-field stimulation (EFS) in isolated RIEVD preparations

The contractions of RIEVD induced by EFS were evaluated in control and L-NAME-treated rats. Briefly, RIEVD strips were submitted to EFS at 60 V for 20 s, at 2–16 Hz in square-wave pulses, 0.3 ms pulse width, and 0.1 ms delay, using a Grass S88 stimulator (Astro-Medical, Industrial Park, RI, USA). In control animals, EFS-induced RIEVD contractions were evaluated in the absence and in the presence of doxazosin (1, 3 and10 nM, 30 min), tamsulosin (0.1, 1 and 10 nM, 30 min), alfuzosin (1 and 10 nM, 30 min), silodosin (10 and 100 nM, 30 min), terazosin (100 and 300 nM, 30 min), prazosin (10 nM, 30 min) or idazoxan (0.01, 0.1 and 1 μM, 30 min). In L-NAME-treated rats, EFS-induced RIEVD contractions were evaluated in the absence and in the presence doxazosin (10 nM, 30 min), tamsulosin (10 nM, 30 min), alfuzosin (10 nM, 30 min), silodosin (100 nM, 30 min), terazosin (300 nM, 30 min), prazosin (10 nM, 30 min) or idazoxan (1 μM, 30 min). In separate RIEVD strips obtained from control and L-NAME-treated rats, the effects of prazosin (1 and 10 nM, 30 min) in preparations previously incubated with idazoxan (1 μM, 30 min) were investigated. The contractile responses were expressed as mN.

2.7. Data analysis

Nonlinear regression analysis to determine the pEC_{50} was carried out using GraphPad Prism (GraphPad Software, version 6.0, San Diego, California, USA) with the constraint that F = 0. All concentration–response data were evaluated for a fit to a logistics function in

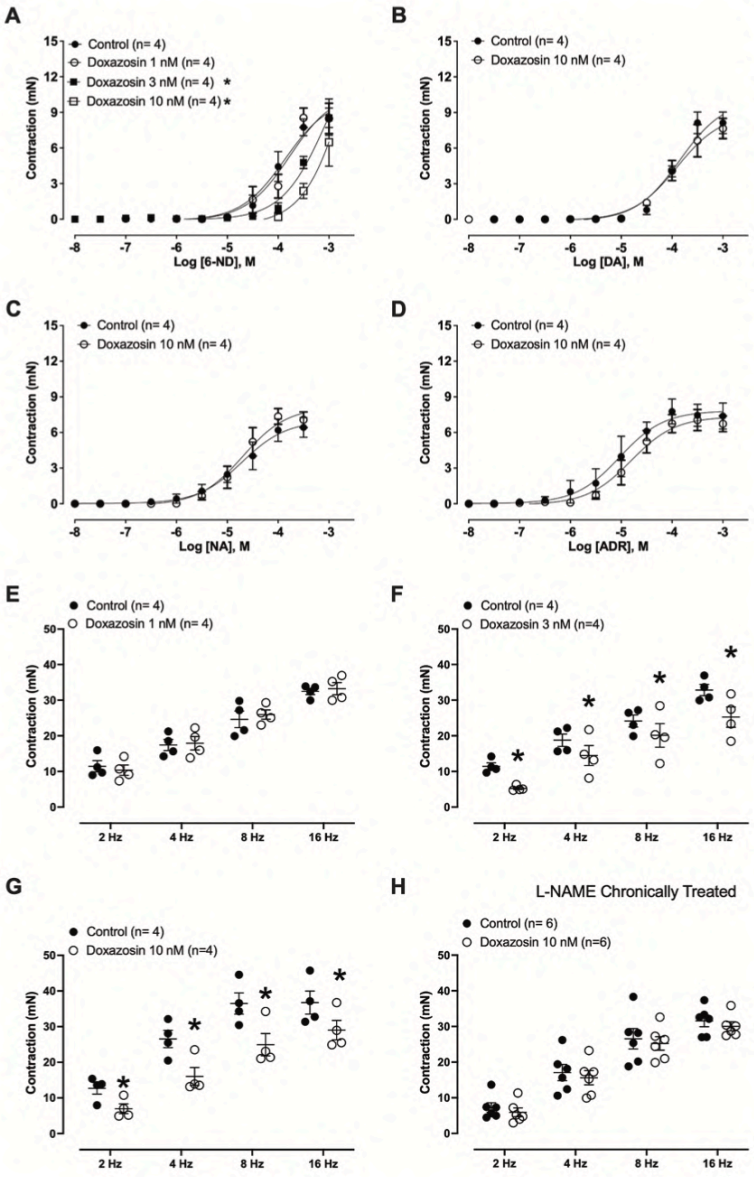


Fig. 1. Effect of doxazosin in the rat isolated epididymal vas deferens (RIEVD). Doxazosin (3 and 10 nM) caused significant concentration-dependent rightward shifts of the concentration-response curves to 6-ND (Panel A). Doxazosin (10 nM) had no effect on the contractions induced by dopamine (Panel B), noradrenaline (Panel C) and adrenaline (Panel D). Doxazosin (1 nM; Panel E) caused no reduction in the contractions induced by EFS but at higher concentrations (3 nM; Panel F) and (10 nM; Panel G) caused significant inhibition of EFS-induced contractions of the RIEVD obtained from control animals. Doxazosin (10 nM) had no effect on the EFS-induced contractions of the RIEVD obtained from animals chronically treated with L-NAME (Panel H). Data are expressed as mean ± SEM. *P < 0.05 compared with respective control values. N means the number of vas deferens strips.

the form: $E = E_{max}/([1 + (10c/10x)^n] + F$, where E represents the increase in response contractile induced by the agonist, E_{max} is the effect agonist maximum, c is the logarithm of concentration of the agonist that produces 50% of E_{max}, x is the logarithm of the concentration of the drug; the exponential term, n, is a curve fitting parameter that defines the slope of the concentration–response line, and F is the response observed in the absence of added drug. The values of pEC_{50} data represent the mean ± standard error of the mean (SEM) of *n* experiments. Values of E_{max} were expressed in mN. One strip was used as the control response and the other strip was incubated with an antagonist/inhibitor. Student's two-tail unpaired *t*-test was employed and the differences between groups. In addition, standard ANOVA, followed by the Newman–Keuls post-test, were used when more than two groups were involved. A p value of less than 0.05 was considered statistically significant. The distribution of the log values (pEC_{50}) was normal for each agonist, confirmed by the Shapiro-Wilk test (Motulsky, 2014). For 6-ND, the pA_2 values of the antagonists were calculated from the intercept on the concentration axis and by application of the equation; pA_2 = log (antagonist concentration) −log (CR-1) -log (antagonist concentration) (Arunlakshana and SCHILD, 1959).

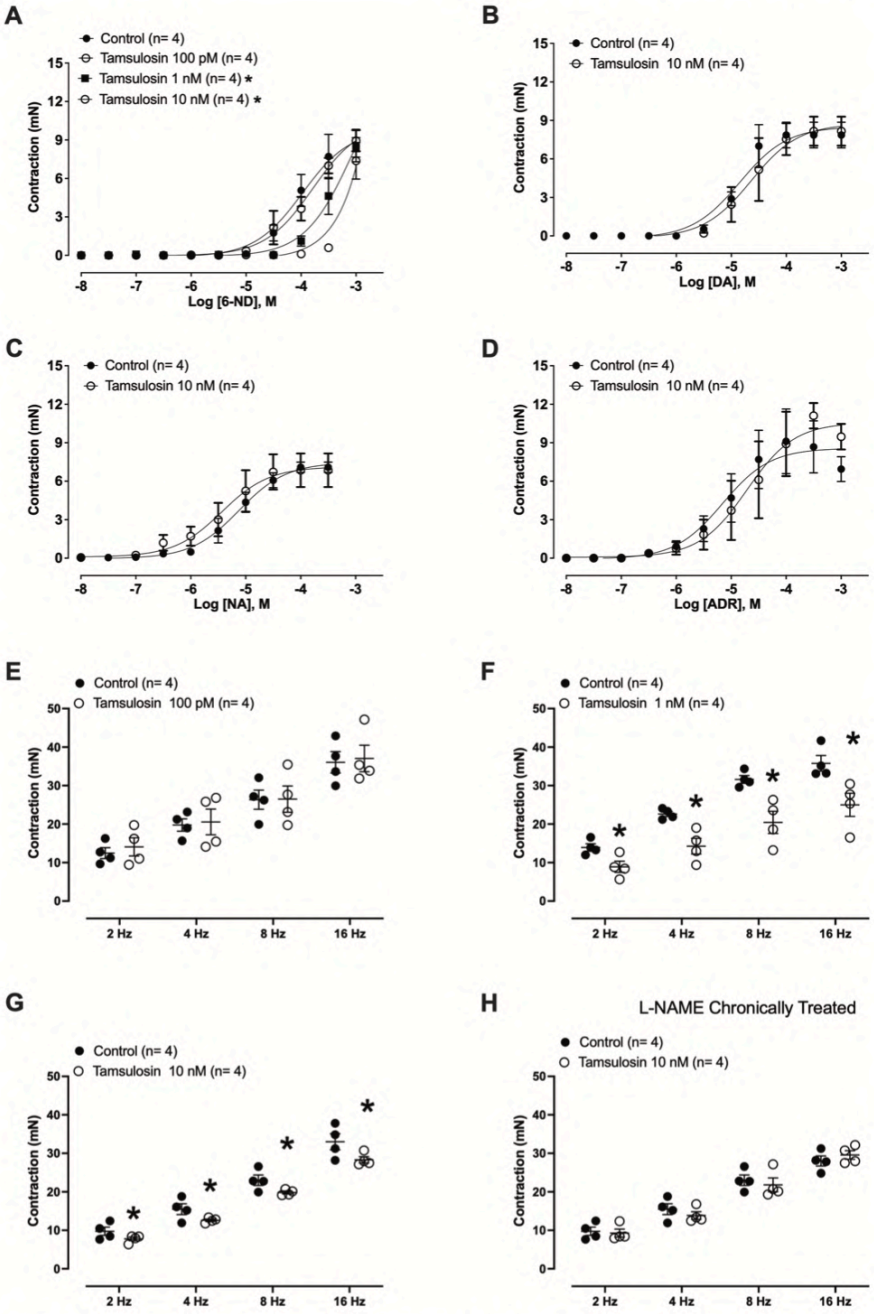


Fig. 2. Effect of tamsulosin in the rat isolated epididymal vas deferens (RIEVD). Tamsulosin (1 and 10 nM) caused significant concentration-dependent rightward shifts of the concentration-response curves to 6-ND (Panel A). Tamsulosin (10 nM) had no effect on the contractions induced by dopamine (Panel B), noradrenaline (Panel C) and adrenaline (Panel D). Tamsulosin (100 pM; Panel E) caused no reduction in the contractions induced by EFS but at higher concentrations (1 nM; Panel F) and (10 nM; Panel G) caused significant inhibition of EFS-induced contractions of the RIEVD obtained from control animals. Tamsulosin (10 nM) had no effect on the EFS-induced contractions of the RIEVD obtained from animals chronically treated with L-NAME (Panel H). Data are expressed as mean ± SEM. *P < 0.05 compared with respective control values. N means the number of vas deferens strips.

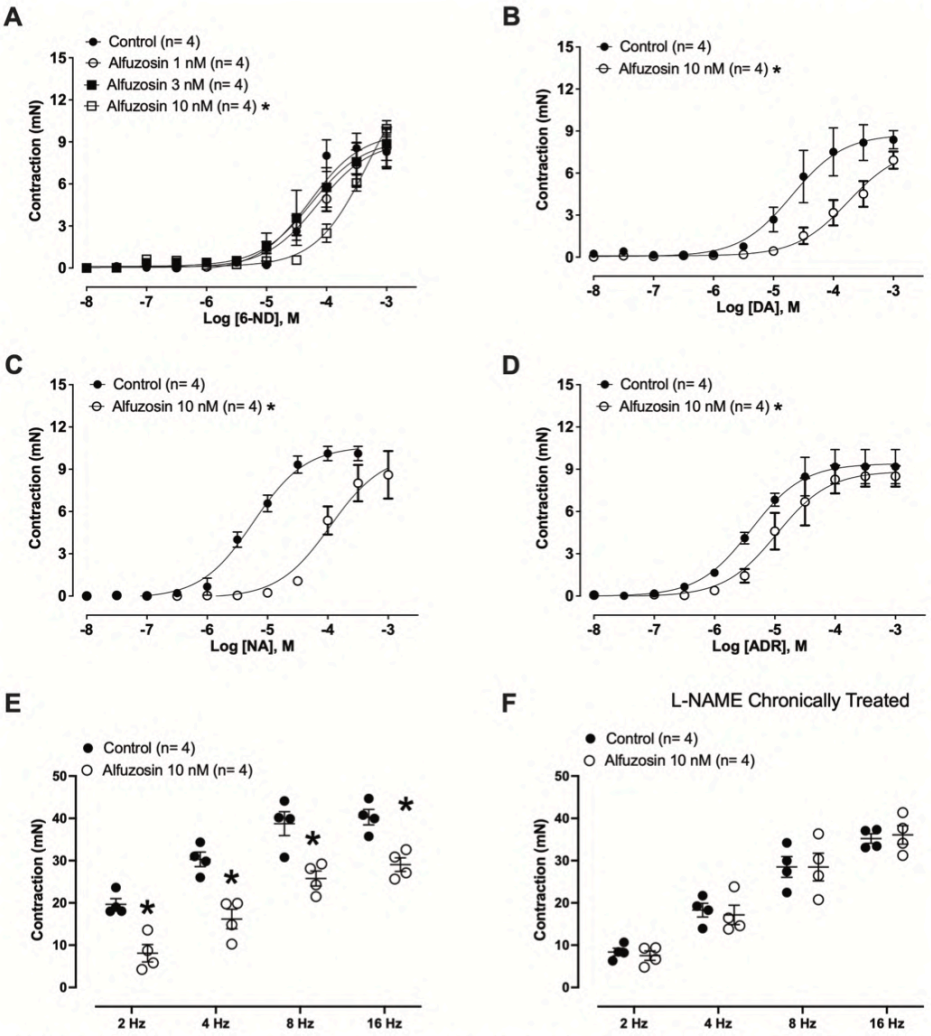


Fig. 3. Effect of alfuzosin in the rat isolated epididymal vas deferens (RIEVD). Alfuzosin (10 nM) caused rightward shifts of the concentration-response curves to 6-ND (Panel A), dopamine (Panel B), noradrenaline (Panel C) and adrenaline (Panel D). Alfuzosin (10 nM) caused reduction in the contractions induced by EFS of the RIEVD obtained from control animals (Panel E) but not in the contractions induced by EFS of the RIEVD obtained from animals chronically treated with L-NAME (Panel F). Data are expressed as mean ± SEM. *P < 0.05 compared with respective control values. N means the number of vas deferens strips.

3. Results

3.1. Effect of doxazosin on RIEVD contractions induced by catecholamines and EFS

Doxazosin (3 and 10 nM) produced concentration-dependent rightward shifts on the concentration-response curves to 6-ND (Fig. 1A; p = 0.0119 and p = 0.0375, respectively) with a pA_2 value of 9.15 ± 0.13 (n = 4). Doxazosin (10 nM) had no effect on the RIEVD contractions induced by dopamine (Fig. 1B; p = 0.3179), noradrenaline (Fig. 1C; p = 0.4708) and adrenaline (Fig. 1D; p = 0.2892).

Doxazosin (1 nM) had no effect on the EFS-induced contractions of RIEVD (Fig. 1E), but at higher concentrations (3 and 10 nM), doxazosin significantly reduced the EFS-induced contractions of the RIEVD in all frequencies tested (Fig. 1F and G, respectively). In RIEVD obtained from L-NAME-treated rats, doxazosin (10 nM) had no effect on the EFS-induced contractions (Fig. 1H).

3.2. Effect of tamsulosin on RIEVD contractions induced by catecholamines and EFS

Tamsulosin (1 and 10 nM) produced concentration-dependent rightward shifts on the concentration-response curve to 6-ND (Fig. 2A; P = 0.0378 and p = 0.0001, respectively) with a pA_2 value of 9.66 ± 0.09 (n = 4). Tamsulosin (10 nM) had no effect on the RIEVD contractions induced by dopamine (Fig. 2B; p = 0.2179), noradrenaline

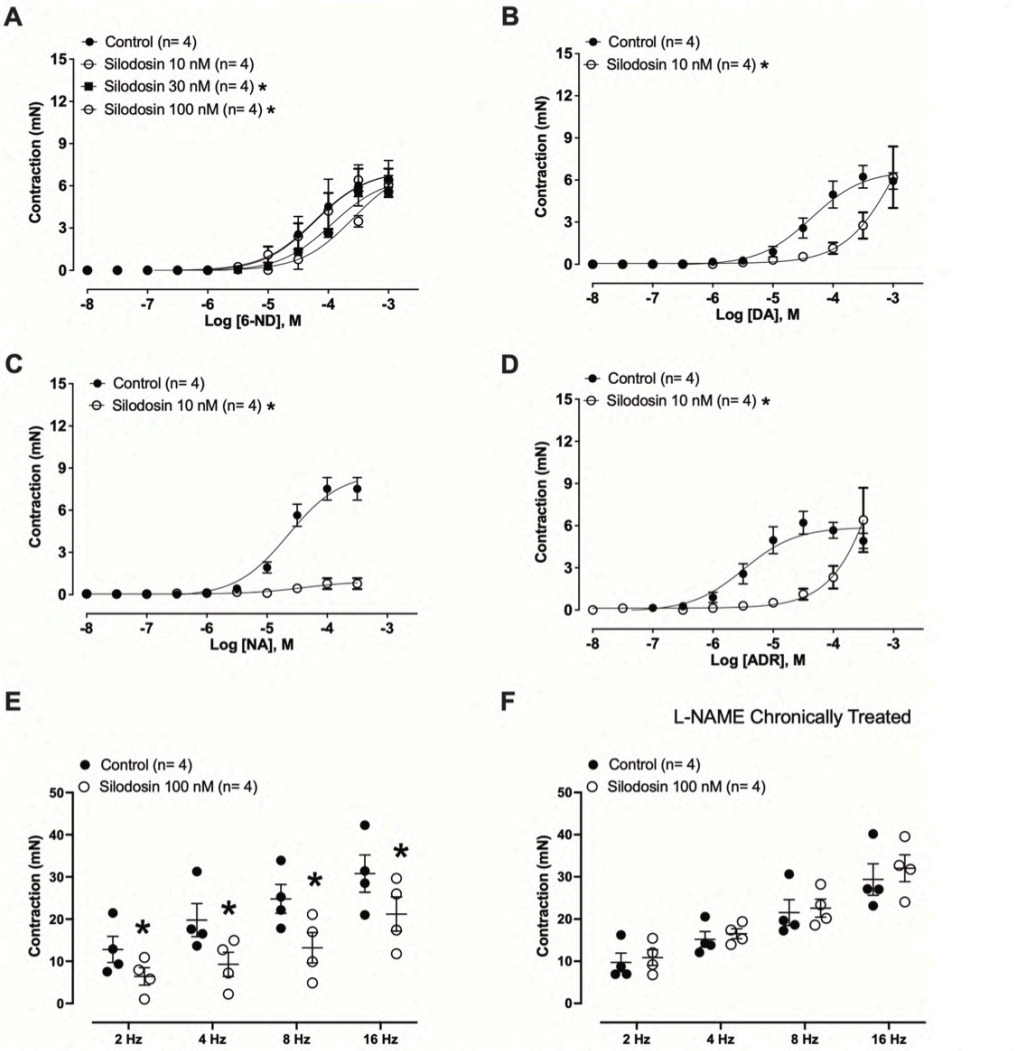


Fig. 4. Effect of silodosin in the rat isolated epididymal vas deferens (RIEVD). Silodosin (30 and 100 nM) induced significant concentration-dependent rightward shifts on the 6-ND induced contractions of the RIEVD (Panel A). Silodosin (10 nM) induced concentration rightward shifts on the contractions induced by dopamine (Panel B) and adrenaline (Panel D) of the RIEVD. Silodosin (10 nM) practically abolished the concentration-response curve to noradrenaline (Panel C) of the RIEVD. Silodosin (100 nM) caused reduction in the contractions induced by EFS of the RIEVD obtained from control animals (Panel E) but not in the contractions induced by EFS of the RIEVD obtained from animals chronically treated with L-NAME (Panel F). Data are expressed as mean ± SEM. *P < 0.05 compared with respective control values. N means the number of vas deferens strips.

(Fig. 2C; p = 0.1988) and adrenaline (Fig. 2D; p = 0.1994).

Tamsulosin (100 pM) had no effect on the EFS-induced contractions of RIEVD (Fig. 2E), but at higher concentrations (1 and 10 nM), it caused significant inhibitions of EFS-induced contractions in all frequencies (Fig. 2F and G, respectively). Tamsulosin (10 nM) failed to affect the EFS-induced contractions when RIEVD were obtained from animals chronically treated with L-NAME (Fig. 2H).

3.3. *Effect of alfuzosin on RIEVD contractions induced by catecholamines and EFS*

Alfuzosin at 10 nM produced a rightward shift on the RIEVD contractions induced by 6-ND (Fig. 3A; p = 0.0004) with a pA_2 value of 8.86 ± 0.07 (n = 4). At 10 nM, alfuzosin also produced rightward shifts in the concentration response curves to dopamine (Fig. 3B; p = 0.0310), noradrenaline (Fig. 3C; p = 0.0001) and adrenaline (Fig. 3D; p = 0.0366).

Alfuzosin (1 nM) had no effect on the EFS-induced contractions of RIEVD (Fig. S01), but at 10 nM caused a significant inhibition of EFS-

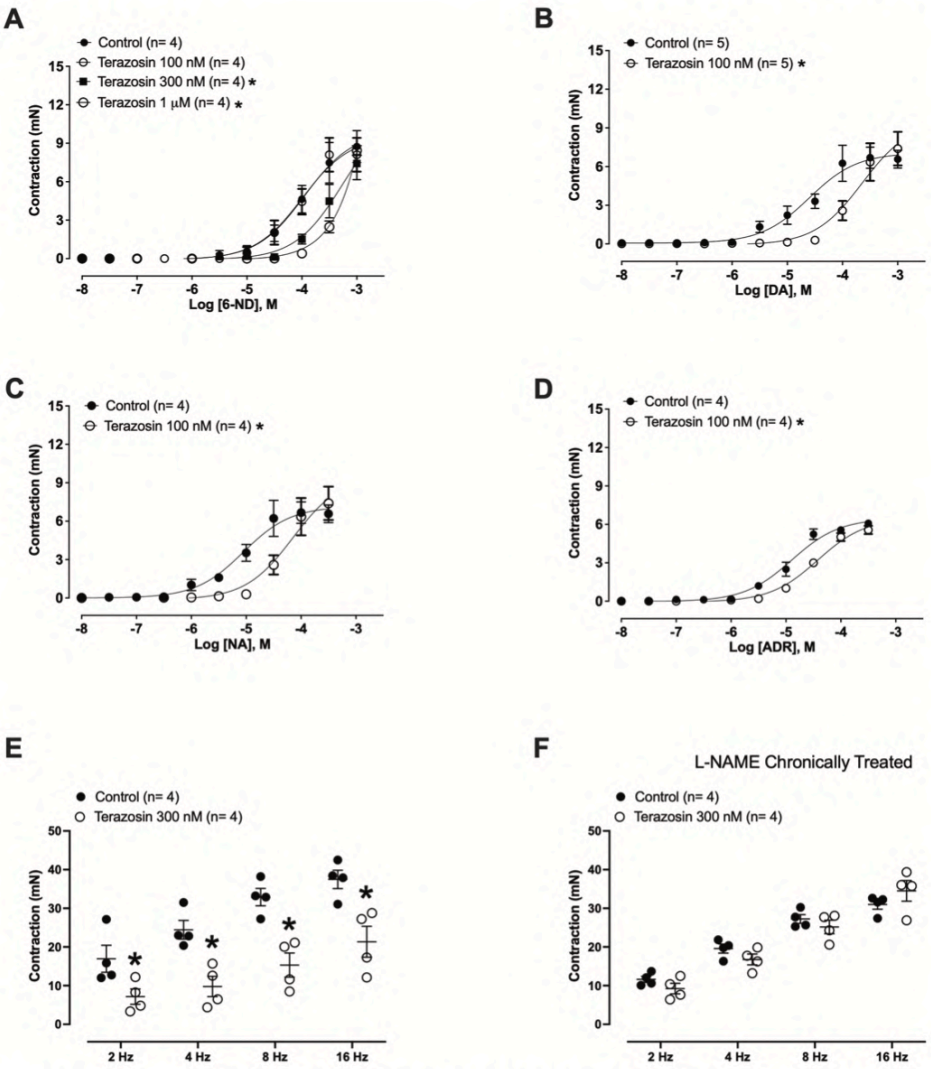


Fig. 5. Effect of terazosin in the rat isolated epididymal vas deferens (RIEVD). Terazosin (300 nM and 1 μM) induced significant concentration-dependent rightward shifts on the 6-ND induced contractions of the RIEVD (Panel A). Terazosin (100 nM) caused rightward shifts of the concentration-response curves dopamine (Panel B), noradrenaline (Panel C) and adrenaline (Panel D). Terazosin (300 nM; Panel E) caused significant ($p < 0.05$) reduction in the contractions induced by EFS of the RIEVD obtained from control animals (Panel E) but not from RIEVD obtained from animals chronically treated with L-NAME (Panel F). Data are expressed as mean ± SEM. *$P < 0.05$ compared with respective control values. N means the number of vas deferens strips.

induced contractions in RIEVD from control (Fig. 3E) but not from L-NAME-treated rats (Fig. 3F).

3.4. Effect of silodosin on RIEVD contractions induced by catecholamines and EFS

Silodosin (30 and 100 nM) produced concentration-dependent rightward shifts on the concentration-response curves to 6-ND (Fig. 4A; $p = 0.0442$ and $p = 0.0142$, respectively). The pA_2 value for silodosin was 7.70 ± 0.18 ($n = 4$). Silodosin (10 nM) induced concentration rightward shifts on the RIEVD contractions induced by dopamine (Fig. 4B; $p = 0.0139$) and adrenaline (Fig. 4D; $p = 0.0310$), and practically abolished the noradrenaline-induced contractions (Fig. 4C; $p = 0.0001$).

Silodosin (10 nM) had no effect on the EFS-induced contractions of RIEVD (Fig. S02). At 100 nM, silodosin significantly inhibited the EFS-induced contractions of the RIEVD from control (Fig. 4E) but not from the L-NAME-treated rats (Fig. 4F).

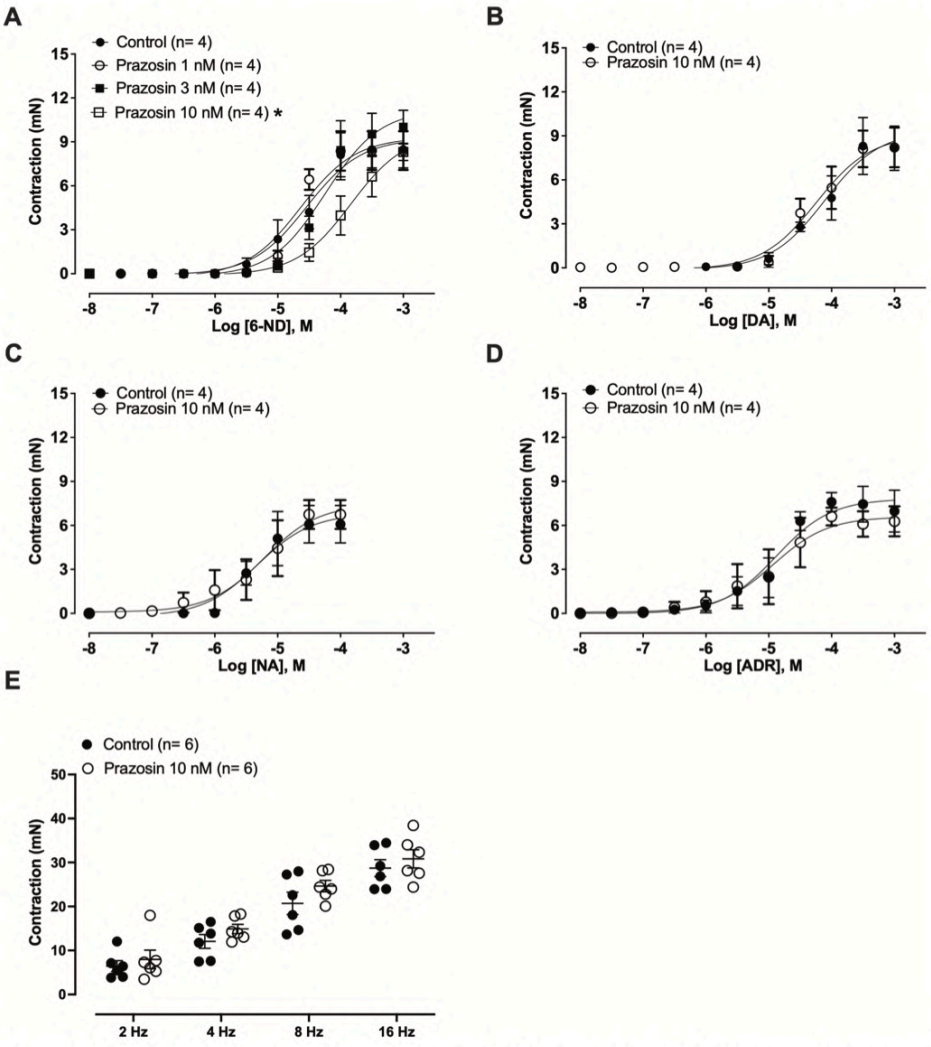


Fig. 6. Effect of prazosin in the rat isolated epididymal vas deferens (RIEVD). Prazosin (10 nM) caused rightward shifts of the concentration-response curves to 6-ND. Prazosin (10 nM) had no effect on the contractions induced by dopamine (Panel B), noradrenaline (Panel C) and adrenaline (Panel D). Prazosin (10 nM) did not cause a significant reduction in the contractions induced by EFS of the RIEVD obtained from control animals (Panel E). *P < 0.05 compared with respective control values. N means the number of vas deferens strips.

3.5. Effect of terazosin on RIEVD contractions induced by catecholamines and EFS

Terazosin (300 nM and 1 μM) induced concentration-dependent rightward shifts on the 6-ND-induced contractions (Fig. 5A; P = 0.0039 and p = 0.0001, respectively) with a pA_2 value of 7.20 ± 0.06 (n = 4). Terazosin (100 nM) induced concentration rightward shifts on the RIEVD contractions induced by dopamine (Fig. 5B; p = 0.0020), noradrenaline (Fig. 5C; p = 0.0023) and adrenaline (Fig. 5D; p = 0.0009).

Terazosin (100 nM) had no effect on the EFS-induced contractions of RIEVD (Fig. S03), but at 300 nM caused a significant inhibition of EFS-induced contractions in RIEVD strips from control (Fig. 5E) but not from L-NAME-treated animals (Fig. 5F).

3.6. Effect of prazosin on RIEVD contractions induced by catecholamines and EFS

Prazosin at 10 nM produced a concentration-dependent rightward shift on the concentration-response curve to 6-ND (Fig. 6A; p = 0.0071). The pA_2 value for prazosin was 8.82 ± 0.07 (n = 4). Prazosin (10 nM) had no effects on the RIEVD contractions induced by dopamine (Fig. 6B; p = 0.4263), noradrenaline (Fig. 6C; p = 0.4649) and adrenaline (Fig. 6D; p = 0.3649) of the RIEVD.

Prazosin (10 nM) had no effect on the EFS-induced contractions of RIEVD (Fig. 6E).

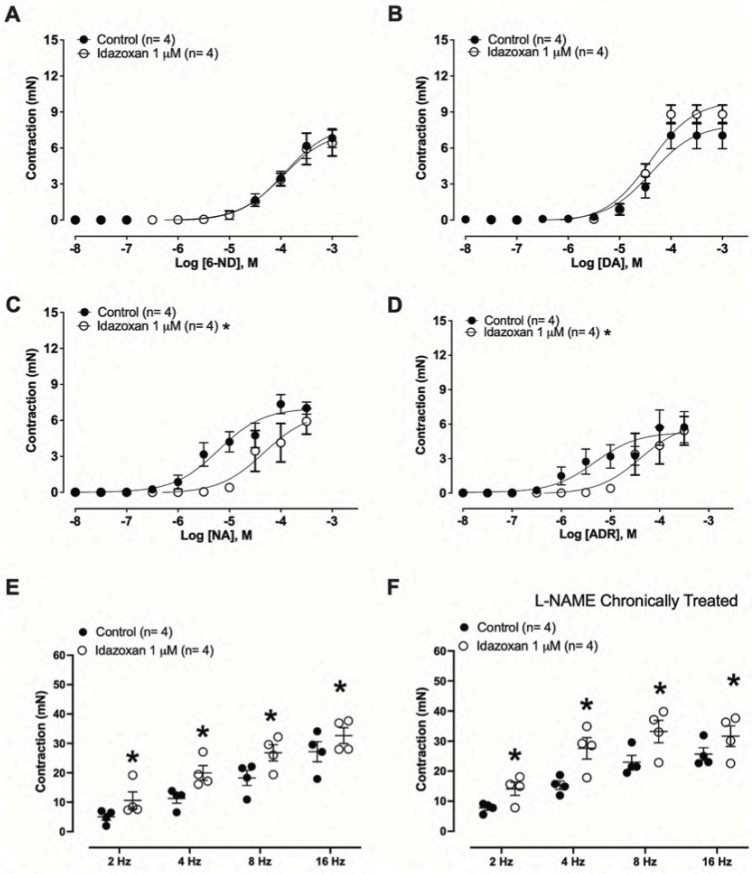


Fig. 7. Effect of idazoxan (1 μM) in the rat isolated epididymal vas deferens (RIEVD) on the concentration-response curves to 6-ND (Panel A), dopamine (Panel B), noradrenaline (Panel C) and adrenaline (Panel D). Idazoxan (1 μM; Panel E) potentiated the contractions induced by EFS of the RIEVD from control and from L-NAME chronically treated rats. (Panel F). *P < 0.05 compared with respective control values. N means the number of vas deferens strips.

3.7. Effect of idazoxan on RIEVD contractions induced by catecholamines and EFS

Idazoxan was investigated at 0.01, 0.1 and 1 μM. At 0.01 μM, it had no effect, at 0.1 μM it caused a 32 ± 7% increase and at 1 μM caused 82 ± 24% increase in the EFS-induced contractions (Fig. S04). Idazoxan (1 μM) had no effect on the contractions of the RIEVD induced by 6-ND (Fig. 7A; p = 0.2532) and dopamine (Fig. 7B; p = 0.3162), but caused significant rightward shifts on the contractions induced by noradrenaline (Fig. 7C; p = 0.0229) and adrenaline (Fig. 7D; p = 0.0220) of the RIEVD.

Idazoxan (1 μM) significantly potentiated the EFS-induced contractions in the RIEVD strips obtained from both control (Fig. 7E) and L-NAME-treated rats (Fig. 7F).

3.8. Effect of prazosin on EFS-induced contractions of RIEVD pre-incubated with idazoxan

Prazosin (1 nM) had no effect on the EFS-induced contractions of RIEVD obtained from control animals which were pre-incubated (30 min) with idazoxan (1 μM; Fig. S05). Prazosin (10 nM) caused a significant inhibition of EFS-induced contractions of the RIEVD preparations pre-incubated (30 min) with idazoxan (1 μM) in all frequencies (Fig. 8A). However, prazosin (10 nM) failed to affect the EFS-induced contractions when the RIEVD were obtained from animals chronically treated with L-NAME which were previously incubated with idazoxan (1 μM; Fig. 8B).

4. Discussion

Our results clearly demonstrate that α_1-adrenergic receptor antagonists cause concentration-dependent antagonism of the contractions induced by 6-ND in the rat isolated epididymal vas deferens. The population of α_1-adrenergic receptors in the rat vas deferens has been extensively characterized through functional, molecular and radioligand binding studies. The α_1-adrenergic receptor subtypes α_{1A}, α_{1B} and α_{1D} are encoded by three different genes (ADRA1A, ADRA1B and ADRA1D) as single-chain protein products (Avellar et al., 2009). Vas deferens, epididymis and other genitourinary tissues present abundant *Adra1a* and *Adra1d* mRNAs and lower levels of *Adra1b* transcripts (Piascik et al., 1997; Queiróz et al., 2008). Although the logical assumption would be that 6-ND is acting as an α_1-adrenergic receptor

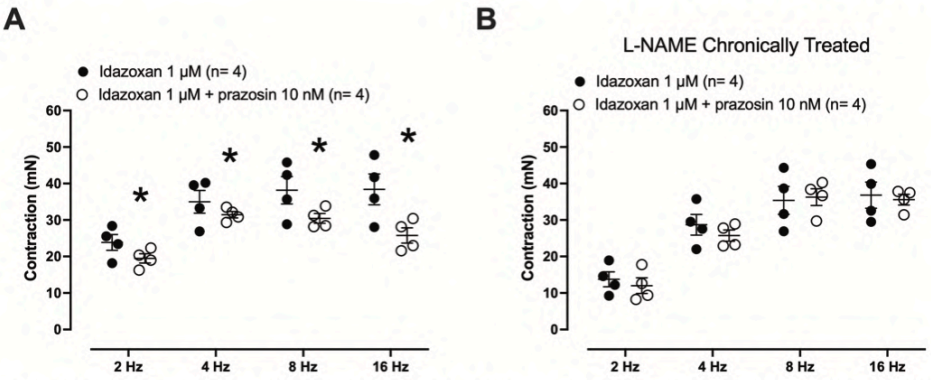


Fig. 8. Effect of prazosin on EFS-induced contractions of RIEVD which were pre-incubated (30 min) with idazoxan. Electric-field stimulation (EFS) caused frequency-dependent contractions of the RIEVD obtained from control animals (Panel A) and from L-NAME chronically treated rats (Panel B). Prazosin (10 nM) caused a significant reduction in the contractions induced by EFS but had no effect on the EFS-induced contractions of the RIEVD obtained from animals chronically treated with L-NAME which were previously incubated (30 min) with idazoxan (1 μM). *P < 0.05 compared with respective control values. N means the number of vas deferens strips.

agonist, several pieces of pharmacological and clinical evidence discussed below do not support this hypothesis.

The first piece of evidence comes from the comparative profile of 6-ND with dopamine, noradrenaline and adrenaline in other tissues. Unlike the latter catecholamines, 6-ND causes no contractions in human umbilical cord vessels (HUCV; Britto-Junior et al., 2021b). Human umbilical cord vessels do express functional α_1-adrenergic receptors since the contractions induced by noradrenaline in the human umbilical artery (Bodelsson and Stjernquist, 1995) and by adrenaline in the human umbilical vein (Errasti et al., 1999) were blocked by the α_1-adrenergic antagonist prazosin. Similar results were observed in *Chelonoidis carbonaria* aortic rings where in contrast to dopamine, noradrenaline and adrenaline, 6-ND had no contractile activity (Britto-Júnior et al., 2021c). In both HUCV and *Chelonoidis carbonaria* aortic rings, 6-ND acts as a selective dopamine D_2-like receptor antagonist.

The second piece of evidence is supported by the results obtained with the α_1-adrenergic receptor antagonists in the EFS-induced RIEVD contractions. The EFS-induced contraction presents a biphasic response, where the first phase is classified as non-adrenoceptor mediated and predominates in the prostatic portion, whereas the second phase is believed until now to be due mainly to noradrenaline release, and predominates in the epididymal portions (Brown et al., 1983). Our results clearly demonstrate that α_1-adrenergicreceptor antagonists only inhibit EFS-induced RIEVD contractions when employed at concentrations that provoke rightward shifts of the concentration response curve to 6-ND, indicating that 6-ND, but not noradrenaline, is the major mediator of this phenomenon. The finding that silodosin at 10 nM abolished noradrenaline-induced contractions but had no effect in either 6-ND- or EFS-induced RIEVD contractions clearly establishes that noradrenaline is not involved in this phenomenon. This is further clarified by the finding that doxasozin at 10 nM has no effect on the noradrenaline-induced contractions; yet, at this concentration, this antagonist inhibits both 6-ND and EFS-induced contractions. The results obtained with prazosin could be considered initially as "non-compliant", since at 10 nM, prazosin inhibited 6-ND-induced contractions and yet, it had no effect on the noradrenaline- and EFS-induced RIEVD contractions. However, prazosin has a high affinity for rodent α_2-adrenergic receptors, but a low affinity for non-rodent ones (Bylund, 1985) and a pre-synaptic α_2-adrenoceptor blocking activity in the rat vas deferens (Vizi et al., 1983). Thus, the lack of effect of prazosin at 10 nM in the EFS-induced contractions could be due to the combination of its inhibitory effects on the 6-ND receptor and the pre-synaptic α_2-adrenergic receptor. Idazoxan is a pre-synaptic α_2-adrenergic receptor antagonist (Doxey et al., 1983) that potentiates electrically induced contractions of the rat vas deferens (Doxey et al., 1985). In idazoxan-pretreated preparations, it is now possible to identify the inhibitory effect of prazosin at 10 nM on the EFS-induced contractions, due to the antagonist action on the 6-ND receptor.

The third piece of evidence is the loss of the inhibitory effect of all α_1-adrenergic antagonists on the EFS-induced contractions in RIEVD obtained from animals chronically treated with L-NAME. The synthesis of 6-ND is coupled to NO synthesis (Britto-Júnior et al., 2021a) and that chronic treatment with L-NAME is associated with reduction by about of 50% of the amounts of 6-noradrenaline extracted from porcine and rat brains (Tsunoda et al., 2007) and more importantly with a 60% reduction of the amounts of 6-ND released from rat isolated vas deferens (Britto-Júnior et al., 2021a). Thus, the loss of the inhibitory effect of the α_1-adrenergic antagonists on the EFS responses could be explained by the reduction of 6-ND synthesis in these animals.

The fourth piece of evidence comes from clinical observations. Both tricyclic antidepressants (Beaumont, 1977; Segraves, 1989) and α_1-adrenergic receptor antagonists (Cavallini, 1995; Hsieh et al., 1999; Debruyne, 2000) cause ejaculation disorders and are therapeutically used for the treatment of premature ejaculation. Although the physiopathology of ejaculation disorders is probably multifactorial, the pharmacological mechanisms of action of both tricyclic antidepressants and α_1-adrenergic receptor antagonists are reasonably established. Tricyclic antidepressants inhibit both serotonin (SERT) and noradrenaline (NET) transporters, responsible for the re-uptake of serotonin and noradrenaline, respectively (Feighner, 1999; López-Muñoz and Alamo, 2009). This mechanism of action causes an increase of both serotonin and noradrenaline in the synaptic cleft. Since α_1-adrenergic receptor antagonists also cause delayed ejaculation and has an opposite pharmacodynamic effect, this apparent paradox indicates that these drugs must be acting in different places to explain its efficacy in the treatment of premature ejaculation. However, an exciting possibility is that α_1-adrenergic antagonists could be acting as 6-ND receptor antagonists in the vas deferens. Since tricyclic antidepressants block 6-ND receptor in the rat vas deferens (Britto-Júnior et al., 2021a), this would establish a common peripheral mechanism by which this class of compounds cause ejaculation abnormalities. Indeed, the finding that both tricyclic antidepressants and α_1-adrenergic antagonists selectively antagonize 6-ND-induced contractions of the rat isolated vas deferens offers the interesting hypothesis that 6-ND must be the main peripheral mediator

of the emission process.

5. Conclusion

In conclusion, the results here presented reinforce the concept that 6-ND is an endogenous modulator of the rat vas deferens. The observation that α_1-adrenergic antagonists act as 6-ND receptor antagonists opens the possibility that many actions previously attributed to noradrenaline could be actually due to the presence of this novel mediator 6-ND.

CRediT authorship contribution statement

José Britto-Júnior: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **André Ribeiro:** Investigation, Methodology. **Luiz Ximenes:** Investigation, Methodology. **Antonio Tiago Lima:** Investigation, Methodology. **Felipe Fernandes Jacintho:** Investigation, Methodology. **Adriano Fregonesi:** Methodology. **Fabíola Z. Mónica:** Methodology, Supervision. **Edson Antunes:** Funding acquisition, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Gilberto De Nucci:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Writing – review & editing, Investigation, Visualization, Writing – original draft.

Declaration of competing interest

The authors declare no competing or financial interests.

Acknowledgment

JBJ & LX thank CAPES for PhD fellowship (001). FFJ thanks FAPESP for PhD fellowship (2018/24971-9). ATL thanks Capes for master's fellowship. EA & FM thank FAPESP (2017/15175-1). GDN thanks FAPESP (2019/16805-4), FAEPEX (2469/21) and CNPq (303839/2019-8).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.174716.

References

Andersson, K.E., Lepor, H., Wyllie, M.G., 1997. Prostatic alpha 1-adrenoceptors and uroselectivity. Prostate 30 (3), 202–215. https://doi.org/10.1002/(sici)1097-0045(19970215)30:3<202::aid-pros9>3.0.co;2-n.

Arunlakshana, O., Schild, H.O., 1959. Some quantitative uses of drug antagonists. Br. J. Pharmacol. Chemother. 14 (1), 48–58. https://doi.org/10.1111/j.1476-5381.1959.tb00928.x. PMID: 13651579.

Avellar, M.C., Lázari, M.F., Porto, C.S., 2009. Expression and function of G-protein-coupled receptors in the male reproductive tract. An. Acad. Bras. Cienc. 81 (3), 321–344. https://doi.org/10.1590/s0001-37652009000300002.

Başar, M.M., Yilmaz, E., Ferhat, M., Başar, H., Batislam, E., 2005. Terazosin in the treatment of premature ejaculation: a short-term follow-up. Int. Urol. Nephrol. 37 (4), 773–777. https://doi.org/10.1007/s11255-005-3616-4.

Beaumont, G., 1977. Sexual side-effects of clomipramine (Anafranil). J. Int. Med. Res. 5 (Suppl. 1), 37–44.

Bodelsson, G., Stjernquist, M., 1995. Characterization of contractile adrenoceptors in the human umbilical artery. Eur. J. Pharmacol. 25;282 (1–3), 95–101. https://doi.org/10.1016/0014-2999(95)00277-r.

Britto-Júnior, J., Ximenes, L., Ribeiro, A., Fregonesi, A., Campos, R., Kiguti, L.R.A., Mónica, F.Z., Antunes, E., De Nucci, G., 2021a. 6-Nitrodopamine is an endogenous mediator of rat isolated epididymal vas deferens contractions induced by electric-field stimulation. Eur. J. Pharmacol. 1 (911), 174544. https://doi.org/10.1016/j.ejphar.2021.174544.

Britto-Júnior, J., Coelho-Silva, W.C., Murari, G.F., Serpellone-Nash, C.E., Mónica, F.Z., Antunes, E., De Nucci, G., 2021b. 6-Nitrodopamine is released by human umbilical cord vessels and modulates vascular reactivity. Life Sci. 1 (276), 119425 https://doi.org/10.1016/j.lfs.2021.119425.

Britto-Júnior, J., Campos, R., Peixoto, M., Lima, A.T., Jacintho, F.F., Mónica, F.Z., Moreno, A.M., Antunes, E., De Nucci, G., 2021c. 6-nitrodopamine is an endogenous selective antagonist of dopamine-induced contractions of chelonoidis carbonaria aorta. Biology Open. Submitted.

Brown, D.A., Docherty, J.R., French, A.M., MacDonald, A., McGrath, J.C., Scott, N.C., 1983. Separation of adrenergic and non-adrenergic contractions to field stimulation in the rat vas deferens. Br. J. Pharmacol. 79 (2), 379–393. https://doi.org/10.1111/j.1476-5381.1983.tb11010.x.

Bylund, D.B., 1985. Heterogeneity of alpha-2 adrenergic receptors. Pharmacol. Biochem. Behav. 22 (5), 835–843. https://doi.org/10.1016/0091-3057(85)90536-2.

Cavallini, G., 1995. Alpha-1 blockade pharmacotherapy in primitive psychogenic premature ejaculation resistant to psychotherapy. Eur. Urol. 1995;28 (2), 126–130. https://doi.org/10.1159/000475036.

Debruyne, F.M., 2000. Alpha blockers: are all created equal? Urology 1;56 (5 Suppl. 1), 20–22. https://doi.org/10.1016/s0090-4295(00)00744-5.

Djavan, B., Chapple, C., Milani, S., Marberger, M., 2004. State of the art on the efficacy and tolerability of alpha1-adrenoceptor antagonists in patients with lower urinary tract symptoms suggestive of benign prostatic hyperplasia. Urology 64 (6), 1081–1088.

Docherty, J.R., 2019. The pharmacology of α_1-adrenoceptor subtypes. Eur. J. Pharmacol. 15 (855), 305–320. https://doi.org/10.1016/j.ejphar.2019.04.047. PMID: 31067439.

Doxey, J.C., Roach, A.G., Smith, C.F., 1983. Studies on RX 781094: a selective, potent and specific antagonist of alpha 2-adrenoceptors. Br. J. Pharmacol. 78 (3), 489–505. https://doi.org/10.1111/j.1476-5381.1983.tb08809.x.

Doxey, J.C., Roach, A.G., Samuel, J., 1985. Effects of desipramine on stimulation-induced contractions of the vas deferens of rats pretreated either chronically with desipramine or acutely with idazoxan. Clin. Sci. (Lond.) 68 (Suppl. 10), 155s–159s. https://doi.org/10.1042/cs068s155.

Dunn, C.J., Matheson, A., Faulds, D.M., 2002. Tamsulosin: a review of its pharmacology and therapeutic efficacy in the management of lower urinary tract symptoms. Drugs Aging 19 (2), 135–161. https://doi.org/10.2165/00002512-200219020-00004. PMID: 11950378.

Elliott, H.L., Meredith, P.A., Sumner, D.J., McLean, K., Reid, J.L., 1982. A pharmacodynamic and pharmacokinetic assessment of a new alpha-adrenoceptor antagonist, doxazosin (UK33274) in normotensive subjects. Br. J. Clin. Pharmacol. 13 (5), 699–703. https://doi.org/10.1111/j.1365-2125.1982.tb01439.x.

Errasti, A.E., Velo, M.P., Torres, R.M., Sardi, S.P., Rothlin, R.P., 1999. Characterization of alpha1-adrenoceptor subtypes mediating vasoconstriction in human umbilical vein. Br. J. Pharmacol. 126 (2), 437–442. https://doi.org/10.1038/sj.bjp.0702320.

Feighner, J.P., 1999. Mechanism of action of antidepressant medications. J. Clin. Psychiatr. 60 (Suppl. 4), 4–11 discussion 12-3. PMID: 10086478.

Frishman, W.H., Eisen, G., Lapsker, J., 1988. Terazosin: a new long-acting alpha 1-adrenergic antagonist for hypertension. Med. Clin. 72 (2), 441–448. https://doi.org/10.1016/s0025-7125(16)30778-7. PMID: 2894488.

Giuliano, F., Clèment, P., 2012. Pharmacology for the treatment of premature ejaculation. Pharmacol. Rev. 64 (3), 621–644. https://doi.org/10.1124/pr.111.004952. PMID: 22679220.

Giuliano, F., Hellstrom, W.J., 2008. The pharmacological treatment of premature ejaculation. BJU Int. 102 (6), 668–675. https://doi.org/10.1111/j.1464-410X.2008.07719.x. PMID: 18485043.

Hellstrom, W.J., Sikka, S.C., 2006. Effects of acute treatment with tamsulosin versus alfuzosin on ejaculatory function in normal volunteers. J. Urol. 176 (4 Pt 1), 1529–1533.

Höfner, K., Claes, H., De Reijke, T.M., Folkestad, B., Speakman, M.J., 1999. Tamsulosin 0.4 mg once daily: effect on sexual function in patients with lower urinary tract symptoms suggestive of benign prostatic obstruction. Eur. Urol. 36 (4), 335–341. https://doi.org/10.1159/000019996. PMID: 10473995.

Hsieh, J.T., Liu, S.P., Hsieh, C.H., Cheng, J.T., 1999. An in vivo evaluation of the therapeutic potential of sympatholytic agents on premature ejaculation. BJU Int. 84 (4), 503–506. https://doi.org/10.1046/j.1464-410x.1999.00173.x. PMID: 10468770.

Keating, G.M., 2015. Silodosin: a review of its use in the treatment of the signs and symptoms of benign prostatic hyperplasia. Drugs 75 (2), 207–217. https://doi.org/10.1007/s40265-014-0344-z. PMID: 25575983.

Lepor, H., Baumann, M., Shapiro, E., 1988. The stereospecificity of LY253352 for alpha 1-adrenoceptor binding sites in the brain and prostate. Br. J. Pharmacol. 95 (1), 139–144. https://doi.org/10.1111/j.1476-5381.1988.tb16557.x. PMID: 2851347; PMCID: PMC1854123.

López-Muñoz, F., Alamo, C., 2009. Monoaminergic neurotransmission: the history of the discovery of antidepressants from 1950s until today. Curr. Pharmaceut. Des. 15 (14), 1563–1586. https://doi.org/10.2174/138161209788168001. PMID: 19442174.

Martin, M.C., 2010. The pharmacological profile of the α1A-adrenoceptor antagonist silodosin. Eur. Urol. Suppl. 9 (4), 486–490.

Monteiro, W.O., Noshirvani, H.F., Marks, I.M., Lelliott, P.T., 1987. Anorgasmia from clomipramine in obsessive compulsive disorder: a controlled trial. Br. J. Psychiatry 151, 107–112.

Motulsky, H., 2014. Normality Tests. In: Intuitive Biostatistics: A Nonmathematical Guide to Statistical Thinking. Oxford University Press, New York, NY, pp. 203–208.

O'Leary, M.P., 2001. Tamsulosin: current clinical experience. discussion 48 Urology 58 (6 Suppl. 1), 42–48. https://doi.org/10.1016/s0090-4295(01)01346-2. PMID: 11750250.

Percie du Sert, N., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W.J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Hurst, V., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E.J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E. S., Silberberg, S.D., Steckler, T., Würbel, H., 2020. Reporting animal research: explanation and elaboration for the ARRIVE guidelines 2.0. PLoS Biol. 14;18 (7), e3000411 https://doi.org/10.1371/journal.pbio.3000411.

Piascik, M.T., Hrometz, S.L., Edelmann, S.E., Guarino, R.D., Hadley, R.W., Brown, R.D., 1997. Immunocytochemical localization of the alpha-1B adrenergic receptor and the contribution of this and the other subtypes to vascular smooth muscle contraction: analysis with selective ligands and antisense oligonucleotides. J. Pharmacol. Exp. Therapeut. 283 (2), 854–868. PMID: 9353407.

Puppo, V., Puppo, G., 2016. Comprehensive review of the anatomy and physiology of male ejaculation: premature ejaculation is not a disease. Clin. Anat. 29 (1), 111–119. https://doi.org/10.1002/ca.22655. PMID: 26457680.

Queiróz, D.B., Porto, C.S., Grossman, G., Petrusz, P., Avellar, M.C., 2008. Immunolocalization of alpha(1A)-adrenoceptors in rat and human epididymis. Cell Tissue Res. 332 (3), 509–522. https://doi.org/10.1007/s00441-008-0576-x. PMID: 18351393.

Ramsay, J.W., Scott, G.I., Whitfield, H.N., 1988. A double-blind controlled trial of a new alpha-1 blocking drug in the treatment of bladder outflow obstruction. Br. J. Urol. 57 (6), 657–659. https://doi.org/10.1111/j.1464-410x.1985.tb07026.x. PMID: 2417649.

Ribeiro, M.O., Antunes, E., De Nucci, G., Lovisolo, S.M., Zatz, R., 1992. Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. Hypertension 20 (3), 298–303. https://doi.org/10.1161/01.hyp.20.3.298. PMID: 1516948.

Roehrborn, C.G., 2009. Efficacy of alpha-adrenergic receptor blockers in the treatment of male lower urinary tract symptoms. Rev. Urol. 11 (Suppl. 1), S1–S8.

Segraves, R.T., 1989. Effects of psychotropic drugs on human erection and ejaculation. Arch. Gen. Psychiatr. 46, 275–284.

Segraves, R., 2007. Sexual dysfunction associated with antidepressant therapy. Urol. Clin. 24, 575–579.

Segraves, R.T., Balon, R., 2014. Antidepressant-induced sexual dysfunction in men. Pharmacol. Biochem. Behav. 121, 132–137. https://doi.org/10.1016/j.pbb.2013.11.003. Epub 2013 Nov 15. PMID: 24239785.

Tsunoda, M., Uchino, E., Imai, K., Hayakawa, K., Funatsu, T., 2007. Determination of nitrocatecholamines in rat brain using high-performance liquid chromatographyperoxyoxalate chemiluminescence reaction detection. J. Chromatogr. A 1164 (1- 2), 162–166. https://doi.org/10.1016/j.chroma.2007.07.005.

U'Prichard, D.C., Charness, M.E., Robertson, D., Snyder, S.H., 1978. Prazosin: differential affinities for two populations of alpha-noradrenergic receptor binding sites. Eur. J. Pharmacol. 1;50 (1), 87–89. https://doi.org/10.1016/0014-2999(78)90258-3. PMID: 28235.

Vizi, E.S., Ludvig, N., Rónai, A.Z., Folly, G., 1983. Dissociation of presynaptic alpha 2-adrenoceptors following prazosin administration: presynaptic effect of prazosin. Eur. J. Pharmacol. 25;95 (3–4), 287–290. https://doi.org/10.1016/0014-2999(83)90648-9. PMID: 6317415.

Wilt, T.J., MacDonald, R., 2006. Doxazosin in the treatment of benign prostatic hypertrophy: an update. Clin. Interv. Aging 1 (4), 389–401. https://doi.org/10.2147/ciia.2006.1.4.389. PMID: 18046916; PMCID: PMC2699642.

Ximenes, L.E., Britto-Júnior, J., Silva-Filho, W., Jacintho, F., Cuppoloni, M., Fregonesi, A., De Nucci, G, 2021. Pd62-12 6-nitrodopamine is released by rat isolated vas deferens and modulates its contractility. J. Urol. 206 (3) e1077–e1077.

Yamagishi, R., Akiyama, K., Nakamura, S., Hora, M., Masuda, N., Matsuzawa, A., Murata, S., Ujiie, A., Kurashina, Y., Iizuka, K., Kitazawa, M., 1966. Effect of KMD-3213, an alpha 1a-adrenoceptor-selective antagonist, on the contractions of rabbit prostate and rabbit and rat aorta. Eur. J. Pharmacol. 7;315 (1), 73–79. https://doi.org/10.1016/s0014-2999(96)00589-4. PMID: 8960867.

Anexo 8

Artigo 10 – β1 and β1/2 -adrenergic receptor antagonist block 6-nitrodopamine-induced contractions of the rat isolated epididymal vas deferens

Autores: Britto-Júnior J, Ribeiro A, Ximenes L, Lima AT, Jacintho FF, Fregonesi A, Mónica FZ, Antunes E, De Nucci G

Revista: Naunyn Schmiedebergs Arch Pharmacol.

Situação: Aceito a publicação em 29 de junho de 2022. Publicado on-line em 08 de julho de 2022

Naunyn-Schmiedeberg's Archives of Pharmacology
https://doi.org/10.1007/s00210-022-02268-6

RESEARCH

β_1- and β_1/β_2-adrenergic receptor antagonists block 6-nitrodopamine-induced contractions of the rat isolated epididymal vas deferens

Antonio Tiago Lima[1] · Amanda Consulin Amorim[1] · José Britto-Júnior[1] · Raquel Rios Campitelli[1] · Adriano Fregonesi[1] · Fabíola Z. Mónica[1] · Edson Antunes[1] · Gilberto De Nucci[1,2,3]

Received: 6 April 2022 / Accepted: 29 June 2022
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2022

Abstract
6-Nitrodopamine (6-ND) is an endogenous modulator of the contractility in the rat isolated epididymal vas deferens (RIEVD) and considered to be the main peripheral mediator of the emission process. Use of selective and unselective β-adrenergic receptor antagonists has been associated with ejaculatory failure. Here, the effects of selective β_1- and β_1/β_2-adrenergic receptor antagonists on RIEVD contractions induced by 6-ND, dopamine, noradrenaline, adrenaline, and electric-field stimulation (EFS) were investigated. The selective β_1-adrenergic receptor antagonists atenolol (0.1 and 1 μM), betaxolol (1 μM), and metoprolol (1 μM) and the unselective β_1/β_2-adrenergic receptor antagonists propranolol (1 and 10 μM) and pindolol (10 μM) caused significant rightward shifts of the concentration–response curve to 6-ND (pA_2 6.41, 6.91, 6.75, 6.47, and 5.74; for atenolol, betaxolol, metoprolol, propranolol, and pindolol), but had no effect on dopamine-, noradrenaline-, and adrenaline-induced contractions. The effects of selective β_1- and β_1/β_2-adrenergic receptor antagonists at a higher concentration (atenolol 1 μM, betaxolol 1 μM, metoprolol 1 μM, propranolol 10 μM, and pindolol 10 μM) also reduced the EFS-induced RIEVD contractions in control, but not in RIEVD obtained from L-NAME-treated animals. The selective β_1-adrenoceptor agonist RO-363, the selective β_2-adrenoceptor agonist salbutamol, and the selective β_3-adrenoceptor agonist mirabegron, up to 300 μM, had no effect on the RIEVD tone. The results demonstrate that β_1- and β_1-/β_2-adrenoceptor receptor antagonists act as 6-ND receptor antagonists in RIEVD, further confirming the main role of 6-ND in the RIEVD contractility.

Keywords Ejaculation disorder · Nitrocatecholamines · EFS · L-NAME

Introduction

6-Nitrodopamine (6-ND) is a novel catecholamine released from vascular tissues such as human umbilical cord vessels (Britto-Jr et al. 2021a), *Chelonoidis carbonaria* aorta (Campos et al. 2020), and from rat vas deferens (Britto-Júnior et al. 2021b, 2022). The synthesis/release of 6-ND is coupled to nitric oxide (NO) synthesis, since it is reduced by the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME).

In the rat epididymal vas deferens, 6-ND has been characterized as a major endogenous modulator of the contractility of this tissue (Britto-Jr et al. 2021b; Britto-Jr et al. 2022). Tricyclic antidepressants such as clomipramine (Millan et al. 2001), desipramine (Cusack et al. 1994), and amitriptyline (Sánchez and Hyttel 1999) and α_1-adrenergic receptor antagonists such as doxazosin (Elliott et al. 1982; Wilt and MacDonald 2006), tamsulosin (Lepor et al. 1988; Dunn et al. 2002), terazosin (Frishman et al. 1988; O'Leary 2001), and alfuzosin (Ramsay et al. 1988) act as 6-ND receptor antagonist in the rat vas deferens (Britto-Jr et al. 2021b; 2022). One known adverse reaction of these two classes of drugs is the impairment of the ejaculatory process

✉ José Britto-Júnior
josebrittojr@gmail.com

[1] Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Rua Tessália Vieira de Camargo, 126 - Cidade Universitária, Campinas, SP 13083-887, Brazil

[2] Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo (USP), São Paulo, SP, Brazil

[3] Faculty of Medical Sciences, Universidade Do Brasil, Fernandópolis, São Paulo, Brazil

Published online: 08 July 2022

(Beaumont 1977; Cavallini 1995; Hsieh et al. 1999; Debruyne 2000). Indeed, both classes of drugs are used for the treatment of premature ejaculation (Hellstrom and Sikka 2006; Basar et al. 2005), indicating a major role for 6-ND in the ejaculatory process. In pre-clinical studies, male rats treated for 16 weeks with the non-selective β-adrenoceptor antagonist propranolol (1.25 mg/day) exhibited an impairment in the ejaculation and copulatory pattern (Srilatha et al. 1999). Subcutaneous administration of the non-selective β-blocker pindolol (4 mg/kg, 30 min) to male rats was also associated with inhibition of the sexual behavior, as evidenced by an increase in mounts, intromissions, and time to ejaculate (Ahlenius and Larsson 1991). In patients with arterial hypertension, coronary artery disease, or heart failure, meta-analytic data have shown that β-blockers are associated with a small, but significant, increase in risk of reported sexual dysfunction, which was not related to the lipid-soluble β-blockers (Ko et al. 2002). The use of the β_1-, β_2-, and α_1-adrenergic receptor antagonist labetalol was associated with ejaculatory failure soon after the initiation of therapy that resolved with drug discontinuation (O'Meara and White 1988). In a double-blind, placebo-controlled trial comprising eighty-six paroxetine-refractory patients, pindolol, at the dose of 7.5 mg/day, increased significantly the mean intravaginal ejaculatory latency time after 6 weeks of treatment (Safarinejad 2008). Thus, both the experimental and clinical observations open the interesting possibility that β-blockers could act as 6-ND receptor antagonists in the vas deferens, as observed with tricyclic antidepressants and α_1-adrenergic receptor antagonists.

Materials and methods

Animals

Adult male Wistar rats (280–320 g) were obtained from the animal care of University of Campinas (UNICAMP; Campinas, São Paulo, Brazil) and Animais de Laboratorio Criação e Com. LTDA (ANILAB; Paulinia, São Paulo, Brazil). All experimental protocols were authorized by the Ethics Committee in Animal Use of UNICAMP (CEUA/UNICAMP, protocol numbers 5952–1/2022 and 5831–1/2021) and followed the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al. 2020). Animals were housed in cages (three per cage) located in ventilated cage shelters with constant humidity of 55% ± 5% and temperature of 24 ± 1 °C under a 12-h light–dark cycle. Animals received filtered water and standard food ad libitum.

Chronic L-NAME treatment

Animals were treated with L-NAME dissolved in the drinking filtered water at a concentration of approximately 20 mg/rat/day for a minimum of 4 weeks (Ribeiro et al. 1992). Control animals received filtered water alone. Vas deferens obtained from these chronically treated animals present lower release of 6-ND, as quantified by LC–MS/MS (Britto-Júnior et al. 2021a, 2021b).

Rat isolated epididymal vas deferens (RIEVD) isolation and preparation

Euthanasia was performed by isoflurane overdose, in which animals were exposed to a concentration greater than 5% until 1 min after the breathing stops. Exsanguination was performed to confirm the euthanasia. The vas deferens was removed and immediately placed in Krebs–Henseleit's solution (KHS). The proximal portion of the vas deferens (close to the epididymis) was surgically dissected (length, 1.5 cm each) for the functional studies (Burnstock and Verkhratsky 2010). The RIEVD strips were suspended vertically between metal hooks in 10-mL custom designed glass chambers containing KHS, continuously gassed with a mixture of 95%O_2:5%CO_2 at 37 °C using a heated circulator (PolyScience, IL, USA). Tissues were allowed to equilibrate under a resting tension of 10 mN, and the isometric tension was registered using a PowerLab system (ADInstruments, Sydney, Australia). Following a 45-min stabilization period, the RIEVD strips were initially contracted with a single concentration of noradrenaline (NA, 10 μM) to verify the tissue viability.

In vitro functional assays in RIEVD preparations

Cumulative concentration–response curves to 6-ND were performed in RIEVD strips in the absence and the presence of atenolol (0.1 and 1 μM, 30 min), betaxolol (0.1 and 1 μM, 30 min), metoprolol (0.1 and 1 μM, 30 min), propranolol (1 and 10 μM, 30 min), or pindolol (1, 3 and 10 μM, 30 min). In separate RIEVD preparations, cumulative concentration–response curves to dopamine, noradrenaline, and adrenaline were performed in the absence and presence of atenolol (1 μM, 30 min), betaxolol (1 μM, 30 min), metoprolol (1 μM, 30 min), propranolol (10 μM, 30 min), or pindolol (10 μM, 30 min).

Cumulative concentration–response curves to selective β1-adrenoceptor agonist RO-3630 (0.001–300 μM),

selective β2-adrenoceptor agonist salbutamol (0.001–300 μM), and selective β3-adrenoceptor agonist mirabegron (0.001–300 μM) were performed in RIEVD strips obtained from control animals.

Electric-field stimulation (EFS) in RIEVD preparations

The EFS-induced contractions from RIEVD were evaluated in control and L-NAME-treated rats. Briefly, RIEVD strips were submitted to EFS (60 V for 20 s, at 2–16 Hz in square-wave pulses, 0.3 ms pulse width, and 0.1 ms delay), using a Grass S88 stimulator (Astro-Medical, Industrial Park, RI, USA). In control animals, EFS-induced contractions were evaluated in the absence and the presence of atenolol (0.1 and 1 μM, 30 min), betaxolol (0.1 and 1 μM, 30 min), metoprolol (0.1 and 1 μM, 30 min), propranolol (1 and 10 μM, 30 min), pindolol (1 and 10 μM, 30 min), or tetrodotoxin (1 μM). In L-NAME-treated rats, EFS-induced contractions were evaluated in the absence and in the presence atenolol (1 μM, 30 min), betaxolol (1 μM, 30 min), metoprolol (1 μM, 30 min), propranolol (10 μM, 30 min), or pindolol (10 μM, 30 min).

Drugs and solutions

Atenolol, dopamine, metoprolol, mirabegron, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), salbutamol, and propranolol were obtained from Sigma-Aldrich Chemicals Co. (St Louis, MO, USA). Adrenaline, betaxolol, noradrenaline, pindolol, and tetrodotoxin were purchased from Cayman Chemical Co (MI, USA). 6-Nitrodopamine was bought from Toronto Research Chemicals Inc (Toronto, Ontario, Canada). RO-363 was provided by MedChem Express (NJ, USA). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$), potassium phosphate monobasic (KH_2PO_4), and glucose were acquired from Merck KGaA (Darmstadt, Germany). The composition of the KHS was in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, and dextrose 5.6.

Data analysis

Nonlinear regression analysis to determine the pEC_{50} was carried out using GraphPad Prism (GraphPad Software, version 9.0, San Diego, CA, USA) with the constraint that $F=0$. All concentration–response data were evaluated for a fit to a logistics function in the form: $E=E_{max}/([1+(10c/10x)^n]+F$, where E represents the increase in response contractile induced by the agonist; E_{max} is the effect agonist maximum; c is the logarithm of concentration of the agonist that produces 50% of E_{max}; x is the logarithm of the concentration of the drug; the exponential term, n, is a curve fitting parameter that defines the slope of the concentration–response line; and F is the response observed in the absence of added drug. The values of pEC_{50} data represent standard deviation (SD) of n experiments. Values of E_{max} were expressed in mN. Each animal provided two epididymal vas deferens (right and left); one strip was used as the control response and the contralateral strip was incubated with an antagonist/inhibitor; n indicates both the number of paired strips (same animal) and the number of rats. Student's two-tail unpaired t-test was employed and the differences between groups. In addition, standard ANOVA, followed by the Newman–Keuls post-test, were used when more than two groups were involved. A p value of less than 0.05 was considered statistically significant. Since the study has an exploratory character, the p values should be considered descriptive (Motulsky 2014; Michel et al. 2020). For 6-ND, the pA_2 values of the antagonists were calculated from the intercept on the concentration axis and by application of the equation: $pA_2=\log$ (antagonist concentration) − log (CR-1) − log (antagonist concentration) (Arunlakshana and Schild 1959).

Results

Effect of atenolol on RIEVD contractions induced by catecholamines and EFS

Atenolol (0.1 and 1 μM) produced concentration-dependent rightward shifts on the concentration–response curves to 6-ND (Fig. 1A; $p=0.0284$ and $p=0.0068$, respectively) with a pA_2 value of 6.51 ± 0.54 ($n=4$). Atenolol (1 μM) had no effect on the RIEVD contractions induced by dopamine (Fig. 1B; $p=0.4540$), noradrenaline (Fig. 1C; $p=0.4234$), and adrenaline (Fig. 1D; $p=0.4570$).

Atenolol (0.1 μM) had no effect on the EFS-induced contractions of RIEVD (Fig. 1F), but at a higher concentration (1 μM), atenolol caused significant reductions on the EFS-induced contractions of the RIEVD in all frequencies tested (Fig. 1F), which was not observed in RIEVD obtained from animals chronically treated with L-NAME (Fig. 1G).

Effect of betaxolol on RIEVD contractions induced by catecholamines and EFS

Betaxolol at 0.1 μM had no effect on 6-ND-induced RIEVD contractions, but at 1 μM it caused a significant rightward shift on the concentration–response curve to 6-ND (Fig. 2A; $p=0.0046$) with a pA_2 value of 6.91 ± 0.03 ($n=4$). Betaxolol (1 μM) had no effect on the RIEVD contractions induced by dopamine (Fig. 2B; $p=0.4608$), noradrenaline (Fig. 2C; $p=0.2830$), and adrenaline (Fig. 2D; $p=0.1571$).

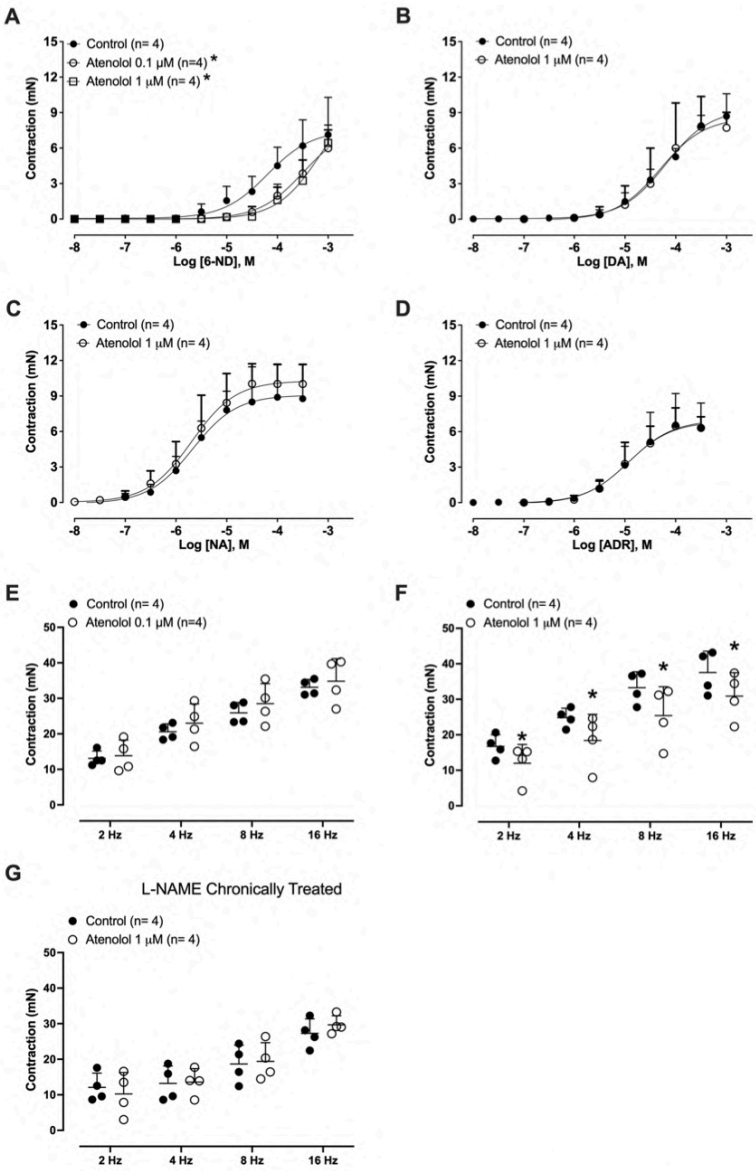


Fig. 1 Effect of atenolol in the rat isolated epididymal vas deferens (RIEVD). Atenolol (0.1 and 1 μM) caused significant concentration-dependent rightward shifts of the concentration–response curves to 6-ND (**A**). Atenolol (1 μM) had no effect on the RIEVD contractions induced by dopamine (DA; **B**), noradrenaline (NA; **C**), and adrenaline (ADR; **D**) concentration–response curves. Atenolol (0.1 μM; **E**) had no effect on the EFS-induced contractions but atenolol (1 μM) reduced the contractions induced by EFS (**F**). Atenolol (1 μM) had no effect on the EFS-induced contractions of the RIEVD obtained from animals chronically treated with L-NAME (**G**). Data are expressed as mean ± SD. $*p < 0.05$ compared with respective control values. ANOVA followed by the Newman–Keuls post-test was applied in **A** whereas the unpaired *t*-test was applied in **B**–**G**. *n* means the number of vas deferens strips

Betaxolol (0.1 μM) had no significant effect on EFS-induced RIEVD contractions (Fig. 2F); however, at 1 μM betaxolol caused significant reductions of the EFS-induced contractions in all frequencies tested (Fig. 2F). In RIEVD obtained from animals chronically treated with L-NAME, betaxolol (1 μM) had no effect on the EFS-induced contractions (Fig. 2G).

Effect of metoprolol on RIEVD contractions induced by catecholamines and EFS

Metoprolol at 0.1 μM had no effect on 6-ND-induced RIEVD contractions, but at 1 μM it caused a significant rightward shift on the concentration–response curve to 6-ND (Fig. 3A; $p = 0.0159$) with a pA_2 value of 6.75 ± 0.08 ($n = 4$). Metoprolol

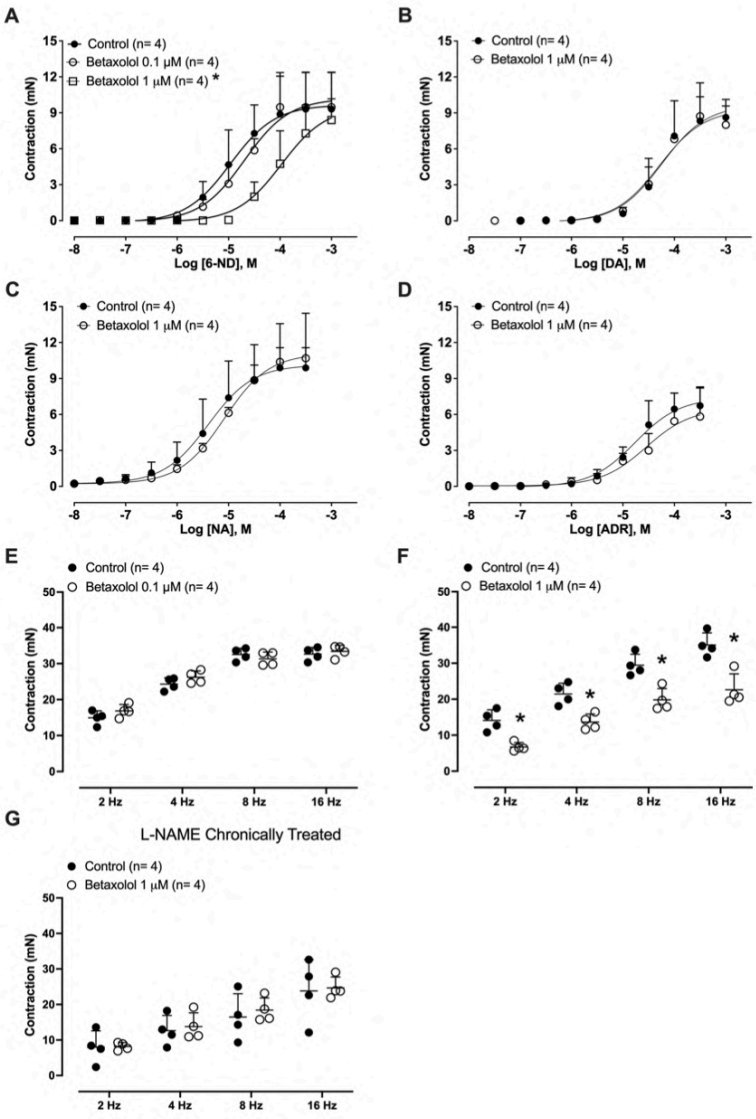


Fig. 2 Effect of betaxolol in the rat isolated epididymal vas deferens (RIEVD). Betaxolol (1 μM) caused significant concentration-dependent rightward shifts of the concentration–response curves to 6-ND (**A**). Betaxolol (1 μM) had no effect on the RIEVD contractions induced by dopamine (DA; **B**), noradrenaline (NA; **C**), and adrenaline (ADR; **D**) concentration–response curves. Betaxolol (0.1 μM) had no effect on the EFS-induced contractions (**E**), but at higher concentration (1 μM) significantly reduced the contractions in all frequencies tested (**F**). In RIEVD obtained from animals chronically treated with L-NAME, betaxolol (1 μM) failed to affect the EFS-induced contractions (**G**). Data are expressed as mean ± SD. $*p < 0.05$ compared with respective control values. ANOVA followed by the Newman–Keuls post-test was applied in **A** whereas the unpaired *t*-test was applied in **B**–**G**. *n* means the number of vas deferens strips

(1 μM) had no effect on the RIEVD contractions induced by dopamine (Fig. 3B; $p = 0.4540$), noradrenaline (Fig. 3C; $p = 0.1887$), and adrenaline (Fig. 3D; $p = 0.3795$).

Metoprolol at 0.1 μM had no significant effect on EFS-induced RIEVD contractions (Fig. 3E); however, at 1 μM metoprolol caused significant reductions of the EFS-induced contractions in all frequencies tested (Fig. 3F). In RIEVD obtained from animals chronically treated with L-NAME, metoprolol (1 μM) had no effect on the EFS-induced contractions (Fig. 3G).

Effect of propranolol on RIEVD contractions induced by catecholamines and EFS

Propranolol (1 and 10 μM) produced concentration-dependent rightward shifts on the concentration–response

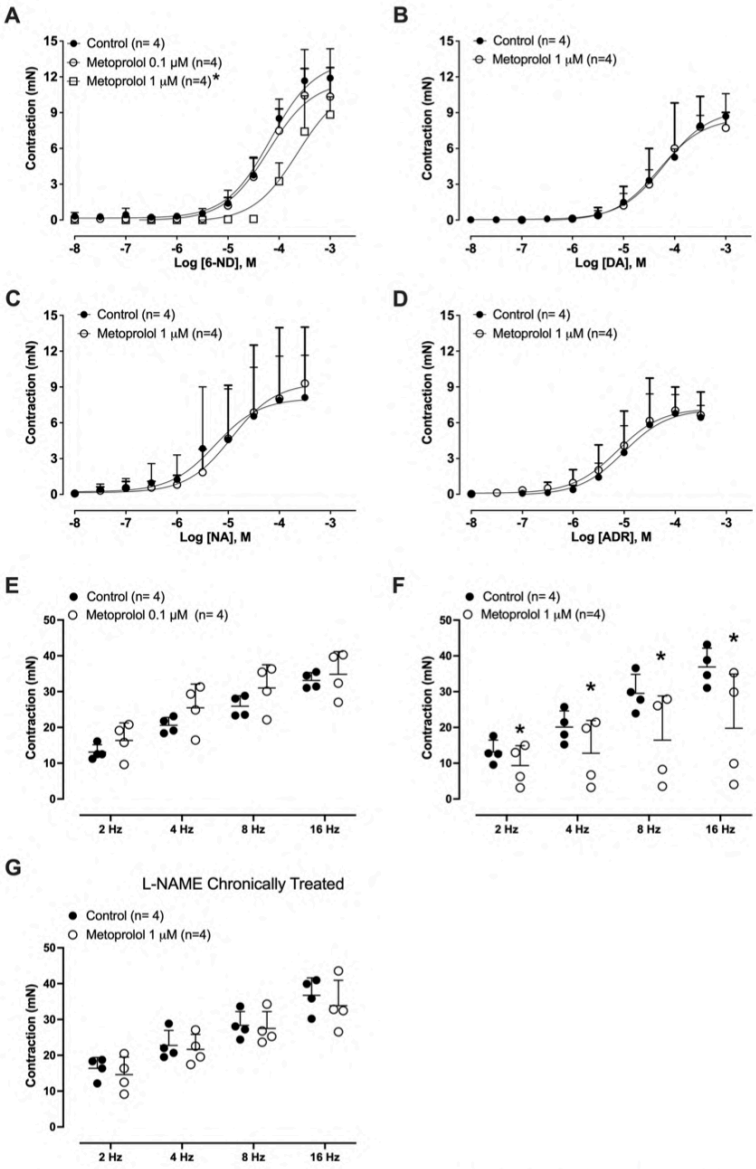


Fig. 3 Effect of metoprolol in the rat isolated epididymal vas deferens (RIEVD). Metoprolol (1 μM) caused significant concentration-dependent rightward shifts of the concentration–response curves to 6-ND (**A**). Metoprolol (1 μM) had no effect on the RIEVD contractions induced by dopamine (DA; **B**), noradrenaline (NA; **C**), and adrenaline (ADR; **D**) concentration–response curves. Metoprolol (0.1 μM) had no effect on the EFS-induced contractions (**E**), but at higher concentration (1 μM) significantly reduced the contractions in all frequencies tested (**F**). In RIEVD obtained from animals chronically treated with L-NAME, metoprolol (1 μM) failed to affect the EFS-induced contractions (**G**). Data are expressed as mean ± SD. *$p < 0.05$ compared with respective control values. ANOVA followed by the Newman–Keuls post-test was applied in **A** whereas the unpaired *t*-test was applied in **B**–**G**. *n* means the number of vas deferens strips

curves to 6-ND (Fig. 4A; $p = 0.029$ and $p = 0.0345$ for 1 and 10 μM, respectively) with a pA_2 value of 6.47 ± 0.35 ($n = 4$). Propranolol (10 μM) had no effect on the RIEVD contractions induced by dopamine (Fig. 4B; $p = 0.1073$), noradrenaline (Fig. 4C; $p = 0.4481$), and adrenaline (Fig. 4D; $p = 0.3986$).

Propranolol at 1 μM had no significant effect on EFS-induced RIEVD contractions (Fig. 4E); however, at 10 μM propranolol caused significant reductions of the EFS-induced contractions at the frequencies of 4 to 16 Hz (Fig. 4F), which was not observed in RIEVD obtained from animals chronically treated with L-NAME (Fig. 4G).

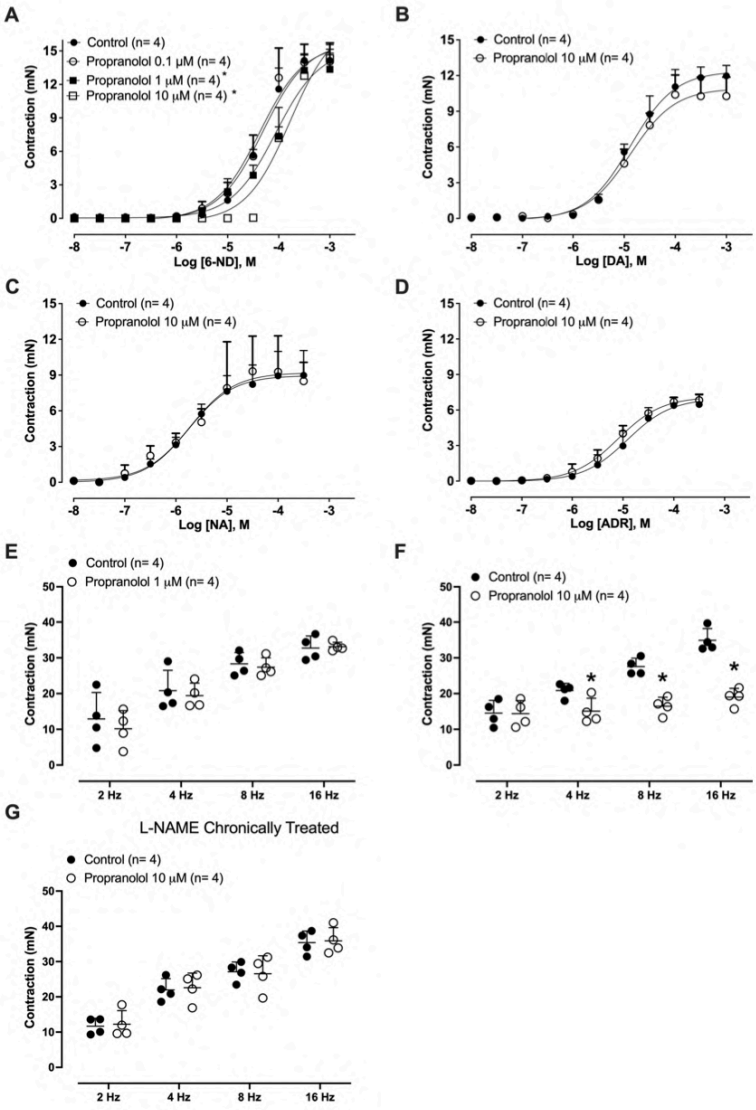


Fig. 4 Effect of propranolol in the rat isolated epididymal vas deferens (RIEVD). Propranolol (1 and 10 μM) caused significant concentration-dependent rightward shifts of the concentration–response curves to 6-ND (**A**). Propranolol (10 μM) had no effect on the RIEVD contractions induced by dopamine (DA; **B**), noradrenaline (NA; **C**), and adrenaline (ADR; **D**) concentration–response curves. Propranolol (1 μM) had no effect on the EFS-induced contractions (**E**), but at higher concentration (10 μM) significantly reduced the contractions in all frequencies tested (**F**). In RIEVD obtained from animals chronically treated with L-NAME, propranolol (10 μM) failed to affect the EFS-induced contractions (**G**). Data are expressed as mean ± SD. *$p < 0.05$ compared with respective control values. ANOVA followed by the Newman–Keuls post-test was applied in **A** whereas the unpaired *t*-test was applied in **B**–**G**. *n* means the number of vas deferens strips

Effect of pindolol on RIEVD contractions induced by catecholamines and EFS

Pindolol (10 μM) caused a rightward shift on the concentration–response curve to 6-ND (Fig. 5A; $p = 0.0184$) with a pA_2 value of 5.74 ± 0.15 ($n = 4$). Lower concentrations of pindolol (1 and 3 μM) had no significant effect on the contractions induced by 6-ND (Fig. 5A). Pindolol (10 μM) had no effect on the RIEVD contractions induced by dopamine (Fig. 5B; $p = 0.2102$), noradrenaline (Fig. 5C; $p = 0.3951$), and adrenaline (Fig. 5D; $p = 0.2394$).

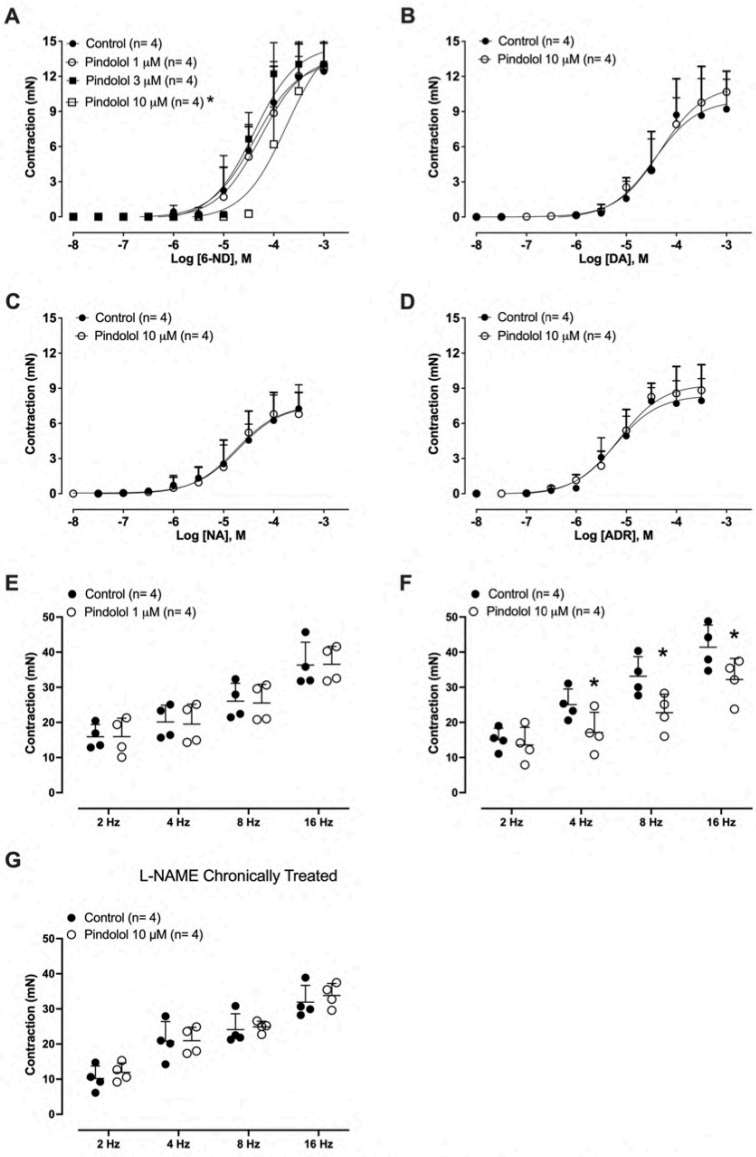


Fig. 5 Effect of pindolol in the rat isolated epididymal vas deferens (RIEVD). Pindolol (10 μM) caused significant concentration-dependent rightward shifts of the concentration–response curves to 6-ND (**A**). Pindolol (10 μM) had no effect on the RIEVD contractions induced by dopamine (DA; **B**), noradrenaline (NA; **C**), and adrenaline (ADR; **D**) concentration–response curves. Pindolol (1 μM) had no effect on the EFS-induced contractions (**E**), but at higher concentration (10 μM) significantly reduced the contractions in all frequencies tested (**F**). In RIEVD obtained from animals chronically treated with L-NAME, pindolol (10 μM) failed to affect the EFS-induced contractions (**G**). Data are expressed as mean ± SD. $*p < 0.05$ compared with respective control values. ANOVA followed by the Newman–Keuls post-test was applied in **A** whereas the unpaired *t*-test was applied in **B**–**G**. *n* means the number of vas deferens strips

Pindolol at 1 μM had no significant effect on EFS-induced RIEVD contractions (Fig. 5E); however, at 10 μM, pindolol caused significant reductions of the EFS-induced contractions at the frequencies of 4 to 16 Hz (Fig. 5F), which was not observed in RIEVD obtained from animals chronically treated with L-NAME (Fig. 5G).

Effect of RO-363, salbutamol, and mirabegron on RIEVD tone

The selective β_1-adrenoceptor agonist RO-363 (Fig. 6A), the selective β_2-adrenoceptor agonist salbutamol (Fig. 6B), and the selective β_3-adrenoceptor agonist mirabegron (Fig. 6C), up to 300 μM, had no effect on the RIEVD tone.

Effect of tetrodotoxin on RIEVD contractions induced by EFS

Pre-treatment (30 min) with tetrodotoxin (TTX; 1 μM) abolished the EFS-induced contractions in the RIEVD (Fig. 7).

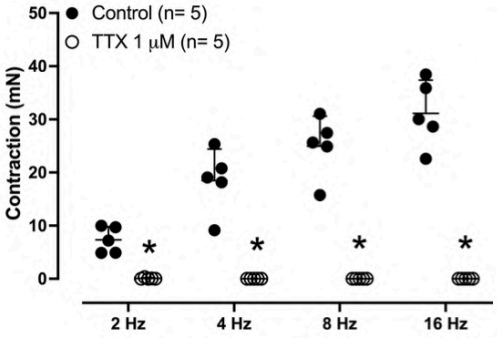


Fig. 7 Electric-field stimulation (EFS) caused frequency-dependent contractions of the isolated rat epididymal vas deferens (RIEVD), which were abolished by pre-treatment with tetrodotoxin (TTX, 1 μM). Data are expressed as mean ± SD. $*p < 0.05$ compared with respective control values in the unpaired *t*-test. *n* means the number of vas deferens strips

Discussion

The results clearly indicate that both selective and non-selective β-blockers can antagonize the contractions of the rat epididymal vas deferens induced by 6-ND, as observed with α_1-adrenergic receptor antagonists and tricyclic depressants. These findings also reinforce the role of 6-ND as the major modulator of rat epididymal vas deferens contractility, since the contractions induced by electric-field stimulation were inhibited by the β-blockers only at the concentrations that caused right-shifts of the 6-ND concentration–response curves. The inhibition of RIEVD contractions by the β-blockers was not observed in the vas deferens obtained from animals chronically treated with L-NAME, further supporting the concept that the inhibition of EFS-induced by β-receptor antagonists is due to blockade of 6-ND action.

β_1-Adrenergic receptors are not considered relevant for contractile activity in the rat vas deferens, since this tissue contains a homogenous population of β_2-adrenoceptors that inhibit field-stimulated contractions (Vohra 1979). Radioligand binding using [^{125}I]-pindolol in the rat vas deferens labeled a single class of high affinity binding sites with properties consistent with a population of β_2-adrenoceptors (May et al. 1985). In the rat vas deferens, β_2-adrenergic antagonists such as carazolol is more potent to displace [^{3}H]-dihydroalprenolol binding compared to β_1-adrenergic antagonists such as atenolol and practolol (Chang and Lotti 1983). Indeed, the finding that the selective β_1-adrenergic receptor agonist RO-363 (Iakovidis et al. 1980) had no contractile activity per se confirms the relative unimportance of modulatory role for this subclass of receptors in the vas deferens. The lack of contractile activity of RO-363 clearly demonstrates that the contractions induced by 6-ND are not due to activation of β_1-adrenergic receptors. Similar results were obtained with the selective β_2-adrenergic

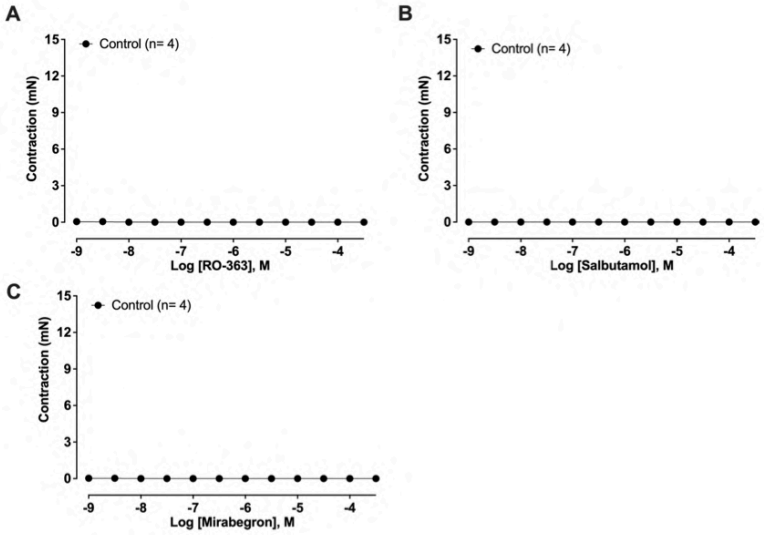


Fig. 6 Effect of the selective β_1-adrenergic receptor agonist RO-363 (**A**), selective β_2-adrenergic agonist salbutamol (**B**), and of the selective β_3-adrenergic agonist mirabegron (**C**) in the rat isolated epididymal vas deferens tone. *n* means the number of vas deferens strips

agonist salbutamol and the selective β_3-adrenergic agonist mirabegron, indicating that the contractile activity induced by 6-ND is independent of β-adrenergic receptor activation. 6-ND has been considered the endogenous mediator of EFS-induced contractions in the rat vas deferens (Britto-Júnior et al. 2021b), and these results further support the concept that 6-ND is acting on a specific 6-ND receptor.

In the rat vas deferens, the adrenergic axons are clearly identified within smooth muscle cells, and some are completely ensheathed in the smooth muscle cells (Furness and Iwayama 1971). Thus, the high concentrations of β-blockers required to inhibit EFS-induced contractions could reflect restricted access to the β-adrenergic receptors located in deeper layers of smooth muscle cells. However, this anatomical hypothesis is unlikely since EFS-induced contractions are inhibited by much lower concentrations of α-adrenergic antagonists (Britto-Júnior et al. 2022).

Although ejaculatory disorders have been reported with the use of selective and unselective β-blockers, the incidence is rather low (reported cases) when compared to α_1-adrenergic receptor antagonists (4–11%; Höfner et al. 1999). This major difference in incidence (Djavan et al. 2004) could be easily attributed to the observed major potency (over 100 times) of α_1-blockers (the pA_2 values are 9.66, 9.15, 8.86, 7.70, 7.20, and 8.82 for tamsulosin, doxazosin, alfuzosin, silodosin, terazosin, and prazosin; Britto-Júnior et al 2022) in blocking 6-ND contractile activity compared to the β-blockers (6.41, 6.91, 6.75, 6.47, and 5.74; for atenolol, betaxolol, metoprolol, propranolol, and pindolol, respectively; this manuscript), further supporting a major role of 6-ND in the ejaculatory process. Although β_1-, β_2-, and β_3-adrenergic receptor agonists per se did not induce contractions in the rat vas deferens, these findings do not exclude a potential modulatory role on vas deferens contractility. The finding that the sodium channel antagonist tetrodotoxin (Narahashi et al. 1967; Lee and Ruben 2008) abolished EFS-induced contractions in the RIEVD (Belevych et al. 1999) does not necessarily indicate that 6-ND is coming from the nerve terminals. Although 6-nitronoradrenaline has been extracted from rat brain (Shintani et al. 1996), it is also possible that some neurotransmitter induces 6-ND release from other cells.

The results here reported extend the findings that tricyclic antidepressants (Britto-Júnior et al. 2021b) and α_1-adrenergic antagonists (Britto-Júnior et al. 2022) inhibited EFS-induced contractions only at the concentrations that inhibited 6-ND-induced contractions and that these drugs had no effect in the EFS-induced contractions of RIEVD obtained from L-NAME chronically treated animals. 6-ND does not act on adrenergic receptors, as demonstrated in human umbilical cord vessels (Britto-Júnior et al. 2021a). All these pieces of evidence support the concept that 6-ND is acting on a specific receptor. Purification and sequencing of the 6-ND receptor, the identification of the metabolic pathways involved in the 6-ND synthesis and the mechanisms responsible for 6-ND storage and release should further clarify its physiological role in the ejaculatory process.

Conclusion

The inhibitory effect of β_1- and β_1/β_2-adrenergic receptor antagonists on the RIEVD contractions induced by both the EFS and 6-ND is due to blockade of the 6-ND receptor.

Author contribution Conceptualization: JBJ, GDN.
Data curation: JBJ, GDN.
Formal analysis: GDN.
Funding acquisition: EA, GDN.
Investigation: ATL, ACA, JBJ, RRC, GDN.
Methodology: ATL, ACA, JBJ, RRC, AF, EA, FZM, GDN.
Project administration: GDN.
Supervision: FZM, EA.
Visualization: AF, EA, GDN.
Writing—original draft: JBJ, AF, EA, GDN.
The authors declare that all data were generated in-house and that no paper mill was used.

Funding ATL thanks FAPESP for PhD fellowship (2021/13593–6).
ACA thanks CAPES for master's fellowship (001).
JBJ thanks CAPES for PhD fellowship (001).
RRC thanks FAPESP for scientific initiation fellowship (2021/14120–4).
EA and FM thank FAPESP (2017/15175–1).
GDN thanks FAPESP (2019/16805–4) and CNPq (303,839/2019–8).

Data availability The authors authorize the availability of any data used in this study.

Declarations

Ethics approval All experimental protocols were authorized by the Ethics Committee in Animal Use of UNICAMP (CEUA/UNICAMP, protocol numbers 5952–1/2022 and 5831–1/2021).

Consent to participate Not applicable.

Consent for publication The authors authorize the submission and publication of this article in *Naunyn–Schmiedeberg's Archives of Pharmacology*.

Competing interests The authors declare no competing interests.

References

Ahlenius S, Larsson K (1991) Opposite effects of 5-methoxy-N, N-dimethyl-tryptamine and 5-hydroxytryptophan on male rat sexual behavior. Pharmacol Biochem Behav 38(1):201–205. https://doi.org/10.1016/0091-3057(91)90611-5

Arunlakshana O, Schild HO (1959) Some quantitative uses of drug antagonists. Br J Pharmacol Chemother 14(1):48–58. https://doi.org/10.1111/j.1476-5381.1959.tb00928.x

Başar MM, Yilmaz E, Ferhat M, Başar H, Batislam E (2005) Terazosin in the treatment of premature ejaculation: a short-term follow-up. Int Urol Nephrol 37(4):773–777. https://doi.org/10.1007/s11255-005-3616-4 (PMID: 16362597)

Beaumont G (1977) Sexual side-effects of clomipramine (Anafranil). J Int Med Res 5(Suppl. 1):37–44

Belevych AE, Zima AV, Vladimirova IA, Hirata H, Jurkiewicz A, Jurkiewicz NH, Shuba MF (1999) TTX-sensitive Na(+) and nifedipine-sensitive Ca(2+) channels in rat vas deferens smooth muscle cells. Biochim Biophys Acta 15;1419(2):343–52. https://doi.org/10.1016/s0005-2736(99)00075-9

Britto-Júnior J, Coelho-Silva WC, Murari GF, Serpellone-Nash CE, Mónica FZ, Antunes E, De Nucci G (2021a) 6-Nitrodopamine is released by human umbilical cord vessels and modulates vascular reactivity. Life Sci 1(276):119425. https://doi.org/10.1016/j.lfs.2021.119425

Britto-Júnior J, Ximenes L, Ribeiro A, Fregonesi A, Campos R, Kiguti LRA, Mónica FZ, Antunes E, De Nucci G (2021b) 6-Nitrodopamine is an endogenous mediator of rat isolated epididymal vas deferens contractions induced by electric-field stimulation. Eur J Pharmacol 1(911):174544. https://doi.org/10.1016/j.ejphar.2021.174544

Britto-Júnior J, Ribeiro A, Ximenes L, Lima AT, Jacintho FF, Fregonesi A, Mónica FZ, Antunes E, De Nucci G (2022) Alpha1-adrenergic antagonists block 6-nitrodopamine contractions on the rat isolated epididymal vas deferens. Eur J Pharmacol 15(915):174716. https://doi.org/10.1016/j.ejphar.2021.174716

Burnstock G, Verkhratsky A (2010) Vas deferens—a model used to establish sympathetic cotransmission. Trends Pharmacol Sci 31(3):131–139. https://doi.org/10.1016/j.tips.2009.12.002

Campos R, Pinheiro DHA, Britto-Júnior J, de Castro HA, Mendes GD, Moraes MO, Moraes MEA, Lopes-Martins RÁB, Antunes NJ, De Nucci G (2020) Quantification of 6-nitrodopamine in Krebs-Henseleit's solution by LC-MS/MS for the assessment of its basal release from Chelonoidis carbonaria aortae in vitro. J Chromatogr B Analyt Technol Biomed Life Sci 22(1173):122668. https://doi.org/10.1016/j.jchromb.2021.122668

Cavallini G (1995) (1995) Alpha-1 blockade pharmacotherapy in primitive psychogenic premature ejaculation resistant to psychotherapy. Eur Urol 28(2):126–130. https://doi.org/10.1159/000475036

Chang RS, Lotti VJ (1983) Characterization of beta-adrenergic receptors in the rat vas deferens using [3H]-dihydroalprenolol binding. Life Sci 30;32(22):2603–9. https://doi.org/10.1016/0024-3205(83)90245-x

Cusack B, Nelson A, Richelson E (1994) Binding of antidepressants to human brain receptors: focus on newer generation compounds. Psychopharmacology 114(4):559–565. https://doi.org/10.1007/BF02244985

Debruyne FM (2000) Alpha blockers: are all created equal? Urology 1;56(5 Suppl 1):20–2. https://doi.org/10.1016/s0090-4295(00)00744-5

Djavan B, Chapple C, Milani MM (2004) State of the art on the efficacy and tolerability of alpha1-adrenoceptor antagonists in patients with lower urinary tract symptoms suggestive of benign prostatic hyperplasia. Urology 64(6):1081–1088

Dunn CJ, Matheson A, Faulds DM (2002) Tamsulosin: a review of its pharmacology and therapeutic efficacy in the management of lower urinary tract symptoms. Drugs Aging 19(2):135–161. https://doi.org/10.2165/00002512-200219020-00004

Elliott HL, Meredith PA, Sumner DJ, McLean K, Reid JL (1982) A pharmacodynamic and pharmacokinetic assessment of a new alpha-adrenoceptor antagonist, doxazosin (UK33274) in normotensive subjects. Br J Clin Pharmacol 13(5):699–703. https://doi.org/10.1111/j.1365-2125.1982.tb01439.x

Frishman WH, Eisen G, Lapsker J (1988) Terazosin: a new long-acting alpha 1-adrenergic antagonist for hypertension. Med Clin North Am 72(2):441–448. https://doi.org/10.1016/s0025-7125(16)30778-7

Furness JB, Iwayama T (1971) Terminal axons ensheathed in smooth muscle cells of the vas deferens. Z Zellforsch Mikrosk Anat 19 113(2):259–70. https://doi.org/10.1007/BF00339420

Hellstrom WJ, Sikka SC (2006) Effects of acute treatment with tamsulosin versus alfuzosin on ejaculatory function in normal volunteers. J Urol 176(4 Pt 1):1529–1533. https://doi.org/10.1016/j.juro.2006.06.004

Höfner K, Claes H, De Reijke TM, Folkestad B, Speakman MJ (1999) Tamsulosin 0.4 mg once daily: effect on sexual function in patients with lower urinary tract symptoms suggestive of benign prostatic obstruction. Eur Urol 36(4):335–41. https://doi.org/10.1159/000019996

Hsieh JT, Liu SP, Hsieh CH, Cheng JT (1999) An in vivo evaluation of the therapeutic potential of sympatholytic agents on premature ejaculation. BJU Int 84(4):503–506. https://doi.org/10.1046/j.1464-410x.1999.00173.x

Iakovidis D, Malta E, McPherson GA, Raper C (1980) In vitro activity of RO363, a beta1-adrenoceptor selective agonist. Br J Pharmacol 68(4):677–685. https://doi.org/10.1111/j.1476-5381.1980.tb10860.x

Ko DT, Hebert PR, Coffey CS, Sedrakyan A, Curtis JP, Krumholz HM (2002) Beta-blocker therapy and symptoms of depression, fatigue, and sexual dysfunction. JAMA 17;288(3):351–7. https://doi.org/10.1001/jama.288.3.351

Lee CH, Ruben PC (2008) Interaction between voltage-gated sodium channels and the neurotoxin, tetrodotoxin. Channels (austin) 6:407–412. https://doi.org/10.4161/chan.2.6.7429

Lepor H, Baumann M, Shapiro E (1988) The stereospecificity of LY253352 for alpha 1-adrenoceptor binding sites in the brain and prostate. Br J Pharmacol 95(1):139–144. https://doi.org/10.1111/j.1476-5381.1988.tb16557.x

May JM, Abel PW, Minneman KP (1985) Binding of agonists and antagonists to beta-adrenoceptors in rat vas deferens: relationship to functional response. Naunyn Schmiedebergs Arch Pharmacol 331(4):324–333. https://doi.org/10.1007/BF00500814

Michel MC, Murphy TJ, Motulsky HJ (2020) New author guidelines for displaying data and reporting data analysis and statistical methods in experimental biology. Mol Pharmacol 97(1):49–60. https://doi.org/10.1124/mol.119.118927

Millan MJ, Dekeyne A, Papp M, La Rochelle CD, MacSweeny C, Peglion JL, Brocco M (2001) S33005, a novel ligand at both serotonin and norepinephrine transporters: II. Behavioral profile in comparison with venlafaxine, reboxetine, citalopram, and clomipramine. J Pharmacol Exp Ther 298(2):581–91

Motulsky HJ (2014) Common misconceptions about data analysis and statistics. Naunyn Schmiedebergs Arch Pharmacol 387(11):1017–1023. https://doi.org/10.1007/s00210-014-1037-6

Narahashi T, Moore JW, Poston RN (1967) Tetrodotoxin derivatives: chemical structure and blockage of nerve membrane conductance. Science 19;156(3777):976–9. https://doi.org/10.1126/science.156.3777.976

O'Leary MP (2001) Tamsulosin: current clinical experience. Urology 58(6 Suppl 1):42–8. https://doi.org/10.1016/s0090-4295(01)01346-2 (discussion 48)

O'Meara J, White WB (1988) Ejaculatory failure and urinary dysfunction secondary to labetalol. J Urol 139(2):371–2. https://doi.org/10.1016/s0022-5347(17)42420-7

Percie du Sert N, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, Emerson M, Garner P, Holgate ST, Howells DW, Hurst V, Karp NA, Lazic SE, Lidster

K, MacCallum CJ, Macleod M, Pearl EJ, Petersen OH, Rawle F, Reynolds P, Rooney K, Sena ES, Silberberg SD, Steckler T, Würbel H (2020) Reporting animal research: explanation and elaboration for the ARRIVE guidelines 2.0. PLoS Biol 14;18(7):e3000411. https://doi.org/10.1371/journal.pbio.3000411

Ramsay JW, Scott GI, Whitfield HN (1988) A double-blind controlled trial of a new alpha-1 blocking drug in the treatment of bladder outflow obstruction. Br J Urol 57(6):657–659. https://doi.org/10.1111/j.1464-410x.1985.tb07026.x

Ribeiro MO, Antunes E, de Nucci G, Lovisolo SM, Zatz R (1992) Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. Hypertension 20(3):298–303. https://doi.org/10.1161/01.hyp.20.3.298

Safarinejad MR (2008) Once-daily high-dose pindolol for paroxetine-refractory premature ejaculation: a double-blind, placebo-controlled and randomized study. J Clin Psychopharmacol 28(1):39–44. https://doi.org/10.1097/jcp.0b013e31816073a5

Sánchez C, Hyttel J (1999) Comparison of the effects of antidepressants and their metabolites on reuptake of biogenic amines and on receptor binding. Cell Mol Neurobiol 19(4):467–489. https://doi.org/10.1023/a:1006986824213

Shintani F, Kinoshita T, Kanba S, Ishikawa T, Suzuki E, Sasakawa N, Kato R, Asai M, Nakaki T (1996) Bioactive 6-nitronorepinephrine identified in mammalian brain. J Biol Chem 271(23):13561–13565. https://doi.org/10.1074/jbc.271.23.13561

Srilatha B, Adaikan PG, Arulkumaran S, Ng SC (1999) Sexual dysfunction related to antihypertensive agents: results from the animal model. Int J Impot Res 11(2):107–113. https://doi.org/10.1038/sj.ijir.3900393

Vohra MM (1979) Evidence for the presence of beta 2-inhibitory adrenoceptors in the rat vas deferens. Gen Pharmacol 10(3):221–225. https://doi.org/10.1016/0306-3623(79)90093-4

Wilt TJ, MacDonald R (2006) Doxazosin in the treatment of benign prostatic hypertrophy: an update. Clin Interv Aging 1(4):389–401. https://doi.org/10.2147/ciia.2006.1.4.389

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Anexo 9

Artigo 11 – 6-Nitrodopamine is an endogenous selective dopamine receptor antagonist in *Chelonoidis carbonaria* aorta.

Autores: Britto-Júnior J, Campos R, Peixoto M, Lima AT, Jacintho FF, Mónica FZ, Moreno RA, Antunes E, De Nucci G.

Revista: Comparative Biochemistry and Physiology, Part C

Situação: Aceito a publicação em 06 de junho de 2022. Publicado on-line em 03 de julho de 2022

Comparative Biochemistry and Physiology, Part C 260 (2022) 109403

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

6-Nitrodopamine is an endogenous selective dopamine receptor antagonist in *Chelonoidis carbonaria* aorta

José Britto-Júnior [a,*], Rafael Campos [b,c], Matheus Peixoto [b], Antonio Tiago Lima [a], Felipe Fernandes Jacintho [a], Fabíola Z. Mónica [a], Ronilson Agnaldo Moreno [a], Edson Antunes [a], Gilberto De Nucci [a,c,d]

[a] Faculty of Medical Sciences, Department of Pharmacology, University of Campinas (UNICAMP), Campinas, Brazil
[b] Superior Institute of Biomedical Sciences, Ceará State University (UECE), Fortaleza, Brazil
[c] Clinical Pharmacology Unit, Drug Research and Development Center, Federal University of Ceará (UFC), Fortaleza, CE, Brazil
[d] Department of Pharmacology, Institute of Biomedical Sciences, USP – University of São Paulo, São Paulo, Brazil

ARTICLE INFO

Edited by Martin Grosell

Keywords:
Endothelium
Dopamine
Nitric oxide
Nitrocatecholamines
Vascular smooth muscle

ABSTRACT

Chelonoidis carbonaria aortic rings present endothelium-derived release of dopamine, noradrenaline, adrenaline and 6-nitrodopamine (6-ND). Here it was investigated whether 6-ND release is coupled to nitric oxide (NO) synthesis and its action on the vascular smooth muscle reactivity.

Basal release of 6-ND from aortic rings in the absence and presence of the NO synthesis inhibitor L-NAME was quantified by LC-MS-MS. Aortic rings were suspended vertically between two metal hooks in 10-mL organ baths containing Krebs-Henseleit's solution and attached to isometric transducers. The tissues were allowed to equilibrate for 1 h before starting the experiments.

The release of 6-ND was significantly reduced by previous incubation with L-NAME. 6-ND (up to 300 μM) had no contractile activity in the aortic rings. 6-ND (1, 3 and 10 μM) produced significant rightward shifts of the concentration-response curves to dopamine in endothelium-intact (pA_2 6.09) and L-NAME pre-treated endothelium-intact (pA_2 7.06) aortic rings. Contractions induced by noradrenaline and adrenaline were not affected by pre-incubation with 6-ND. The EFS (16 Hz)-induced aortic contractions were significantly inhibited by incubation with 6-ND (10 μM).

In the thromboxane A_2 mimetic U-46619 (30 nM) pre-contracted endothelium intact aortic rings, 6-ND (1 nM–1 μM) and the dopamine D_2-receptor antagonist haloperidol (1 nM-1 μM) induced concentration-dependent relaxations. The relaxations were not present in endothelium-removed aortic rings but they were not affected by incubation with L-NAME in endothelium-intact aortic rings.

The results indicate that the synthesis of this novel catecholamine in *Chelonoidis carbonaria* aortic rings is coupled to NO release and that 6-ND acts as a highly selective dopamine D_2-like receptor antagonist.

1. Introduction

Nitrocatecholamines such as 6-nitrodopamine (6-ND) and 6-nitronoradrenaline (6-NN) can be generated *in vitro* when dopamine and noradrenaline are exposed to nitric oxide (NO) in oxygenated phosphate buffer (d'Ischia & Costantini, 1995). 6-nitronoradrenaline has been extracted from porcine brain (Shintani et al., 1996) and acts as a catechol-*O*-methyl transferase inhibitor (Huotari et al., 2001) whereas 6-ND is devoid of the biological activity of dopamine (Daveu et al., 1997).

Chelonoidis carbonaria aorta (Campos et al., 2021) and human umbilical cord vessels (HUCV; Britto-Júnior et al., 2021a) present basal release of 6-ND, which is dependent on the integrity of the endothelium. Basal release of 6-ND has also been detected by mass spectrometry in the rat isolated vas deferens (Ximenes et al., 2021; Britto-Júnior et al., 2021b). The basal release of 6-ND was coupled to NO release, since it was inhibited when the HUCV and/or the rat vas deferens were incubated with the NO synthase inhibitor L-NAME. Although NO can nitrate dopamine *in vitro* (Palumbo et al., 1999), it is not known whether the

* Corresponding author at: University of Campinas (Unicamp), Faculty of Medical Sciences, Department of Pharmacology, Rua Tessália Vieira de Camargo, 126 - Cidade Universitária, 13083-887 Campinas, São Paulo, Brazil.
E-mail address: josebrittojr@gmail.com (J. Britto-Júnior).

https://doi.org/10.1016/j.cbpc.2022.109403
Received 21 May 2022; Received in revised form 17 June 2022; Accepted 26 June 2022
Available online 3 July 2022
1532-0456/© 2022 Elsevier Inc. All rights reserved.

nitrosation/nitration of the catecholamines *in vivo* is dependent on an enzymatic or a chemical process.

In HUCV, 6-ND was devoid of contractile activity, but it antagonized the contractions induced by dopamine in both artery and vein rings (Britto-Júnior et al., 2021a). In the rat isolated vas deferens, 6-ND caused contraction of the strips and that this action was selectively blocked by tricyclic antidepressants and other tricyclic compounds such as cyclobenzaprine and carbamazepine, revealing the presence of a novel 6-ND receptor in that tissue (Ximenes et al., 2021; Britto-Júnior et al., 2021b).

Here it is demonstrated that the basal release of 6-ND from *Chelonoidis carbonaria* aorta is inhibited by incubation with L-NAME. In contrast to other catecholamines such as dopamine, noradrenaline and adrenaline, 6-ND induced no contractions of the *Chelonoidis carbonaria* aortic rings. As reported in HUCV (Britto-Júnior et al., 2021a), 6-ND selectively antagonized dopamine-induced contractions of *Chelonoidis carbonaria* aorta, without affecting the contractions induced by either noradrenaline or adrenaline. Electrical-field stimulation (EFS) caused frequency-dependent contractions of *Chelonoidis carbonaria* aortic rings (Campos et al., 2020), and this contraction is dependent on endothelium-derived dopamine release (Britto-Júnior et al., 2021c). 6-ND inhibited EFS-induced contractions of *Chelonoidis carbonaria* aortic rings. In aortic rings pre-contracted by the thromboxane mimetic U-46619 (Ojeda et al., 1976), 6-ND caused endothelium-dependent but L-NAME-independent relaxations of *Chelonoidis carbonaria* aortic rings. These results indicate that this novel endothelium-derived catecholamine modulates smooth muscle reactivity of *Chelonoidis carbonaria* aorta, acting as a highly selective dopamine antagonist.

2. Material and methods

2.1. Animals

All experimental procedures using *Chelonoidis carbonaria* of either sex (weight varied from 2 to 7 kg, 31 tortoises) were approved by the Institutional Animal Care and Use Committee (CEUA/UNICAMP: 3907-1) and followed the ARRIVE guidelines (Percie du Sert et al., 2020). The use of *Chelonoidis carbonaria* was authorized by the Brazilian Institute for Environment (Sisbio; number 20910) and the animals were provided by the Parque Ecológico do Tietê (São Paulo, SP, Brazil) and CRAS-Univap (São José dos Campos, SP, Brazil).

2.2. Chemical and reagents

Dopamine, adrenaline, noradrenaline, prazosin, ascorbic acid, acetylcholine, adenosine 5′-triphosphate (ATP), 1H-[1,2,4]-oxadiazolo-[4,3-a] quinoxalin-1-one (ODQ) and N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) were acquired from Sigma-Aldrich Chemicals Co. (St Louis, Missouri, USA). 6-nitrodopamine and 6-nitrodopamine-d_4 were purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). U-46619 was obtained from Tocris (Bristol, UK). Haloperidol was provided by Vamsi Labs Ltd. (Maharashtra, India). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$), potassium phosphate monobasic (KH_2PO_4) and glucose were acquired from Merck KGaA (Darmstadt, Germany). Acetonitrile and methanol were bought from J.T. Baker (Phillipsburg, NJ, USA) and formic acid from Mallinckrodt (St Louis, MO, USA).

2.3. Basal release of 6-nitrodopamine from Chelonoidis carbonaria aortic rings

Two aortic rings per animal (15 mm, right aorta near the heart) from *Chelonoidis carbonaria* were suspended in 5 mL organ baths containing Krebs Henseleit's solution (KHS) and O_2/CO_2 mixture (95 %/5 %) at 27 °C for 30 min. The aortic rings were incubated in the absence and in the presence of the NO synthase inhibitor N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 100 μM). Two aliquots of 2 mL of the supernatant were transferred to two tubes and stored at −20 °C until analysis (Britto-Júnior et al., 2020; Britto-Júnior et al., 2021a).

2.4. Extraction and quantification of 6-nitrodopamine by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

The extraction and quantification of 6-ND in KHS were performed as reported previously (Campos et al., 2021). Briefly, 6-ND was extracted from 1 mL of KHS by solid phase extraction (SPE). Calibrators and quality controls (QCs) prepared in blank KHS and the KHS samples obtained from aortic ring for 30 min were spiked with 50 μL of the internal standard (IS) solution (6-ND-d_4, 100 ng/mL). Extraction cartridges were conditioned with 1 mL of methanol and then equilibrated with 2 mL of deionized water. The samples were transferred to the extraction cartridges and washed three times with deionized water. The samples were eluted with 0.9 mL methanol/deionized water (90/10, *v*/v) plus 0.1 % formic acid and followed by evaporation under N_2 flow at 50 °C. The dry residues were dissolved with 100 μL acetonitrile/deionized water (50/50, v/v) plus 0.1 % formic acid, transferred to vials and submitted to chromatographic analysis.

The separation of 6-ND was performed on a 150 mm × 3.0 mm Shimpack GIST-HP C_{18} column, 3-μm particle size (Shimadzu, Duisburg, Germany) held at 65 °C. A 75 % of mobile phase A consisting of deionized water with 0.1 % formic acid (*v*/v) and 25 % of mobile phase B consisting of acetonitrile/deionized water (90/10, v/v) plus 0.1 % formic acid at a flow rate of 350 μL/min in an isocratic mode were used. The detection of 6-ND and IS was carried out by a LC-MS-8060 triple quadrupole mass spectrometer (MS/MS) (Shimadzu, Kyoto), operating in positive ionization mode. The analyses were performed in the multiple reaction monitoring (MRM) mode. The protonated ions $[M + H]^+$ and their respective ion products monitored were 199.10 > 181.95 and 203.10 > 186.00 for 6-ND and 6-ND-d4, respectively.

2.5. Aortic ring preparations and isometric tension recordings

The tortoises were anesthetized with ketamine and propofol (40 mg/kg IM and 15 mg/kg IV in the jugular vein, respectively) after sedation with midazolam (2 mg/kg; IM). The animals were euthanized by exsanguination. A segment of aorta was removed and immediately placed in oxygenated (95%O_2/5%CO_2) KHS at 27 °C (Campos et al., 2019). Subsequently, aortic rings (3 mm) were suspended vertically between two metal hooks in 10-mL organ baths containing KHS (mM): NaCl (118), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (1.2), $NaCO_3$ (25), KH_2PO_4 (1.2) and glucose (5.6), gassed with a mixture of 95 % O_2: 5 % CO_2 (pH 7.4) at 27 °C. Isometric force was recorded using a PowerLab 400TM data acquisition system (Software Chart, version 7.0, AD Instrument, MA, USA). The tissues were allowed to equilibrate for 1 h before starting the experiments. Following a 60-min stabilization period, rings were pre-contracted with acetylcholine (ACh, 3 μM). The integrity of the endothelium in the aortic rings was evaluated through ATP-induced relaxation (10 μM; Knight and Burnstock, 1995; Campos et al., 2020).

2.6. Concentration-response curves to dopamine

Cumulative concentration-response curves to dopamine (1 nM–1 mM) were performed in endothelium-intact aortic rings in the absence and in the presence of L-NAME (100 μM), as well as in preparations where the endothelium was mechanically removed by gently rubbing the arteries with forceps. Cumulative concentration-response curves to dopamine (1 nM–1 mM) in the above-described conditions were carried out in the presence of the 6-ND (1, 3 and 10 μM). Cumulative concentration-response curves to dopamine (1 nM–1 mM) in endothelium-intact aortic rings treated with L-NAME (100 μM) were also conducted in the absence and presence of the α_1 adrenergic receptor

antagonist prazosin (10, 30 and 100 nM).

2.7. Concentration-response curves to noradrenaline and adrenaline

Cumulative concentration-response curves to noradrenaline (1 nM–1 mM) and adrenaline (1 nM–1 mM) were performed in endothelium-intact aortic rings treated with L-NAME (100 μM, 30 min) in the absence and in the presence of either 6-ND (1, 3 and 10 μM) or prazosin (10, 30 and 100 nM).

2.8. Electrical-field stimulation (EFS)-induced aorta contractions

Aortic rings pretreated with L-NAME (100 μM) were submitted to EFS at 60 V for 30 s, at 16 Hz in square-wave pulses, 0.3 ms pulse width and 0.1 ms delay, using a Grass S88 stimulator (Astro-Medical, Industrial Park, RI, USA). Electrical-field simulations (16 Hz; Kirby and Burnstock, 1969) were performed in aortic rings in the absence and presence of 6-ND (10 μM). Potassium chloride (KCl, 80 mM) was added at the beginning and at the end of the experimental protocols to evaluate the tissue reactivity after EFS (Campos et al., 2020; Britto-Júnior et al., 2021c).

2.9. Relaxing responses to 6-ND in pre-contracted aortic rings

Aortic rings were pre-contracted with the thromboxane A_2 (TXA_2) mimetic U-46619 (30 nM) in the absence and presence of L-NAME (100 μM). Endothelium-denuded aortic rings were also pre-contracted with U-46619 (30 nM). After a sustained contraction was obtained, cumulative concentration-response curves to either 6-ND (1 nM–1 μM) or the D2 like-receptor antagonist haloperidol (1 nM–1 μM nM) were performed. The relaxation responses were expressed as percentage of the contractile response.

2.10. Data analysis

Nonlinear regression analysis to determine the pEC_{50} was carried out using GraphPad Prism (GraphPad Software, version 6.0, San Diego, CA, USA) with the constraint that F = 0. All concentration–response data were evaluated for a fit to a logistics function in the form: $E = E_{max}/([1 + (10c/10x)n] + F)$, where E represents the increase in response contractile induced by the agonist, E_{max} is the effect agonist maximum, c is the logarithm of concentration of the agonist that produces 50 % of E_{max}, x is the logarithm of the concentration of the drug; the exponential term, n, is a curve fitting parameter that defines the slope of the concentration–response line, and F is the response observed in the absence of added drug. The pA_2 values were calculated from the intercept on the concentration axis and by application of the equation; pA_2 = log (antagonist concentration) − log (CR-1) − log (antagonist concentration) (Arunlakshana and Schild, 1959). The values of EC_{50} data represent the mean ± standard error of the mean (SEM) of *n* experiments. Values of E_{max} were expressed in mN.

Data are expressed as mean ± standard error of the mean (SEM) of the number of experiments. In the pharmacological experiments, the number of experiments in expressed is x/y, where x represents the number of aortas (animal) and y the number of rings employed in the experiment. The contractions were quantified in milli-Newtons (mN). One ring was used as the control response and the other ring was incubated with an antagonist/inhibitor. Student's two-tail unpaired *t*-test was employed and the differences between groups at $p < 0.05$ were considered significant. For E_{max} and $pEC_{50\ analysis}$, unpaired Student's *t*-test was used. When EFS was performed in the absence and in the presence of an antagonist/inhibitor (the first contraction being the control and second the treated response), Student's paired *t*-test was employed for statistical analysis.

3. Results

3.1. Basal release of 6-ND in Chelonoidis carbonaria aortic rings by LC-MS-MS

The calibration curve of 6-ND was linear for concentrations of 0.1–10 ng/mL, with a correlation coefficient higher than 0.99. The limit of quantification was 0.1 ng/mL. The basal release of 6-ND was decreased by pre-treatment of the aortic rings with L-NAME (100 μM; Fig. 1).

3.2. Effect of 6-ND on the dopamine-induced Chelonoidis carbonaria aortic contractions

The addition of 6-ND (0.001–300 μM) alone had no contractile activity on endothelium-intact aortic rings (not shown). The lack of contractile activity was also observed in the presence of either L-NAME (100 μM) or of the heme-site inhibitor of soluble-guanylate cyclase ODQ (100 μM; data not shown). In endothelium-intact aortic rings, pre-incubation (30 min) with 6-ND (1, 3 and 10 μM) produced a significant concentration-dependent rightward shift of the concentration-response curves to dopamine (Fig. 2A; Table 1). At 1 μM, 6-ND also significantly reduced the E_{max} values to dopamine (Fig. 2A; Table 1). Similar results were observed in L-NAME pre-treated endothelium-intact aortic rings (Fig. 2B; Table 1). In endothelium-denuded aortic rings, 6-ND caused no significant rightward shift of the concentration-response curves to dopamine, but it did produce a significant reduction in the E_{max} (Fig. 2C; Table 1). The calculated pA_2 values for 6-ND in endothelium-intact aortic rings were 6.09 ± 0.23 and for L-NAME pre-treated endothelium-intact aortic rings were 7.06 ± 0.27.

3.3. Effect of 6-ND on the contractions induced by noradrenaline and adrenaline

Noradrenaline (Fig. 3A; Table 2) and adrenaline (Fig. 3B; Table 2) induced concentration-dependent contractions of endothelium-intact and L-NAME pre-treated aortic rings. In contrast to dopamine, pre-incubation (30 min) with 6-ND (1, 3 and 10 μM) had no effect on either the pEC_{50} or E_{max} of the concentration-response curves to noradrenaline (Fig. 3A, Table 2) or adrenaline (Fig. 3B, Table 2).

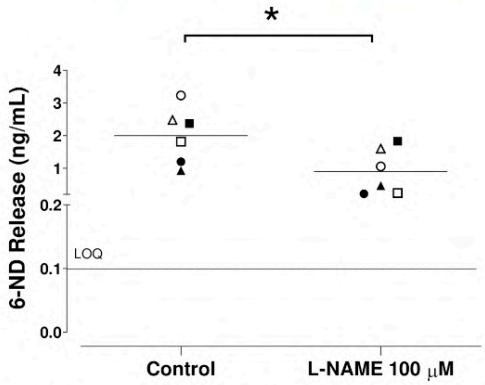


Fig. 1. Basal release of 6-nitrodopamine in aortic rings of *Chelonoidis carbonaria*. The basal release of 6-nitrodopamine was measured by LC-MS/MS following a 30 min-period incubation in Krebs-Henseleit's solution. Treatment with L-NAME (100 μM) reduced the 6-nitrodopamine release in in aortic rings of *Chelonoidis carbonaria* ($n = 4/6$). * indicates $p < 0.05$. Each individual symbol represents a ring before and after treatment.

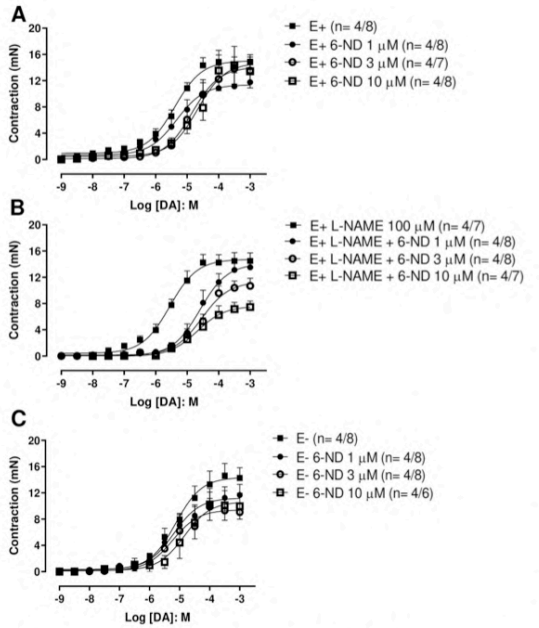


Fig. 2. Concentration-response curves (1 nM–1 mM) to dopamine in aortic rings of *Chelonoidis carbonaria*. Endothelium-intact (panel A), endothelium-intact in presence of L-NAME 100 μM (panel B) and endothelium-denuded rings (panel C) were contracted in the presence of 6-nitrodopamine (1, 3 and 10 μM). Data are expressed as mean ± S.E.M.

Table 1
The potency (pEC_{50}) and maximum response (E_{max}) of the concentration-response curves to dopamine in the absence and presence of 6-ND.

Agonist	pEC_{50} (log[M])	E_{max} (mN)	n
Dopamine			
E+	5.35 ± 0.09	14.83 ± 0.78	4/8
E+ 6-ND 1 μM	5.06 ± 0.20*	11.76 ± 1.91*	4/8
E+ 6-ND 3 μM	4.81 ± 0.19*	13.52 ± 1.75	4/7
E+ 6-ND 10 μM	4.66 ± 0.08*	13.44 ± 2.55	4/8
E+ L-NAME	5.61 ± 0.15	14.49 ± 1.22	4/7
E+ L-NAME 6-ND 1 μM	4.63 ± 0.14*	13.53 ± 1.19	4/8
E+ L-NAME 6-ND 3 μM	4.65 ± 0.17*	10.70 ± 1.16*	4/8
E+ L-NAME 6-ND 10 μM	4.54 ± 0.19*	7.47 ± 0.90*	4/7
E−	5.28 ± 0.22	15.51 ± 1.15	4/8
E− 6-ND 1 μM	4.98 ± 0.23	11.67 ± 1.64*	4/8
E− 6-ND 3 μM	5.26 ± 0.18	9.06 ± 1.29*	4/8
E− 6-ND 10 μM	4.87 ± 0.20	9.98 ± 1.99*	4/6

pEC50 is defined as the negative logarithm of the EC_{50}; E_{max} is the maximal effect at high drug; n means the number of vas deferens strips.
* $P < 0.05$ compared with respective control values.

3.4. Effect of 6-ND in EFS-induced contractions

In L-NAME pre-treated endothelium-intact aortic rings, the EFS (16 Hz)-induced aortic contractions were significantly reduced ($p < 0.05$) by incubation with the 6-ND (10 μM, 30 min; 4.39 ± 0.86 and 1.96 ± 0.40 mN for control and 6-ND, respectively; Fig. 4A and B).

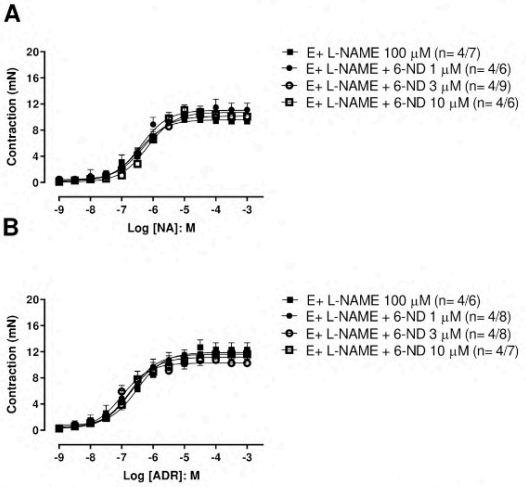


Fig. 3. Concentration-response curves (1 nM–1 mM) to noradrenaline (panel A) and adrenaline (panel B) in aortic rings of *Chelonoidis carbonaria*. Endothelium-intact aortic rings in presence of L-NAME 100 μM were contracted in presence of the 6-nitrodopamine (1, 3 and 10 μM). Data are expressed as mean ± S.E.M.

Table 2
The potency (pEC_{50}) and maximum response (E_{max}) of the concentration-response curves to noradrenaline and adrenaline in the absence and presence of 6-ND.

Agonist	pEC_{50} (log[M])	E_{max} (mN)	N
Noradrenaline			
E+ L-NAME	6.46 ± 0.10	9.34 ± 0.38	4/7
E+ L-NAME 6-ND 1 μM	6.39 ± 0.12	11.13 ± 0.99	4/6
E+ L-NAME 6-ND 3 μM	6.32 ± 0.10	10.08 ± 0.97	4/9
E+ L-NAME 6-ND 10 μM	6.19 ± 0.08	10.71 ± 1.08	4/6
Adrenaline			
E+ L-NAME	6.50 ± 0.13	12.68 ± 1.13	4/6
E+ L-NAME 6-ND 1 μM	6.71 ± 0.10	11.71 ± 0.67	4/8
E+ L-NAME 6-ND 3 μM	6.99 ± 0.13	10.76 ± 1.20	4/8
E+ L-NAME 6-ND 10 μM	6.73 ± 0.09	11.63 ± 0.77	4/7

pEC50 is defined as the negative logarithm of the EC_{50}; E_{max} is the maximal effect at high drug; n means the number of vas deferens strips.

3.5. Effect of prazosin on the contractions induced by the dopamine, noradrenaline, and adrenaline

In L-NAME pre-treated endothelium-intact aortic rings, pre-incubation (30 min) with the selective α_1-adrenergic antagonist prazosin (10, 30 and 100 nM) produced significant concentration-dependent rightward shifts of the concentration-response curves to dopamine (Fig. 5A; Table 3), noradrenaline (Fig. 5B; Table 3) and adrenaline (Fig. 5A; Table 3). Prazosin (30 and 100 nM) also produced a significant reduction in the E_{max} of the concentration-response curves to noradrenaline (Table 3). In L-NAME pre-treated endothelium intact aortic rings, the calculated pA_2 values for prazosin were 9.01 ± 0.13, 8.60 ± 0.39 and 8.05 ± 0.24 for dopamine, noradrenaline and adrenaline, respectively.

3.6. Effect of 6-ND in pre-contracted aortic rings

The aortic rings were initially pre-contracted with the TXA_2 mimetic U46619 (30 nM). Addition of 6-ND (0.001–1 μM; Fig. 6A and C) and

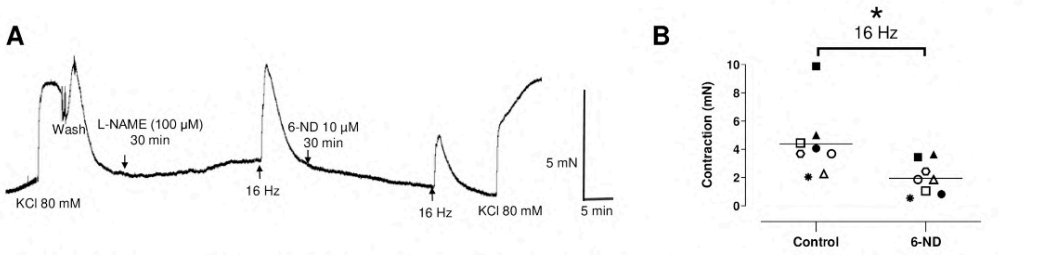


Fig. 4. 6-ND showing significant reduction in EFS-induced contractions in aortic rings from *Chelonoidis carbonaria* (panel A; control and 6-ND 10 μM). Scatter plot shows the individual values and mean ± S.E.M of the EFS-induced contractions in aortic rings from *Chelonoidis carbonaria* (panel B; n = 4/8 for 16 Hz).

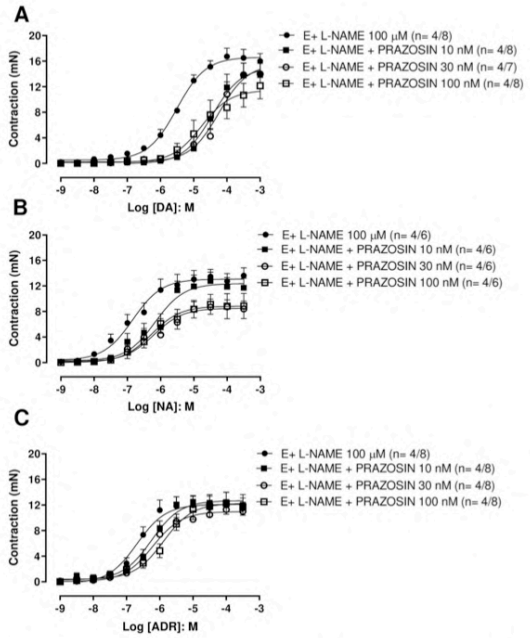


Fig. 5. Concentration-response curves to catecholamines in presence of L-NAME (100 μM) and Prazosin in *Chelonoidis carbonaria* aortic rings. Concentration-response curves to dopamine – (panel A), noradrenaline – (panel B) adrenaline – (panel C) were also performed in presence and absence of the α_1-adrenergic receptor antagonist prazosin (10, 100 and 300 nM). Data are expressed as mean ± S.E.M.

haloperidol (0.001–1 μM; Fig. 6B and D) caused concentration-dependent relaxation in endothelium-intact aortic rings. The relaxations were markedly reduced in endothelium-denuded aortic rings (Fig. 6A and B), but they were not affected by pre-treatment with L-NAME (Fig. 6C and D).

4. Discussion

The decrease of 6-ND release by the NO synthesis inhibitor L-NAME has been described in human umbilical cord vessels (HUCV; Britto-Júnior et al., 2021a) and rat isolated vas deferens (Ximenes et al., 2021; Britto-Júnior et al., 2021b). Chronic treatment with L-NAME causes significant reduction of 6-noradrenaline levels in rat brain (Tsunoda et al., 2007). In both *Chelonoidis carbonaria* aortic and HUCV rings, the release of 6-ND was also reduced by mechanical removal of the endothelium layer, indicating that endothelial cells are responsible for 6-ND production. Whether the nitrosation/nitration of dopamine occurs *via* chemical or enzymatic reaction is under current investigation.

Table 3
The potency (pEC_{50}) and maximum response (E_{max}) of the concentration-response curves to dopamine, noradrenaline and adrenaline in the absence and presence of prazosin.

Agonist	pEC_{50} (log[M])	E_{max} (mN)	n
Dopamine			
E+ L-NAME	5.55 ± 0.14	16.01 ± 1.18	4/8
E+ L-NAME prazosin 10 nM	4.49 ± 0.09*	13.83 ± 1.82	4/8
E+ L-NAME prazosin 30 nM	4.34 ± 0.18*	13.98 ± 1.98	4/7
E+ L-NAME prazosin 100 nM	4.68 ± 0.20*	12.15 ± 2.05	4/8
Noradrenaline			
E+ L-NAME	6.67 ± 0.27	13.59 ± 1.23	4/6
E+ L-NAME prazosin 10 nM	6.29 ± 0.23*	11.70 ± 1.50	4/6
E+ L-NAME prazosin 30 nM	6.16 ± 0.38*	8.40 ± 1.21*	4/6
E+ L-NAME prazosin 100 nM	6.30 ± 0.22*	8.86 ± 1.96*	4/6
Adrenaline			
E+ L-NAME	6.60 ± 0.11	11.45 ± 1.01	4/8
E+ L-NAME prazosin 10 nM	6.30 ± 0.12*	11.90 ± 1.74	4/8
E+ L-NAME prazosin 30 nM	6.27 ± 0.15*	11.02 ± 1.09	4/8
E+ L-NAME prazosin 100 nM	6.05 ± 0.18*	11.74 ± 1.35	4/8

pEC50 is defined as the negative logarithm of the EC_{50}; E_{max} is the maximal effect at high drug; n means the number of vas deferens strips.
* $P < 0.05$ compared with respective control values.

6-ND caused concentration-dependent rightward shifts of dopamine-induced contractions of *Chelonoidis carbonaria* aortic rings (pA_2 = 6.1 and 7.1 for L-NAME untreated and treated, respectively). The dopamine D_2-like receptor antagonists haloperidol (Thornburg and Moore, 1975), risperidone (Leysen et al., 1988) and quetiapine (Campbell et al., 1991) caused significant rightward shifts of dopamine-induced contractions of *Chelonoidis carbonaria* aortic rings, suggesting the presence of functional dopamine D_2-like receptors (Britto-Júnior et al., 2021c). Although haloperidol (pA_2 7.2; Simon and Van Maanen, 1976) and risperidone (pA2 8.9; Millan et al., 2008) are somewhat more potent than 6-ND, they are not very selective for the dopaminergic D_2-like receptors, since they also bind to α_1 adrenergic receptors. Indeed, haloperidol K_i values are 1.4 and 4.7 nM (Schmidt et al., 2001), risperidone Ki are 2.2 and 1.4 nM (Schmidt et al., 2001) and quetiapine K_i are 160 and 7 nM (Bymaster et al., 1996) for D_2 and α_1 receptors, respectively. In contrast, 6-ND behaves as an extremely selective antagonist for D_2-like receptors, since it affected neither noradrenaline nor adrenaline-induced contractions of *Chelonoidis carbonaria* aortic rings. This high selectivity for D_2-like dopamine receptors was also present in both human umbilical artery and vein (Britto-Júnior et al., 2021a).

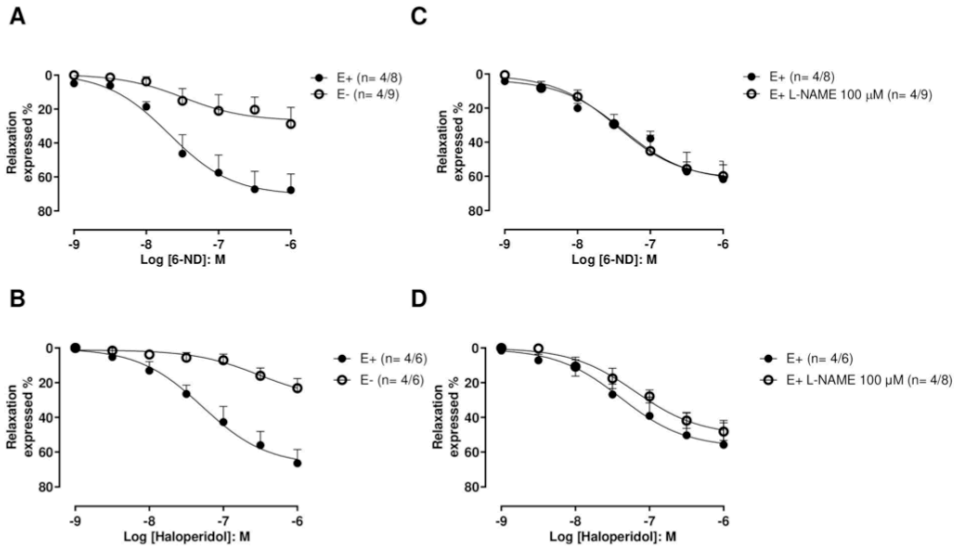


Fig. 6. In U-46619 pre-contracted (30 nM) *Chelonoidis carbonaria* aortic rings 6-ND (panel A) and Haloperidol (panel B) caused concentration-dependent relaxation of *Chelonoidis carbonaria* aortic rings, which were abolished by mechanical removal of the endothelium. L-NAME failed to affect the 6-ND (panel C) and haloperidol (panel D)-induced relaxation. Data are expressed as mean ± S.E.M.

Electrical-field stimulation contracts *Chelonoidis carbonaria* aortic rings, which were sensitive to the dual α_1 and α_2 adrenergic receptor antagonist phentolamine, but not to the selective α_1 receptor antagonist prazosin or the selective α_2 receptor antagonist idazoxan, suggesting that phentolamine was possibly acting on dopaminergic receptors (Campos et al., 2020). The finding that 6-ND inhibited EFS-induced contractions confirms that endothelium-derived dopamine acting on D_2-like receptors is the major modulator of *Chelonoidis carbonaria* aortic ring contractility following electrical stimulation.

The contractions of *Chelonoidis carbonaria* aortic rings induced by noradrenaline were inhibited by prazosin, indicating that this tissue expresses functional α1-adrenergic receptors. The pA_2 value obtained for α1-adrenergic antagonist prazosin in noradrenaline-induced contractions of *Chelonoidis carbonaria* aortic rings (8.60 ± 0.39) was in the same range as those reported for rabbit thoracic aorta (8.82 ± 0.03), rat (9.89 ± 0.03) and guinea-pig aorta (8.45 ± 0.17; Muramatsu et al., 1990) (Scivoletto et al., 1976). The contractions induced by adrenaline in *Chelonoidis carbonaria* aortic rings were also due to activation of α_1-adrenergic receptors since they are blocked by prazosin. Vascular smooth muscle expresses both α_1- and α_2-post-synaptic adrenergic receptors, capable of causing vasoconstriction (Timmermans and Van Zwieten, 1981). Although adrenaline can act as an post-synaptic α_2-adrenergic receptor agonist (Brown and Werman, 1990), the contractions induced by adrenaline in human umbilical veins were affected neither by the β_1,β_2-blocker propranolol (McNeill, 1964) nor by the α_2 blocker rauwolscine (Kohli and De, 1956), but were inhibited by prazosin, revealing the presence of prazosin-sensitive functional α_1 (but not β_1, β_2 or α_2) adrenoceptors (Errasti et al., 1999). Adrenaline-induced vasoconstriction of human internal mammary artery strips is also due to activation of prazosin-sensitive functional α_1-adrenergic receptors (Bevilacqua et al., 1991).

The contractions induced by dopamine in vas deferens can also be explained by activation of functional α_1-adrenergic receptors, since they are also inhibited by prazosin. In the rat vas deferens, the pA_2 values for prazosin against noradrenaline and dopamine were 9.09 ± 0.12 and 8.79 ± 0.27, respectively (Miranda et al., 1990). Since 6-ND does not present contractile activity in *Chelonoidis carbonaria* aortic rings, our results clearly demonstrate that it does not act on α_1 adrenergic receptors, as observed in human umbilical cord vessels (Britto-Júnior et al., 2021a).

The finding that 6-ND causes NO-independent relaxation in U-46619 pre-contracted aortic rings reinforces the modulatory role of dopamine in vascular reactivity. Dopamine acts on selective G-protein coupled receptors, which comprise the D_1-like (D_1R and D_5R) subfamily, that activates adenylate cyclase, and the D_2-like (D_2R, D_3R and D_4R) subfamily, which inhibits adenylate cyclase (Missale et al., 1998). They have been identified in both vascular smooth muscle (Amenta et al., 1990) and endothelium (Amenta, 1997). Since dopamine also contracts endothelium-denuded *Chelonoidis carbonaria* aortic rings, it is likely that 6-ND acts by blocking D_2-like receptors in the vascular smooth muscle. The use of specific agonists/antagonists coupled to immunohistochemical detection should clarify which subtypes of dopamine receptors are present in the *Chelonoidis carbonaria* aorta.

What is the possible physiological relevance of 6-ND in *Chelonoidis carbonaria* circulatory system? The findings that both dopamine (Britto-Júnior et al., 2021c) and 6-ND are released by the endothelium, and the release of 6-ND is coupled to NO synthesis, indicate that vascular tonus can be influenced by a dynamic equilibrium between the vascular contractile activity of dopamine and the vasodilator action of 6-ND. Indeed, *Chelonoidis carbonaria* aortic rings do not present autonomic innervation, as demonstrated by the lack of immunoreactivity to S-100 (Campos et al., 2020). Although the aorta is considered a conduit vessel (Eelen et al., 2020), and therefore not responsible for vascular resistance (Kannenkeril et al., 2018), morphological evidence of autonomic innervation such as sympathetic nerve terminals in the microcirculation is very difficult to find, suggesting that endothelium-dependent catecholamine release could be the major mechanism for controlling local blood flow.

5. Conclusion

6-ND is a novel catecholamine that is released by *Chelonoidis*

carbonaria aortic rings and modulate vascular reactivity by antagonizing the action of dopamine in D_2-like receptors in the vascular smooth muscle.

Declaration of competing interest

The authors declare no competing or financial interests.

Acknowledgment

JBJ thanks CAPES for PhD fellowship (001). ATL and FFJ thank FAPESP for PhD fellowship (2021/13593-6; 2018/24971-9) MP thank CAPES for master's degree fellowship (001). EA & FM thank FAPESP (2017/15175-1). GDN thanks FAPESP (2019/16805-4) and CNPq (303839/2019-8).

References

Amenta, F., Collier, W.L., Ricci, A., 1990. Autoradiographic localization of vascular dopamine receptors. Am. J. Hypertens. 34S–36S.

Amenta, F., 1997. Light microscope autoradiography of peripheral dopamine receptor subtypes. Clin. Exp. Hypertens. 19, 27–41.

Arunlakshana, O., Schild, H.O., 1959. Some quantitative uses of drug antagonists. Br. J. Pharmacol. Chemother. 14 (1), 48–58.

Bevilacqua, M., Vago, T., Monopoli, A., Baldi, G., Forlani, A., Antona, C., Biglioli, P., Scrofani, R., Norbiato, G., 1991. Alpha 1 adrenoceptor subtype mediates noradrenaline induced contraction of the human internal mammary artery: radioligand and functional studies. Cardiovasc. Res. 25 (4), 290–294.

Britto-Júnior, J., Antunes, N.J., Campos, R., Sucupira, M., Mendes, G.D., Fernandes, F., Moraes, M.O., Moraes, M.E.A., De Nucci, G., 2020. Determination of dopamine, noradrenaline, and adrenaline in Krebs-Henseleit solution by liquid chromatography coupled with tandem mass spectrometry and measurement of their basal release from *Chelonoidis carbonaria* aortae *in vitro*. Biomed. Chromatogr. 35 (2), e4978.

Britto-Júnior, J., Coelho-Silva, W.C., Murari, G.F., Serpellone, C.N., Mónica, F.Z., Antunes, E., De Nucci, G., 2021a. 6-Nitrodopamine is released by human umbilical cord vessels and modulates vascular reactivity. Life Sci. 26 (276), 119425.

Britto-Júnior, J., Ximenes, L., Ribeiro, A., Fregonesi, A., Campos, R., Kiguti, L.R.A., Mónica, F.Z., Antunes, E., De Nucci, G., 2021b. 6-Nitrodopamine is an endogenous mediator of rat isolated epididymal vas deferens contractions induced by electric-field stimulation. Eur.J.Pharmacol. 1 (911), 174544.

Britto-Júnior, J., Fernandes, Jacintho F., Campos, R., Pinheiro, D.H.A., Figueiredo Murari, G.M., de Souza, V.B., Schenka, A.A., Mónica, F.Z., Moreno, R.A., Antunes, E., De Nucci, G., 2021c. The basal release of endothelium-derived catecholamines regulates the contractions of Chelonoidis carbonaria aorta caused by electrical-field stimulation. Biol. Open 20;10 (1), bio057042.

Brown, C.G., Werman, H.A., 1990. Adrenergic agonists during cardiopulmonary resuscitation. Resuscitation 19 (1), 1–17.

Bymaster, F.P., Calligaro, D.O., Falcone, J.F., Marsh, R.D., Moore, N.A., Tye, N.C., Seeman, P., Wong, D.T., 1996. Radioreceptor binding profile of the atypical antipsychotic olanzapine. Neuropsychopharmacology 14 (2), 87–96.

Campos, R., Justo, A.F.O., Jacintho, F.F., Mónica, F.Z., Rojas-Moscoso, J.A., Moreno, R. A., Napolitano, M., Cogo, J.C., De Nucci, G., 2019. Pharmacological and transcriptomic characterization of the nitric oxide pathway in aortic rings isolated from the tortoise Chelonoidis carbonaria. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 222, 82–89.

Campos, R., Jacintho, F.F., Britto-Júnior, J., Mónica, F.Z., Justo, A.F.O., Pupo, A.S., Moreno, R.A., de Souza, V.B., Schenka, A.A., Antunes, E., De Nucci, G., 2020. Endothelium modulates electrical field stimulation-induced contractions of Chelonoidis carbonaria aortic rings. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 233, 108763.

Campos, R., Pinheiro, D.H.A., Britto-Júnior, J., de Castro, H.A., Mendes, G.D., Moraes, M. O., Moraes, M.E.A., Lopes-Martins, R.A.B., Antunes, N.J., De Nucci, G., 2021. Quantification of 6-nitrodopamine in Krebs-Henseleit's solution by LC-MS/MS for the assessment of its basal release from Chelonoidis carbonaria aortae in vitro. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 22 (1173), 122668.

Campbell, A., Yeghiayan, S., Baldessarini, R.J., Neumeyer, J.L., 1991. Selective antidopaminergic effects of S(+)N-n-propylnoraporphines in limbic versus extrapyramidal sites in rat brain: comparisons with typical and atypical antipsychotic agents. Psychopharmacology (Berl.) 103 (3), 323–329.

d'Ischia, M., Costantini, C., 1995. Nitric oxide-induced nitration of catecholamine neurotransmitters: a key to neuronal degeneration? Bioorg. Med. Chem. 3 (7), 923–927.

Daveu, C., Servy, C., Dendane, M., Marin, P., Ducrocq, C., 1997. Oxidation and nitration of catecholamines by nitrogen oxides derived from nitric oxide. Nitric Oxide 1 (3), 234–243.

Eelen, G., Treps, L., Li, X., Carmeliet, P., 2020. Basic and therapeutic aspects of angiogenesis updated. Circ. Res. 127 (2), 310–329.

Errasti, A.E., Velo, M.P., Torres, R.M., Sardi, S.P., Rothlin, R.P., 1999. Characterization of alpha1-adrenoceptor subtypes mediating vasoconstriction in human umbilical vein. Br. J. Pharmacol. 126 (2), 437–442.

Huotari, M., Passlin, M., Nordberg, H.L., Forsberg, M., Kotisaari, S., Tuomisto, L., Shintani, F., Tanaka, K.F., Reenilä, I., Laitinen, K., Männistö, P.T., 2001. Effect of intracerebral 6-nitronoradrenaline, an endogenous catechol-O-methyltransferase (COMT) inhibitor, on striatal dopamine metabolism in anaesthetised rats. J. Neurosci. Methods 109 (1), 47–52, 15.

Kannenkeril, D., Harazny, J.M., Bosch, A., Ott, C., Michelson, G., Schmieder, R.E., Friedrich, S., 2018. Retinal vascular resistance in arterial hypertension. Blood Press. 27 (2), 82–87. https://doi.org/10.1080/08037051.2017.1393311.

Kirby, S., Burnstock, G., 1969. Comparative pharmacological studies of isolated spiral strips of large arteries from lower vertebrates. Comp. Biochem. Physiol. 28 (1), 307–319.

Knight, G.E., Burnstock, G., 1995. Responses of the aorta of the garter snake (Thamnophis sirtalis parietalis) to purines. Br. J. Pharmacol. 114 (1), 41–48. https://doi.org/10.1111/j.1476-5381.1995.tb14903.x.

Kohli, J.D., De, N.N., 1956. Pharmacological action of Rauwolscine. Nature 23;177 (4521), 1182.

Leysen, J.E., Gommeren, W., Eens, A., de Chaffoy de Courcelles, D., Stoof, J.C., Janssen, P.A., 1988. Biochemical profile of risperidone, a new antipsychotic. J. Pharmacol. Exp. Ther. 247 (2), 661–670.

Mcneill, R.S., 1964. Effect of a BETA-adrenergic-blocking agent, propranolol, on asthmatics. Lancet 21;2 (7369), 1101–1102.

Millan, M.J., Mannoury la Cour, C., Novi, F., Maggio, R., Audinot, V., Newman-Tancredi, A., Cussac, D., Pasteau, V., Boutin, J.A., Dubuffet, T., Lavielle, G.M., 2008. S33138 [N-[4-[2-[(3aS,9bR)-8-cyano-1,3a,4,9b-tetrahydro[1]-benzopyrano[3,4-c] pyrrol-2(3H)-yl) ethyl]phenylacetamide], a preferential dopamine D3 versus D2 receptor antagonist and potential antipsychotic agent: I. Receptor-binding profile and functional actions at G-protein-coupled receptors. J. Pharmacol. Exp. Ther. 324 (2), 587–599.

Miranda, H.F., Wolstenholme, W.W., Moreu, G.M., Cuevas, N., Bustamante, D., 1990. Evidence that dopamine is acting at alpha-adrenoceptors of the rat vas deferens. J. Recept. Res. 10 (5–6), 317–332.

Missale, C., Nash, S.R., Robinson, S.W., Jaber, M., Caron, M.G., 1998. Dopamine receptors: from structure to function. Physiol. Rev. 78 (1), 189–225.

Muramatsu, I., Ohmura, T., Kigoshi, S., Hashimoto, S., Oshita, M., 1990. Pharmacological subclassification of alpha 1-adrenoceptors in vascular smooth muscle. Br. J. Pharmacol. 99 (1), 197–201.

Ojeda, S.R., Jameson, H.E., McCann, S.M., 1976. Further studies on prostaglandin E2 (PGE2)-induced gonadotropin release: comparison with the effect of 15-methyl PGE2, PGAs, PGBs and prostaglandin endoperoxide analogs. Prostaglandins 12 (2), 281–301.

Palumbo, A., Napolitano, A., Barone, P., d'Ischia, M., 1999. Nitrite- and peroxide-dependent oxidation pathways of dopamine: 6-nitrodopamine and 6-hydroxydopamine formation as potential contributory mechanisms of oxidative stress- and nitric oxide-induced neurotoxicity in neuronal degeneration. Chem. Res. Toxicol. 12 (12), 1213–1222.

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W. J., Clark, A., Cuthill, I.C., Dirnagl, U., Hurst, V., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W.J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Karp, N.A., Lazic, S.E., Lidster, K., CJ, MacCallum, Macleod, M., Pearl, E.J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E. S., Silberberg, S.D., Steckler, T., Würbel, H., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E.J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E.S., Silberberg, S.D., Steckler, T., Würbel, H., 2020. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. PLoS Biol. 18 (7), e3000410, 14.

Schmidt, A.W., Lebel, L.A., Howard, H.R., Zorn, S.H., 2001. Ziprasidone: a novel antipsychotic agent with a unique human receptor binding profile. Eur. J. Pharmacol. 425 (3), 197–201, 17.

Scivoletto, R., Toledo, A.J., Gomes da Silva, A.C., Nigro, D., 1976. Mechanism of the hypotensive effect of prazosin. Arch. Int. Pharmacodyn. Ther. 223 (2), 333–338.

Shintani, F., Inoshita, T., Kanba, S., Ishikawa, T., Suzuki, E., Sasakawa, N., Kato, R., Asai, M., Nakaki, T., 1996. Bioactive 6-nitronorepinephrine identified in mammalian brain. J. Biol. Chem. 271 (23), 13561–13565, 7.

Simon, A., Van Maanen, E.F., 1976. Dopamine receptors and dopaminergic nerves in the vas deferens of the rat. Arch. Int. Pharmacodyn. Ther. 222 (1), 4–15.

Thornburg, J.E., Moore, K.E., 1975. Supersensitivity to dopaminergic agonists induced by haloperidol. Natl. Inst. Drug Abuse Res. Monogr. Ser. 3, 23–28.

Timmermans, P.B., Van Zwieten, P.A., 1981. Mini-review. The postsynaptic alpha 2-adrenoreceptor. J. Auton. Pharmacol. 1 (2), 171–183.

Tsunoda, M., Uchino, E., Imai, K., Hayakawa, K., Funatsu, T., 2007. Determination of nitrocatecholamines in rat brain using high-performance liquid chromatography-peroxyoxalate chemiluminescence reaction detection. J. Chromatogr. A 14;1164 (1–2), 162–166.

Ximenes, L.E., Britto-Júnior, J., Silva-Filho, W., Jacintho, F., Cuppoloni, M., Fregonesi, A., De Nucci, G., 2021. PD62-12 6-nitrodopamine is released by rat isolated vas deferens and modulates its contractility. J. Urol. 206 (Supplement 3) e1077-e1077.

Anexo 10

Artigo 12 – 6-nitrodopamine is a major endogenous modulator of human vas deferens contractility.

Autores: Britto-Júnior J, da Silva-Filho WP, Amorim AC, Campos R, Moraes MO, Moraes MEA, Fregonesi A, Monica FZ, Antunes E, De Nucci G

Revista: Andrology

Situação: Aceito a publicação em 30 de julho de 2022. Publicado on-line em 04 de agosto de 2022

Received: 17 June 2022 | Revised: 30 July 2022 | Accepted: 4 August 2022

DOI: 10.1111/andr.13263

ANDROLOGY WILEY

ORIGINAL ARTICLE

6-nitrodopamine is a major endogenous modulator of human vas deferens contractility

José Britto-Júnior[1] | Walter Pinto da Silva-Filho[1] | Amanda Consulin Amorim[1] | Rafael Campos[2,3] | Manoel Odorico Moraes[2] | Maria Elisabete A. Moraes[2] | Adriano Fregonesi[1] | Fabiola Z. Monica[1] | Edson Antunes[1] | Gilberto De Nucci[1,2,4,5]

[1]Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas, Campinas, São Paulo, Brazil

[2]Clinical Pharmacology Unit, Drug Research and Development Center, Federal University of Ceará, Fortaleza, Ceará, Brazil

[3]Superior Institute of Biomedical Sciences, Ceará State University, Fortaleza, Ceará, Brazil

[4]Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, São Paulo, Brazil

[5]Deparment of Pharmacology, Faculty of Medicine, Metropolitan University of Santos, Santos, São Paulo, Brazil

Correspondence
José Britto-Júnior, Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas, 126 Tessália Vieira de Camargo St, 13083–887 – Campinas, Sao Paulo, Brazil.
Email: j229793@dac.unicamp.br

Funding information
Coordination for the Improvement of Higher Education Personnel (CAPES), Grant/Award Numbers: 001, 23067.050073/2018-19; Brazilian Ministry of Health, Grant/Award Number: 23067.050073/2018-19; Sao Paulo Research Foundation (FAPESP), Grant/Award Numbers: 2017/15175-1, 2019/16805-4; National Council for Scientific and Technological Development (CNPq), Grant/Award Number: 303839/2019-8

Abstract

Background: Rat isolated *vas deferens* releases 6-nitrodopamine (6-ND), and the spasmogenic activity of this novel catecholamine is significantly reduced by tricyclic compounds such as amitriptyline, desipramine, and carbamazepine and by antagonists of the α_1-adrenergic receptors such as doxazosin, tamsulosin, and prazosin.

Objectives: To investigate the liberation of 6-ND by human epididymal vas deferens (HEVDs) and its pharmacological actions.

Methods: The in vitro liberation of 6-ND, dopamine, noradrenaline, and adrenaline from human vas deferens was evaluated by LC-MS/MS. The contractile effect of the catecholamines in HEVDs was investigated in vitro. The action of tricyclic antidepressants was evaluated on the spasmogenic activity ellicited by the catecholamines and by the electric-field stimulation (EFS). The tissue was also incubated with the inhibitor of nitric oxide (NO) synthase L-NAME and the release of catecholamines and the contractile response to EFS were assessed.

Results: 6-ND is the major catecholamine released from human vas deferens and its synthesis/release is inhibited by NO inhibition. The spasmogenic activity elicited by EFS in the human vas deferens was blocked by tricyclic antidepressants only at concentrations that selectively antagonize 6-ND induced contractions of the human vas deferens, without affecting the spasmogenic activity induced by dopamine, noradrenaline, and adrenaline in this tissue. Incubation of the vas deferens with L-NAME reduced both the 6-ND release and the contractions induced by EFS.

Discussion and conclusion: 6-ND should be considered a major endogenous modulator of human vas deferens contractility and possibly plays a pivotal role in the emission process of ejaculation. It offers a novel and shared mechanism of action for tricyclic antidepressants and α_1-adrenergic receptor antagonists.

KEYWORDS
ejaculation, nitric oxide, tricyclic antidepressants

© 2022 American Society of Andrology and European Academy of Andrology.

1 | INTRODUCTION

Emission occurs following a sympathetic spinal cord reflex (T10-L2) stimuli following the activation of sensory receptors located in the glans (Krause-Finger corpuscles). The physiological process of the male ejaculation initiates with the increase of the contractile activity of the duct that extends from the rete testis to the epididymis (*vasa efferentia*).[1] Contractions of the vas deferens also have an important role in the propulsion of the spermatozoa and occur near-simultaneously with the contractions of the seminal glands.[2] 6-nitrodopamine (6-ND) is a new catecholamine that has been recently shown to be released from rat isolated vas deferens[3] and atria[4] and from human umbilical cord vessels.[5] The synthesis/release of 6-nitrodopamine is reduced (but not abolished) when these tissues are incubated with the nitric oxide (NO) synthase inhibitor L-NAME. 6-nitrodopamine can ben synthesized in vitro by incubation dopamine with nitric acid,[6] which generates NO or peroxynitrite.[6] Thus, nitrosation of dopamine is apparently an essential step in the formation of 6-nitrodopamine. Whether this process in vivo is a chemical, or enzymatic process is yet to be determined. Since 6-nitrodopamine is a potent vasodilator,[5,7] the formation of this novel catecholamine rather than stimulation of soluble guanlylate cyclase[8] may be an important mechanism of action for NO. The contractions caused by 6-ND in the rat isolated vas deferens are selectively antagonized by tricyclic antidepressants such as amitriptyline, desipramine and clomipramine[3] and by α_1-adrenergic receptor blockers such as doxazosin, tamsulosin and silodosin.[9]

The clinical use of tricyclic antidepressants has been associated with delayed ejaculation,[10] and the tricyclic antidepressant clomipramine has therapeutic indication in the control of premature ejaculation in man.[11–13] Lower urinary tract symptoms (LUTSs) are generally treated with α_1-adrenergic receptor blockers, but reduced ejaculate volume, absence of ejaculate, and retrograde ejaculation are observed in 4%–11% of patients.[14–15]

Since both tricyclic antidepressants and α_1-adrenergic receptor antagonists are potent and selective 6-ND receptor antagonists in the rat vas deferens, these findings open the exciting possibility that these drugs could have similar mechanism in the human vas deferens. Thus, we have investigated whether 6-ND is released by human vas deferens and its actions in this tissue.

2 | MATERIALS AND METHODS

2.1 | Study participants

Participants who underwent vasectomy surgery from Hospital e Maternidade Salvalus were asked to sign an informed consent approved by the University of Sao Paulo Institutional Review Board (protocol number 4.468.508). The human epididymal vas deferens (HEVDs) were obtained from 96 participants aged 28–53 years. The vasectomy[16] was performed under local anaesthesia, and the excised segment (2-cm long, taken approximately at 9 cm from the cauda epididymis) is illustrated in Figure 1.

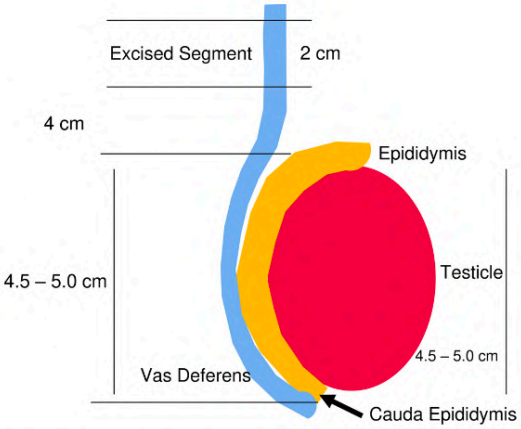


FIGURE 1 The vasectomy illustration

2.2 | Catecholamine basal release

Two HEVD strips (15 mm length) were suspended in a 5-ml glass chamber containing warmed (37°C) and oxygenated (95% O_2 / 5% CO_2) Krebs-Henseleit's solution (KHS) containing ascorbic acid (3 mM) to prevent catecholamine oxidation.[17] The NO synthase inhibitor L-NAME (100 μM) was incubated for 30 min. Samples (2 ml) of the KHS were transferred to amber Eppendorf vials and frozen at −20°C until analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

2.3 | Determination of catecholamine concentrations by mass spectrometry

The method employed for 6-ND quantification[18] was modified to allow the measurement of the four catecholamines in a single chromatographic run. Briefly, the extraction of the catecholamines from KHS (1 ml) was performed by solid phase extraction. To 1 ml of KHS was added 50 μl (100 ng/ml) of the deuterated catecholamines used as internal standards, and the samples were homogenized for 10 s. The Strata-X 33 mm Polymeric Reversed Solid Phase Extraction (SPE) cartridges were prewashed with MeOH (1 ml) followed by deionized H_2O (2 ml). After sample introduction into the cartridge, the cartridge was subsequently washed three times with deionized H_2O. The catecholamines were then eluted with 900 μl MeOH/H_2O (90/10, v/v) with formic acid (0.1%). The eluate was evaporated under N_2 flow at 50°C. The residue was dissolved with 100 μl of acetonitrile/H_2O (50/50, v/v) with 0.1% formic acid and transferred to vials ready for injection into the mobile phase (75% A composed of deionized H_2O with 0.1% formic acid (v/v) and 25% B composed of acetonitrile/H_2O (90/10, v/v) with 0.1% formic acid. The mobile phase perfused an LC ADVp

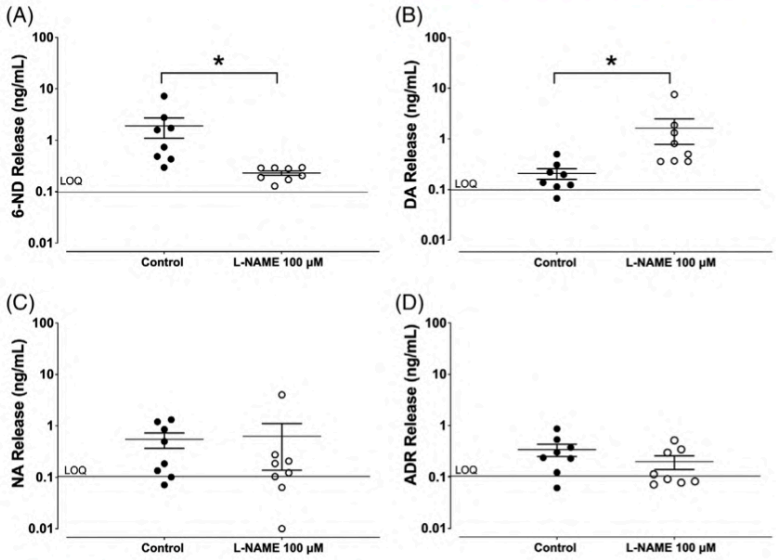


FIGURE 2 Basal release of 6-nitrodopamine (6-ND) (A), dopamine (B), noradrenaline (C), and adrenaline (D) were detected in the human epididymal vas deferens (HEVD). The basal release was measured by LC-MS/MS following a 30 min period incubation in Krebs-Henseleit's solution in the absence and the presence of L-NAME (100 μM). *$p < 0.05$ control versus L-NAME

Liquid Chromatograph Shimadzu System coupled to a Shimadzu 8060 triple quadrupole mass spectrometer operating in ESP^+ mode at 350 μl/min. The dissolved residues were injected by a SIL-30AC autoinjector, at a temperature of 8°C. The transitions monitored by electrospray multiple reaction monitoring, injection volume, run-time, and limit of quantitation were described elsewhere.[18] The linearity was given in a range of 0.1–20 ng/ml, and the method validation was carried following the Food and Drug Adminisration (FDA) guidelines for bioanalytical methods.[19]

2.4 | HEVD functional assays

Each HEVD strip was suspended vertically between metal hooks in 10-ml glass chamber containing heated (37°C) and oxygenated (95%O_2: 5%CO_2) KHS. Tissues were maintained at 10 mN, and the isometric tension was recorded by a PowerLab system. After a stabilization interval (45-min), the HEVD strips were initially contracted with potassium chloride (KCl, 80 mM) to assess the tissue viability. After KCl removal and return to the baseline (15 min approximately), cumulative concentration-response curves to 6-ND were performed in control HEVD strips and in tissues preincubated with amitriptyline (30-300 nM, 30 min), desipramine (30-300 nM, 30 min) and/or carbamazepine (30–300 nM, 30 min).

Cumulative concentration-response curves to dopamine, noradrenaline, and adrenaline were performed in HEVD pre-incubated or not with amitriptyline (100 nM, 30 min), desipramine (100 nM, 30 min), and carbamazepine (100 nM, 30 min).

2.5 | Electric-field stimulation in HEVD preparations

The HEVD strips were submitted to electric-field stimulation (EFS) (60 V for 20 sec, at 2–32 Hz in square-wave pulses, 0.3 ms pulse width, and 0.1 ms delay), using a Grass S88 stimulator. The EFS-induced HEVD contractions were conducted in tissues control and tissues preincubated with amitriptyline (100 nM, 30 min), desipramine (100 nM, 30 min), and carbamazepine (100 nM, 30 min).

2.6 | Data analysis

Nonlinear regression analysis to determine the pEC_{50} was carried out using GraphPad Prism (GraphPad Software, version 9.0, San Diego, California, USA) with the constraint that F = 0. All concentration–response data were evaluated for a fit to a logistics function in the form: $E = E_{max}/([1 + (10c / 10x)^n] + F$, where E represents the increase in response contractile induced by the agonist, E_{max} is the effect agonist maximum, c is the logarithm of concentration of the agonist that produces 50% of E_{max}, x is the logarithm of the concentration of the drug; the exponential term, n, is a curve fitting parameter that defines the slope of the concentration–response line, and F is the response observed in the absence of added drug. The values of pEC_{50} data represent the mean ± standard error of the mean of *n* experiments. Values of E_{max} were expressed in mN. One strip was used as the control response, and the other strip was incubated with an antagonist/inhibitor. Student's two-tail unpaired *t*-test was employed and

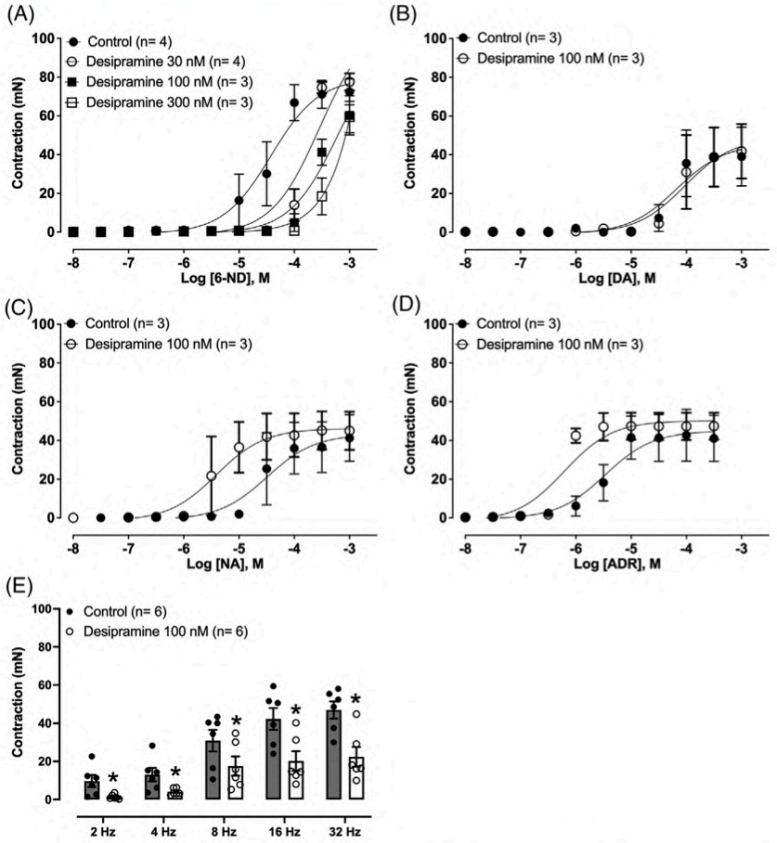


FIGURE 3 Effect of desipramine in the human epididymal vas deferens (HEVD). Desipramine (30-300 nM) causes a significant concentration-dependent rightward shifts on the concentration-response curves to 6-nitrodopamine (6-ND) (A). Desipramine (100 nM) had no effect on the contractions induced by dopamine (B) but caused a significant leftward shift on the concentration-response curves to noradrenaline (C) and adrenaline (D). Electric-field stimulation (EFS) caused frequency-dependent contractions of the HEVD (E). Desipramine (100 nM) caused a significant reduction in the contractions induced by EFS in HEVD (E). *$p < 0.05$. *n* means the number of the HEVD.

the differences between groups. In addition, standard analysis of variance (ANOVA), followed by the Newman–Keuls post-test, were used when more than two groups were involved. A *p* value of less than 0.05 was considered statistically significant. For 6-ND, the pA_2 values of the antagonists were calculated from the intercept on the concentration axis and by application of the equation; pA_2 = log (antagonist concentration) −log (CR-1) -log (antagonist concentration).[20]

3 | RESULTS

3.1 | Basal release of catecholamines

6-nitrodopamine (Figure 2A), dopamine (Figure 2B), noradrenaline (Figure 2C), and adrenaline (Figure 2D) were detected by LC-MS/MS in HEVD strips. Incubation (30 min) of the HEVD strips with L-NAME (100 μM) significantly reduced the 6-ND levels (1.91 ± 0.82 and 0.23 ± 0.03 ng/ml, for control and L-NAME respectively; Figure 2A), and an equivalent increase in basal dopamine release was observed (0.20 ± 0.05 and 1.63 ± 0.86 ng/ml, for control and L-NAME, respectively; Figure 2B). Incubation (30 min) of the HEVD strips with L-NAME (100 μM) had no effect in the levels of noradrenaline (Figure 2C) and adrenaline (Figure 2D).

3.2 | Effect of desipramine

Pretreatment (30 min) with desipramine (30-300 nM) caused significant rightward shifts on the concentration-response curves to 6-ND (pEC_{50} 4.48 ± 0.21; Figure 3A; $p = 0.0015$) with a pA_2 value of 9.46 ± 0.07($n = 4$). Desipramine (100 nM) did not affect the HEVD contractions induced by dopamine (Figure 3B; pEC_{50} 4.18 ± 0.16

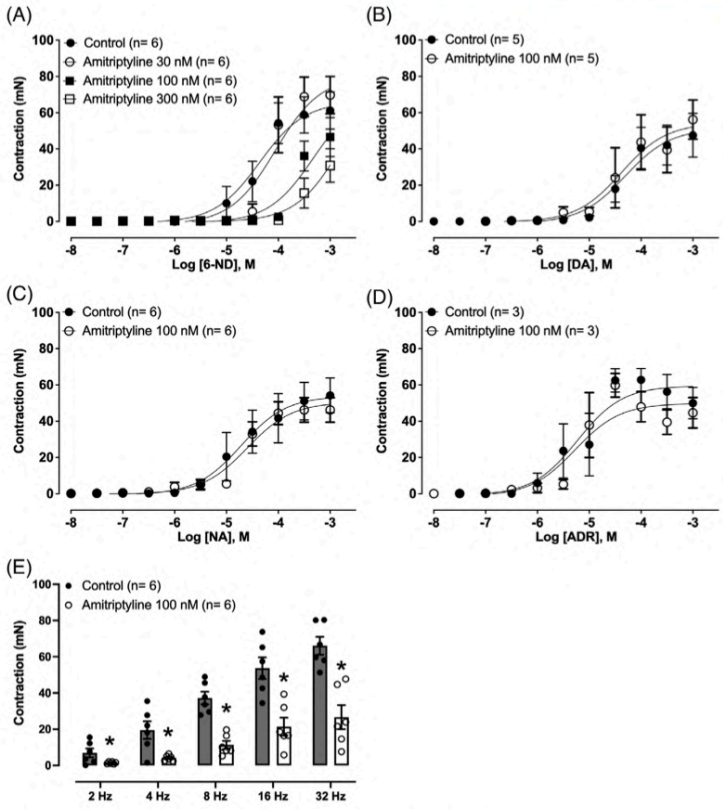


FIGURE 4 Effect of amitriptyline in the human epididymal vas deferens (HEVD). Amitriptyline (100 nM) produced concentration-dependent rightward shifts on the concentrationresponse curves to 6-ND (A) and at 300 nM, amitriptyline markedly reduced the maximal responses to 6-nitrodopamine (6-ND). Amitriptyline (100 nM) had no effect on the contractions induced by dopamine, noradrenaline (C), and adrenaline (D). Electric-field stimulation (EFS) caused frequency-dependent contractions of the HEVD (E). Amitriptyline (100 nM) caused a significant reduction in the contractions induced by EFS in HEVD (E). $^*p < 0.05$. *n* means the number of the HEVD.

and 4.03 ± 0.16, for control and treated; respectively; $p = 0.4795$) but caused significant leftward shifts on the concentration-response curves to noradrenaline (Figure 3C; pEC_{50} 4.49 ± 0.16 and 5.41 ± 0.15, for control and treated, respectively; $p = 0.014$) and adrenaline (Figure 3D; pEC_{50} 5.47 ± 0.13 and 6.21 ± 0.08, for control and treated, respectively; $p = 0.0095$).

Desipramine (100 nM) significantly antagonized the HEVD contractions induced by EFS (2–32 Hz; Figure 3E).

3.3 | Effect of amitriptyline

Pretreatment (30 min) with amitriptyline (100 and 100 nM) caused significant rightward shifts on the concentration-response curves to 6-ND (pEC_{50} 4.33 ± 0.14; Figure 4A; $p = 0.0031$) with a pA_2 value of 8.86 ± 0.09($n = 6$). However, at 300 nM, amitriptyline markedly reduced the maximal responses to 6-ND (Emax; 61.17 ± 7.54 and 30.87 ± 12.60 mN, for control and amitriptyline 300 nM, respectively; $p = 0.0026$), making the pEC_{50} calculation inaccurate. Amitriptyline (100 nM) did not alter the HEVD contractions induced by dopamine (Figure 4B; pEC_{50} 4.31 ± 0.08 and 4.38 ± 0.11, for control and treated; $p = 0.6081$), noradrenaline (Figure 4C; pEC_{50} 4.72 ± 0.09 and 4.60 ± 0.05, for control and treated; $p = 0.2925$) and adrenaline (Figure 4D; pEC_{50} 5.23 ± 0.11 and 5.27 ± 0.12, for control and treated; $p = 0.7911$).

Amitriptyline (100 nM) significantly reduced the HEVD contractions induced by EFS (2–32 Hz; Figure 4E).

3.4 | Effect of carbamazepine

Pretreatment (30 min) with carbamazepine (100 nM) caused significant rightward shifts on the concentration-response curves to 6-ND (pEC_{50} 4.36 ± 0.09; Figure 5A; $p = 0.0148$) with a pA_2 value of

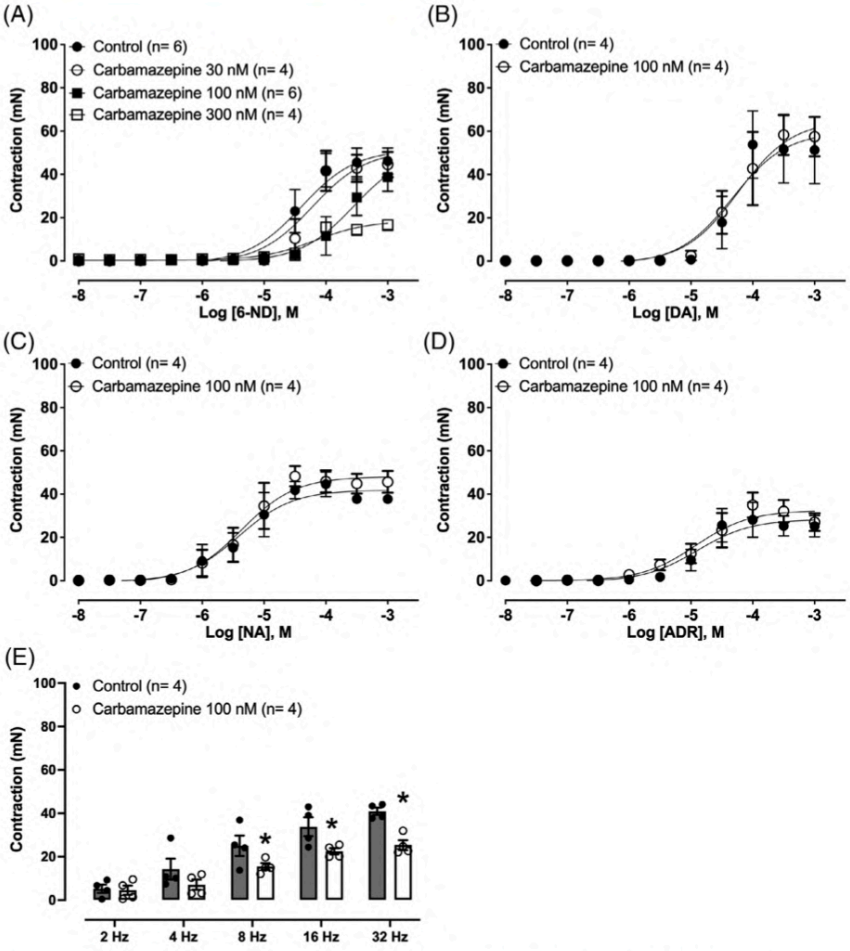


FIGURE 5 Effect of carbamazepine in the human epididymal vas deferens (HEVD). Carbamazepine (100 nM) produced concentration-dependent rightward shifts on the concentration-response curves to 6-nitrodopamine (6-ND) (A) and at 300 nM, carbamazepine markedly reduced the maximal responses to 6-ND. Carbamazepine (100 nM) had no effect on the contractions induced by dopamine, noradrenaline (C), and adrenaline (D). Electric-field stimulation (EFS) caused frequency-dependent contractions of the HEVD (E). Carbamazepine (100 nM) caused a significant reduction in the contractions induced by EFS in HEVD (E). *$p < 0.05$. *n* means the number of the HEVD.

8.77 ± 0.067(n = 6). However, at 300 nM, carbamazepine markedly reduced the maximal responses to 6-ND (Emax; 46.1 ± 6.1 and 16.7 ± 2.72 mN, for control and carbamazepine 300 nM, respectively; $p = 0.0028$), making the pEC_{50} calculation inaccurate. Carbamazepine (100 nM) did not affect the HEVD contractions induced by dopamine (Figure 5B; pEC_{50} 4.33 ± 0.10 and 4.23 ± 0.08, for control and treated; $p = 0.4883$), noradrenaline (Figure 5C; pEC_{50} 5.41 ± 0.08 and 5.36 ± 0.07, for control and treated; $p = 0.6912$) and adrenaline (Figure 5D; pEC_{50} 4.90 ± 0.09 and 4.92 ± 0.08, for control and treated; $p = 0.8309$).

Carbamazepine (100 nM) significantly reduced the HEVD contractions induced by EFS (2–32 Hz; Figure 5E).

3.5 | Effect of L-NAME

EFS caused frequency-dependent contractions of the HEVDs. Pretreatment (30 min) with L-NAME (100 μM) significantly inhibited the contractions of the HEVD induced by EFS (2–32 Hz; Figure 6).

4 | DISCUSSION

This is the first demonstration that 6-ND is the major catecholamine released from human vas deferens. The release of 6-ND was reduced by pretreatment of the vas deferens with L-NAME, indicating that the

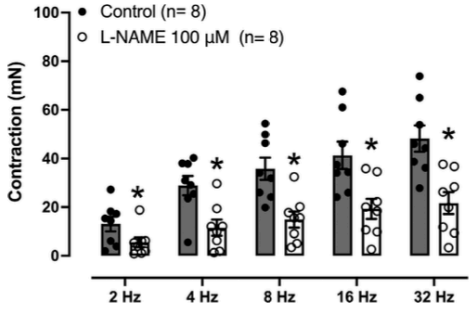


FIGURE 6 Electric-field stimulation (EFS) caused frequency-dependent contractions of the human epididymal vas deferens (HEVD). The pretreatment with L-NAME (100 μM) caused a significant reduction in the contractions induced by EFS in HEVD. $^*p < 0.05$. *n* means the number of the HEVD.

synthesis/release of 6-ND is coupled to NO synthesis. Pretreatment of human umbilical cord vessels[5] and rat vas deferens[3] with L-NAME is accompanied by decrease of 6-ND synthesis/release. Chronic treatment of rats with L-NAME also decreases the 6-ND release by vas deferens.[3] However, the effect of L-NAME pretreatment in the human vas deferens has two distinct characteristics of the rat, namely, in the rat vas deferens, although incubation with L-NAME decreased the basal release of 6-ND, it did not affect the contractions induced by EFS, an indication that 6-ND is probably stocked in vesicles in the animal species. Another interesting difference is that in contrast to the rat vas deferens, inhibition of 6-ND in human vas deferens is accompanied by an equivalent increase of dopamine levels. This difference cannot be attributed to species differences (man and rat), since the inhibition of 6-ND synthesis/release by L-NAME in human cord vessels was not accompanied by increase in dopamine levels. Thus, EFS in human vas deferens must stimulate 6-ND synthesis. The finding that L-NAME pretreatment does not abolish 6-ND synthesis/release could be due to a synthetic pathway independent of NO synthase activation. For instance, 6-ND and 6-hydroxydopamine can be formed by nitrite- and peroxide-dependent oxidation pathways of dopamine.[6] Another possibility is the existence of another enzymatic pathway for the synthesis of 6-ND independent of NO synthase.

Tricyclic antidepressants increase both serotonin and noradrenaline levels in the synaptic cleft[21,22] due to inhibition of the reuptake of these monoamines following the binding of the tricyclic antidepressants to serotonin reuptake transporter (SERT)[23] and norepinephrine transporter (NET).[24] Based on this proposed mechanism of action, it is surprising that the use of tricyclic antidepressants is associated with delayed ejaculation, since the increase in noradrenaline levels should result in increased contractility of the vas deferens. Amitriptyline, nortriptyline and imipramine bind to α_{1A}-, α_{1B}-, and α_{1D}- adrenergic receptors in HEK-293 cells expressing human α_1- receptor subtypes.[25] They also bind to α_1-adrenergic native receptors in rat vas deferens; however, they were much less potent in the competition for the specific binding of [^{3}H]-prazosin,[25] indicating that this antagonism is unlikely to overcome the increase of noradrenaline resulting from NET inhibition. Amitriptyline (100 nM–10 μM) inhibited neurogenic induced contractions in human vas deferens, although at 10 μM it also attenuated the contractions elicited by KCl, indicating a nonspecific mechanism at higher concentration.[26] At 100 nM, we did not find inhibition by amitriptyline of noradrenaline-induced contractions of the human vas deferens, indicating that the inhibition of the neurogenic induced contractions was not due to blockade of the α_1-adrenergic receptors in this tissue.

As observed in the rat vas deferens[3] dopamine and 6-ND have similar pEC_{50} values in the human vas deferens (4.28 $\pm$ 0.12 and 4.11 $\pm$ 0.10 for dopamine and 6-ND, respectively). Yet, L-NAME pretreatment caused a significant inhibition of EFS-induced contractions, although the inhibition of 6-ND synthesis/release was accompanied by a similar increase in dopamine synthesis/release. Since 6-ND and dopamine cause vas deferens contraction by acting in different receptors, one possible explanation for this apparent paradox could be a distinct location of these receptors. Purification and sequence of the 6-ND receptor should provide further information on the interactions of this novel mediator with the classical catecholamines in human vas deferens.

EFS causes NO release in human corpus cavernosum.[27] Since NO promotes smooth muscle relaxation,[28] the finding that L-NAME inhibits EFS-induced HEVD contractions demonstrates that endogenously produced 6-ND, and not NO, acts as a direct mediator of HEVD contractility.

5 | CONCLUSION

The identification of 6-ND as a novel mediator of HEVD contractility provides a new insight on the physiological process of the male ejaculation and presents a promising therapeutic target for ejaculatory disorders.

ACKNOWLEDGMENTS

This study was supported by coordination for the Improvement of Higher Education Personnel (CAPES) grants 001 (JBJ and ACA), 23067.050073/2018-19 (RC); Brazilian Ministry of Health grant 23067.050073/2018-19; Sao Paulo Research Foundation (FAPESP); 2017/15175-1 (EA), 2019/16805-4 (GDN); National Council for Scientific and Technological Development (CNPq) grant 303839/2019-8 (GDN).

FUNDING INFORMATION

Coordination for the Improvement of Higher Education Personnel (CAPES), Grant Numbers: 001 and 23067.050073/2018-19; Brazilian Ministry of Health, Grant Number: 23067.050073/2018-19; Sao Paulo Research Foundation (FAPESP), Grant Number: 2017/15175-1 and 2019/16805-4; National Council for Scientific and Technological Development (CNPq), Grant Number: 303839/2019-8

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Conceptualization, data curation, investigation, methodology, writing – original draft, writing – review and editing: JBJ. *Investigation and methodology*: WPSF, ACA. RC, MOM, and MEAM. *Methodology*: AF. *Methodology and supervision*: FZM. *Funding acquisition, methodology, supervision, visualization, writing – original draft, writing – review and editing*: EA. *Conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, writing – review and editing, investigation, visualization, writing – original draft*: GDN.

ORCID

José Britto-Júnior https://orcid.org/0000-0003-0250-8468

REFERENCES

1. Motofei IG, Rowland DL. Neurophysiology of the ejaculatory process: developing perspectives. *BJU Int*. 2005;96(9):1333-1338.
2. Puppo V, Puppo G. Comprehensive review of the anatomy and physiology of male ejaculation: premature ejaculation is not a disease. *Clin Anat*. 2016;29(1):111-119.
3. Britto-Júnior J, Ximenes L, Ribeiro A, et al. 6-nitrodopamine is an endogenous mediator of rat isolated epididymal vas deferens contractions induced by electric-field stimulation. *Eur J Pharmacol*. 2021;15;911:174544.
4. Britto-Júnior J, Oliveira MG, Gati CR et al. NitroDopamine is an endogenous modulator of rat heart chronotropism. *Life Sci*. Forthcoming 2022.
5. Britto-Júnior J, Coelho-Silva WC, Murari GF, et al. 6-nitrodopamine is released by human umbilical cord vessels and modulates vascular reactivity. *Life Sci*. 2021;1:276:119425.
6. Palumbo A, Napolitano A, Barone P, d'Ischia M. Nitrite- and peroxide-dependent oxidation pathways of dopamine: 6-nitrodopamine and 6-hydroxydopamine formation as potential contributory mechanisms of oxidative stress- and nitric oxide-induced neurotoxicity in neuronal degeneration. *Chem Res Toxicol*. 1999;12(12):1213-1222.
7. Britto-Júnior J, Campos R, Peixoto M, et al. 6-nitrodopamine is an endogenous selective dopamine receptor antagonist in Chelonoidis carbonaria aorta. *Comp Biochem Physiol C Toxicol Pharmacol*. 2022;3:109403.
8. Murad F, Waldman S, Molina C, Bennett B, Leitman D. Regulation and role of guanylate cyclase-cyclic GMP in vascular relaxation. *Prog Clin Biol Res*. 1987;249:65-76.
9. Britto-Júnior J, Ribeiro A, Ximenes L, Lima AT, et al. Alpha1-adrenergic antagonists block 6-nitrodopamine contractions on the rat isolated epididymal vas deferens. *Eur J Pharmacol*. 2022;15;915:174716.
10. Rothmore J. Antidepressant-induced sexual dysfunction. *Med J Aust*. 2020;212(7):329-334.
11. Waldinger MD, Zwinderman AH, Olivier B. On-demand treatment of premature ejaculation with clomipramine and paroxetine: a randomized, double- blind fixed-dose study with stopwatch assessment. *Eur Uro*. 2004;46(4):510-515.
12. Waldinger MD. Drug treatment options for premature ejaculation. *Expert Opin Pharmacother*. 201;19(10):1077-1085.
13. Saleh R, Majzoub A, Abu El-Hamd M. An update on the treatment of premature ejaculation: a systematic review. *Arab J Urol*. 2021;19(3):281-302.
14. Hofner K, Claes H, De Reijke TM, et al. Tamsulosin 0.4 mg once daily: effect on sexual function in patients with lower urinary tract symptoms suggestive of benign prostatic obstruction. *Eur Urol*. 1999;36(4):335-341.
15. Djavan B, Chapple C, Milani S, et al. State of the art on the efficacy and tolerability of alpha1-adrenoceptor antagonists in patients with lower urinary tract symptoms suggestive of benign prostatic hyperplasia. *Urology*. 2004;64(6):1081-1088.
16. Rogers MD, Kolettis PN Vasectomy. *Urol Clin North Am*. 2013;40(4):559-568.
17. Van Oene JC, Sminia P, Mulder AH, Horn AS. The purported dopamine agonist DPI inhibits [3H]noradrenaline release from rat cortical slices but not [3H]dopamine and [14C]acetylcholine release from rat striatal slices in-vitro. *J Pharm Pharmacol*. 1983;35(12):786-792.
18. Campos R, Pinheiro DHA, Britto-Júnior J, et al. Quantification of 6-nitrodopamine in Krebs-Henseleit's solution by LC-MS/MS for the assessment of its basal release from Chelonoidis carbonaria aortae in vitro. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2021;22:122668.
19. Meesters R, Voswinkel S. Bioanalytical method development and validation: from the USFDA 2001 to the USFDA 2018 guidance for industry. *J Appl Bioanal*. 2018;4(3):67-73.
20. Arunlakshana O, Schild HO. Some quantitative uses of drug antagonists. *Br J Pharmacol Chemother*. 1959;14(1):48-58.
21. Feighner JP. Mechanism of action of antidepressant medications. *J Clin Psychiatr*. 1999;60(Suppl. 4):4-11
22. López-Munoz F, Alamo C. Monoaminergic neurotransmission: the history of the discovery of antidepressants from 1950s until today. *Curr Pharmaceut*. 2009;15(14):1563-1586.
23. Sarker S, Weissensteiner R, Steiner I, et al. The high-affinity binding site for tricyclic antidepressants resides in the outer vestibule of the serotonin transporter. *Mol Pharmacol*. 2010;78(6):1026-1035.
24. Nijenhuis CM, Horst PG, Berg LT, et al. Disturbed development of the enteric nervous system after in utero exposure of selective serotonin re-uptake inhibitors and tricyclic antidepressants. Part 1: literature review. *Br J Clin Pharmacol*. 2012;73(1):16-26
25. Nojimoto FD, Mueller A, Hebeler-Barbosa F, et al. The tricyclic antidepressants amitriptyline, nortriptyline and imipramine are weak antagonists of human and rat alpha1B-adrenoceptors. *Neuropharmacology*. 2010;59(1-2):49-57.
26. Medina P, Segarra G, Ballester R, et al. Effects of antidepressants in adrenergic neurotransmission of human vas deferens. *Urology*. 2000;55(4):592-597.
27. Teixeira CE, de Oliveira JF, Baracat JS, et al. Nitric oxide release from human corpus cavernosum induced by a purified scorpion toxin. *Urology*. 2004;63(1):184-189.
28. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980;288(5789):373-376.

How to cite this article: Britto-Júnior J, da Silva-Filho WP, Amorim AC, et al. 6-nitrodopamine is a major endogenous modulator of human vas deferens contractility. *Andrology*. 2022;1-8. https://doi.org/10.1111/andr.13263

Anexo 11

Artigo 13 – 6-NitroDopamine is an endogenous modulator of rat heart chronotropism.

Autores: Britto-Júnior J, de Oliveira MG, Dos Reis Gati C, Campos R, Moraes MO, Moraes MEA, Mónica FZ, Antunes E, De Nucci G.

Revista: Life Sciences

Situação: Aceito a publicação em 08 de agosto. Publicado on-line em 10 de agosto de 2022

Life Sciences 307 (2022) 120879

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

6-NitroDopamine is an endogenous modulator of rat heart chronotropism

José Britto-Júnior [a], Mariana Gonçalves de Oliveira [a], Carolina dos Reis Gati [a], Rafael Campos [b,c], Manoel Odorico Moraes [c], Maria Elisabete A. Moraes [c], Fabíola Z. Mónica [a], Edson Antunes [a], Gilberto De Nucci [a,c,d,e,*]

[a] Faculty of Medical Sciences, Department of Pharmacology, University of Campinas (UNICAMP), Campinas, Brazil
[b] Superior Institute of Biomedical Sciences, Ceará State University (UECE), Fortaleza, Brazil
[c] Clinical Pharmacology Unit, Drug Research and Development Center, Federal University of Ceará (UFC), Fortaleza, CE, Brazil
[d] Department of Pharmacology, Institute of Biomedical Sciences, USP – University of São Paulo, Brazil
[e] Metropolitan University of Santos (UNIMES), Santos, Brazil

ARTICLE INFO

Keywords:
β_1-Adrenoceptor antagonist
Nitric oxide
Tetrodotoxin
Atropine
ODQ

ABSTRACT

6-Nitrodopamine (6-ND) is released by rat vas deferens and exerts a potent contractile response that is antagonized by tricyclic antidepressants and α_1-, β_1- and β_1/β_2-adrenoceptor antagonists. The release of 6-ND, noradrenaline, adrenaline and dopamine from rat isolated right atria was assessed by tandem mass spectrometry. The effects of the catecholamines were evaluated in both rat isolated right atria and in anaesthetized rats. 6-ND was the major catecholamine released from the isolated atria and the release was significantly reduced in nitric oxide synthase inhibitor L-NAME pre-treated atria or in atria obtained from L-NAME chronically treated animals, but unaffected by tetrodotoxin. 6-ND (1 pM) significantly increased the atrial frequency, being 100 times more potent than noradrenaline and adrenaline. Selective β_1-blockers reduced the atrial frequency only at concentrations that prevented the increases in atrial frequency induced by 6-ND 1pM. Conversely, β_1-blockade did not affect dopamine (10 nM), noradrenaline (100 pM) or adrenaline (100 pM) effect. The reductions in atrial frequency induced by the β_1-adrenoceptor antagonists were absent in L-NAME pre-treated atria and in atria obtained from chronic L-NAME-treated animals. Tetrodotoxin did not prevent the reduction in atrial frequency induced by L-NAME or by β_1-blockers treated preparations. In anaesthetized rats, at 1 pmol/kg, only 6-ND caused a significant increase in heart rate. Inhibition of 6-ND synthesis by chronic L-NAME treatment reduced both atrial frequency and heart rate. The results indicate that 6-ND is a major modulator of rat heart chronotropism and the reduction in heart rate caused by β_1-blockers are due to selective blockade of 6-ND receptor.

1. Introduction

The nitrocatecholamines nitronoradrenaline and nitroadrenaline have been extracted from the rat brain [1] and release of 6-nitroadrenaline was observed in microdialysates of the rat spinal cord dorsal horn [2]. Basal release of 6-nitrodopamine (6-ND) was detected in human umbilical cord vessels (HUCV) [3], *Chelonoidis carbonaria* aortic rings [4], as well as rat [5] and human [6] vas deferens. In HUCV, 6-ND is released by the endothelium and acts as a selective antagonist of the dopamine D_2-like receptors [3]. In vas deferens, 6-ND is released from nerve terminals and exerts contractile activity, which is selectively blocked by tricyclic compounds such as tricyclic antidepressants, carbamazepine [5,6] and by α_1-adrenoceptor antagonists [7].

Interestingly, in the rat vas deferens, the β_1-adrenoceptor (β_1-AR) antagonists atenolol, betaxolol and metoprolol, at concentrations that selectively block the contractile activity of 6-ND, also significantly reduced the contractions induced by electric-field stimulation [8]. These results indicate that β_1-AR blockers can also act as selective antagonists of the 6-ND receptor [8].

Catecholamines are the most used vasoactive agents in the intensive care, and among them noradrenaline is the first-line therapy in most clinical conditions [9]. Intravenous administration of either noradrenaline or adrenaline causes dose-dependent increases in blood pressure [10] due to vasoconstriction and β-adrenoceptor stimulation [11]. β_1-Adrenoceptors predominate in the heart, accounting for approximately 80 % of the cardiac β-adrenoceptors [12–14]. β1-Adrenoceptor

* Corresponding author at: University of Campinas (Unicamp), Faculty of Medical Sciences, Department of Pharmacology, Rua Tessália Vieira de Camargo, 126 - Cidade Universitária, 13083-887 Campinas, São Paulo, Brazil.
E-mail address: denucci@unicamp.br (G. De Nucci).

https://doi.org/10.1016/j.lfs.2022.120879
Received 27 June 2022; Received in revised form 27 July 2022; Accepted 8 August 2022
Available online 10 August 2022
0024-3205/© 2022 Elsevier Inc. All rights reserved.

activation is the primary responsible for increasing heart rate and ventricular contractility (positive chronotropic and inotropic effect) and the current dogma is that its activation is caused by the release of noradrenaline by heart adrenergic nerve terminals [12–14]. Since β_1-AR antagonists block the action of 6-ND in the vas deferens, we have investigated whether 6-ND is released by the heart and whether it had any effect in the rat isolated atria and in the anaesthetized rat. The effects of β_1-AR antagonists on 6-ND action on isolated atria pre-treated with L-NAME or obtained from animals chronically treated with L-NAME were also evaluated.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (280 to 320 g) were provided by the Central Animal House of the University of Campinas (CEMIB-UNICAMP; São Paulo, Brazil) and Animais de Laboratório Criação e Com. (ANILAB; São Paulo, Brazil). All experimental protocols were approved by the Ethics Committee for Animal Use of the UNICAMP (CEUA; Protocol No. 5746-1/2021; 5831-1/2021) following the Brazilian Guidelines for the Production, Maintenance and Use of Animals for Teaching or Research from the National Council of Control in Animal Experimentation (CONCEA) [15], as well as by the ARRIVE guidelines [16]. Animals were housed in cages (three per cage) located in ventilated cage shelters with a constant humidity of 55 ± 5 % and temperature of 24 ± 1 °C under a 12-hour light-dark cycle. Animals received filtered water and standard rodent food *ad libitum*.

2.2. Chronic L-NAME treatment

Animals were treated with the nitric oxide (NO) synthase inhibitor L-NAME dissolved in the drinking filtered water at a concentration that resulted in a final dose of approximately 20 mg/rat/day [17]. Control animals received filtered water. Throughout the treatment, the systolic blood pressure (SBP) and heart rate (HR) were monitored using a non-invasive NIBP System (AD Instruments, Sidney, Australia). All the animals were acclimatized to the restrainer and the tail-cuff before the recording of baseline parameters and experimentation. Systolic blood pressure (SBP) and HR measurements were carried out three times a week, on alternate days, for 4 weeks, and expressed as a weekly average.

2.3. Isolation of right atrium from rats

Euthanasia was performed by isoflurane overdose, in which animals were exposed to a concentration >5 % until 1 min after the breathing stops. Exsanguination was performed to confirm euthanasia. After euthanasia, the heart was removed, and the right atrium was isolated. The right atrium was mounted between two metal hooks in 10 mL organ baths containing heated (37 °C), oxygenated (95 % O_2/5 % CO_2) Krebs-Henseleit's solution (KHS) coupled to a PowerLab Acquisition System (AD Instruments, Sidney, Australia). The atria were allowed to equilibrate for 1 h under a resting tension of 10 mN [18].

2.4. Basal release of 6-nitrodopamine, dopamine, noradrenaline and adrenaline from rat right atrium

Isolated right atria from each rat were suspended separately in a 5 mL organ bath containing KHS and ascorbic acid (3 mM) continuously gassed with a mixture (95 % O_2/5 % CO_2) at 37 °C for 20 min, in the absence and the presence of either L-NAME (100 μM) or the voltage-gated sodium channel blocker tetrodotoxin (TTX; 1 μM).Two aliquots of 2 mL of the supernatant were transferred to black Eppendorf tubes and stored at −20 °C until analysis [19]. Release of all catecholamines by atria was also evaluated by tandem mass spectrometry (see below) in preparations treated or not with L-NAME (100 μM), TTX (1 μM) or in those obtained from animals chronically treated with L-NAME.

2.5. Determination of catecholamine concentrations in KHS by tandem mass spectrometry (LC-MS/MS)

The 6-ND LC-MS/MS method [4] was modified to allow the measurement of the four catecholamines in a single chromatographic run. Briefly, the extraction of the catecholamines from 1 mL of KHS was performed by solid phase extraction. To 1 mL of KHS was added 50 μL of the internal standards (100 ng/mL of 6-nitrodopamine-d_4, dopamine-d_3, noradrenaline-d_6, and adrenaline-d_6). The samples were homogenized for 10 s. The Strata™-X 33 mm Polymeric Reversed SPE cartridges were pre-conditioned with 1 mL of methanol and then balanced by 2 mL of deionized water. The samples were injected into the cartridge, and the cartridge was subsequently washed 3 times with deionized water. The samples were then eluted with 0.9 mL methanol/water (90/10, *v*/v) with 0.1 % formic acid. The eluate was evaporated under N_2 flow at 50 °C. The residue was dissolved with 100 μL of acetonitrile/water (50/50, v/v) with 0.1 % formic acid and transferred to vials ready for injection. The LC–MS/MS system consisted of LC ADVp Liquid Chromatograph Shimadzu System (Shimadzu Corporation, Kyoto, Japan) coupled to an 8060 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan) operating in electrospray positive-ionization mode. Samples were injected into the system by means of a SIL-30AC autoinjector, at a temperature of 8 °C. The chromatography separation was performed at room temperature using a GIST-HP C_{18} column (150 mm × 3.0 mm, 3 mm) column (Shimadzu, Duisburg, Germany). A 75 % mobile phase A consisting of deionized water with 0.1 % formic acid (*v*/v) and 25 % mobile phase B consisting of acetonitrile/water (90/10, v/v) with 0.1 % formic acid at a flow rate of 0.35 mL/min were used. The electrospray was configured for multiple reaction monitoring (MRM) to monitor the transitions 199.10 > 181.95 for 6-nitrodopamine, 203.10 > 186.00 for 6-nitrodopamine-d_4, 154.00 > 91.15 for dopamine, 157.00 > 93.00 for dopamine-d_3, 170.10 > 107.10 for noradrenaline, 176.10 > 158.10 for noradrenaline-d_6, 184.20 > 107.00 for adrenaline, and 190.00 > 171.95 for adrenaline-d_6. The injection volume was 3 μL of each sample. The total run-time was 3.5 min. The limit of quantitation was 0.1 ng/mL for each catecholamine and linearity was given in a range of 0.1–20 ng/mL with a correlation coefficient of $r = 0.9998$ for 6-nitrodopamine, $r = 0.9995$ for dopamine, $r = 0.9994$ for noradrenaline, and r = 0.9994 for adrenaline. The method validation was carried out according to the United States Food and Drug Administration (FDA) [20] bioanalytical method validation guidelines.

2.6. Atrial rate analysis and experimental design

After the equilibration period, single concentrations of 6-ND (0.1, 1, 10 and 100 pM), dopamine (0.1, 1, 10 and 100 nM), noradrenaline (1, 10, 100 and 1000 pM) or adrenaline (1, 10, 100 and 1000 pM) were added to the organ bath and the changes in atrial rate were monitored for 30 min. In some experiments, after 30-min incubation with the catecholamine, the KHS was changed (to washout the agonist) and the atrial rate was monitored for a further 30 min period.

A single concentration of either L-NAME (100 μM), its inactive isomer D-NAME (100 μM), the soluble guanylate cyclase (sGC) heme site inhibitor ODQ (1H-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one (100 μM), TTX (1 μM) or the non-selective muscarinic receptor antagonist atropine (100 nM) was added to the organ bath and the changes in atrial rate monitored for 30 min. In atria pre-treated with ODQ (100 μM; 30 min), the effect of L-NAME (100 μM) was evaluated for an additional 30 min. In atria pre-treated with TTX (1 μM; 30 min), single concentrations of L-NAME (100 μM), atropine (100 nM) or 6-ND (1 pM) were added to the organ bath and the atrial rate was monitored for 30 min. To atropine-pre-treated atria (100 nM; 30 min), a single concentration of TTX (1 μM) was applied to the organ bath and the atrial rate was then monitored for 30 min.

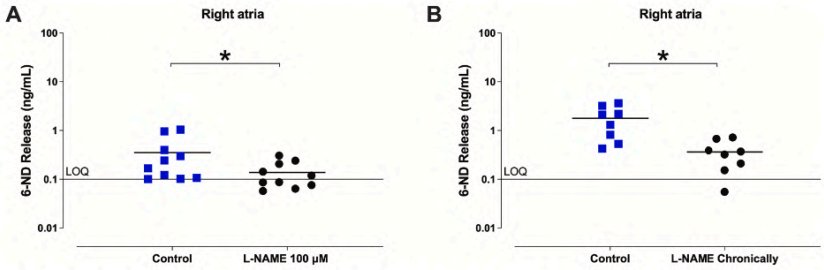


Fig. 1. Basal release of 6-nitrodopamine (6-ND) in isolated right atria. Rat isolated right atria (A and B) were incubated in Krebs-Henseleit's solution. The basal release of 6-ND was measured by LC-MS/MS after a 20 min incubation period. Incubation with L-NAME (100 µM; 20 min) caused a significant reduction of the 6-ND release (A). The basal release of 6-ND was also reduced in atria (B) obtained from animals chronically treated with L-NAME (20 mg/kg/day, 4 weeks). *$P < 0.05$.

In untreated isolated atria preparations, the β_1-AR antagonists atenolol (0.1 and 1 µM), betaxolol (0.1 and 1 µM) or metoprolol (0.1 and 1 µM) were applied to the organ bath after the equilibration period (30 min), and changes in atrial rate were monitored for a further 30 min period. The effect of these β_1-AR antagonists (1 µM) was also evaluated in atria pre-treated with L-NAME (100 µM; 30 min), D-NAME (100 µM; 30 min) or TTX (1 µM; 30 min), and in atria obtained from animals chronically treated with L-NAME. In another set of experiments, the increase of atrial frequency induced by single concentrations of 6-ND (1 pM), dopamine (10 nM), noradrenaline (100 pM) or adrenaline (100 pM) was evaluated in the absence and the presence of either atenolol, betaxolol or metoprolol (1 µM each).

In all protocols detailed above, one atrium was always used for a single drug and concentration.

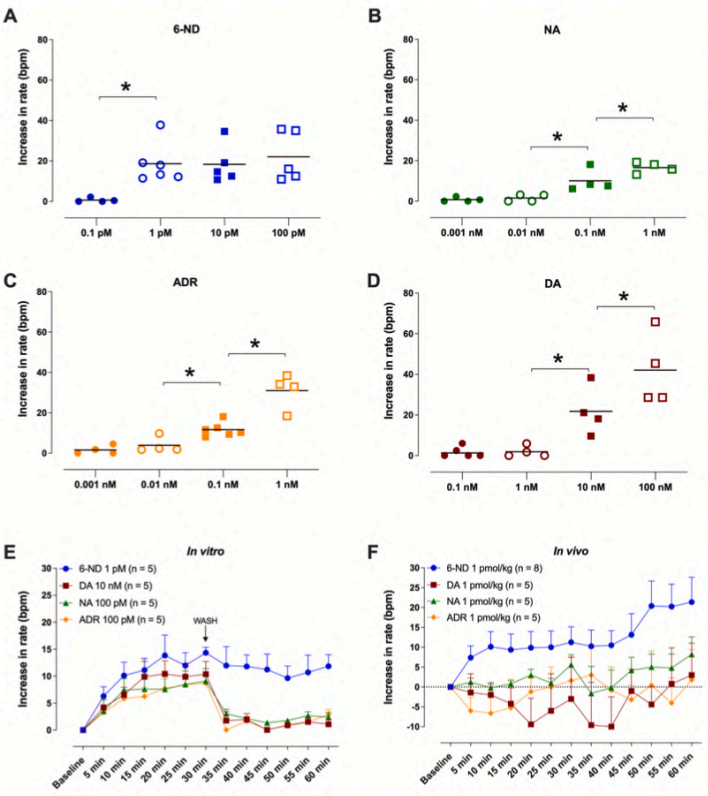


Fig. 2. Concentration-response curves of catecholamines in the rat isolated right atrial rate. 6-Nitrodopamine (6-ND; 0.1–100 pM; A) exhibited higher potency, and the maximal response was achieved at 1 pM with no further increases at higher concentrations (10 and 100 pM) whereas noradrenaline (NA; 0.001–1 nM; B), adrenaline (ADR; 0.001–1 nM; C) and dopamine (DA; 0.1–100 nM; D) presented clear concentration-dependent increases in the atrial rate. In the rat isolated atrium, note that the minimum concentration to increase the atrial frequency for 6-ND was 1 pM, whereas for noradrenaline and adrenaline was 100 pM and for dopamine 10 nM (E). At the dose of 1 pmol/kg, only 6-ND induced an increase in the heart rate of anaesthetized rat (F). Note also that the effect of 6-ND was very prolonged either *in vitro* (E) or *in vivo* (F). *$P < 0.05$.

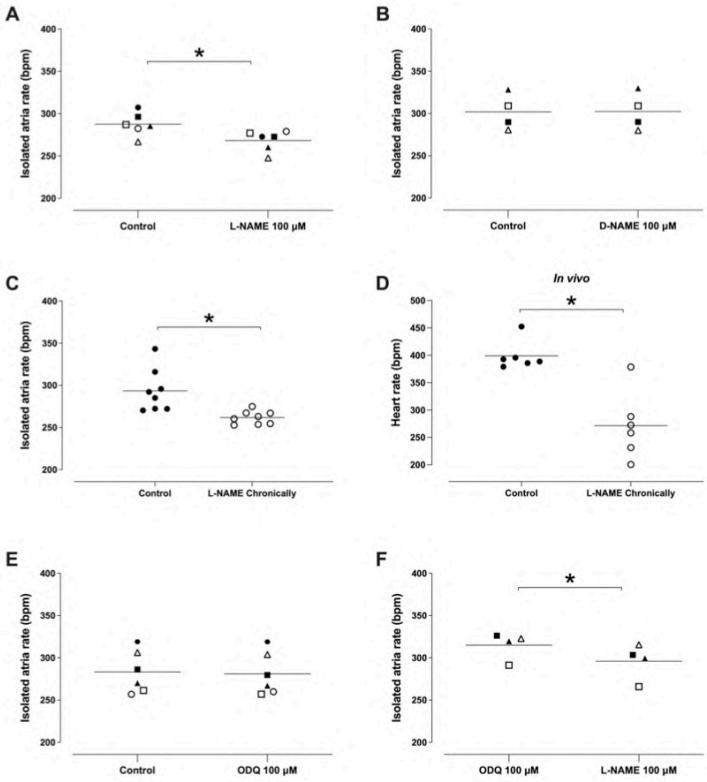


Fig. 3. Effects of L-NAME and ODQ in the rat isolated right atria. Pre-treatment of the atria with L-NAME (100 µM, 30 min; A), but not D-NAME (100 µM, 30 min; B), caused a significant decrease in the atrial rate. Atria obtained from animals chronically treated with L-NAME also showed reduced atrial rate compared to that observed in atria obtained from control animals (C). In conscious animals chronically treated with L-NAME, the heart rate, measured by tail plethysmography, was significantly lower compared to control animals (D). Pre-treatment of the atria with the heme site inhibitor of the soluble guanylate cyclase ODQ (100 µM; 30 min) affected neither the atrial rate (E) nor prevented the fall induced by L-NAME (100 µM, 30 min; F). *$P < 0.05$.

2.7. Measurement of mean arterial blood pressure (MABP) and heart rate (HR) of anaesthetized rats

Animals were anaesthetized with sodium thiopental (40 mg/kg, ip). Body temperature was maintained with a heating pad and monitored with a rectal probe. A 2-cm skin incision was performed on the ventral middle line of the neck. The left carotid was exposed by dissection of the muscular layers and a distal ligature was performed, after which a polyethylene 24G catheter filled with heparinised saline solution (20 IU/mL heparin in 0.9 % saline) was inserted and fixed with the proximal ligature. The catheter was attached to a pressure transducer (MLT1199 BP Transducer, AD Instruments, Sidney, Australia) for continuously BP monitoring and flushed with 0.1 mL of heparinized saline every 15–30 min to prevent clot formation. Mean arterial blood pressure (MABP) was calculated from the pulsatile arterial pressure signal, as the diastolic blood pressure plus one-third of the difference between systolic and diastolic blood pressure. The heart rate (HR) was derived from the pulse pressure and expressed as beats per minute (bpm). A second 24G catheter was inserted into the jugular vein for administration of either saline or drugs.

After 30 min of basal recordings, animals received 6-nitrodopamine (1 pmol/kg), noradrenaline (1 pmol/kg), adrenaline (1 pmol/kg) or dopamine (1 pmol/kg) through intravenous bolus (1 mL/kg). Changes in MABP and HR were monitored for an additional 60 min after drug administration. One animal was used for a single catecholamine. At the end of the experiments, the rats were euthanized with an overdose of anaesthetic. Exsanguination was performed to confirm euthanasia.

2.8. Statistical analysis

Data represent the mean ± standard error of the mean (SEM). Comparison between baseline values to values obtained during drug stimulation in the same sample was performed by paired Student's *t*-test. Comparison between two groups was performed by unpaired Student's *t*-test. Comparisons among three or more groups were evaluated using one-way analysis of variance (ANOVA), followed by the Newman-Keuls post-test. $P < 0.05$ was taken as significant. Data of atrial rate are presented as beats per minute (bpm) before and after the respective stimulation or as the delta increase of atrial rate.

2.9. Chemical and reagents

Adrenaline, atenolol, atropine, dopamine, metoprolol, N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and N^{ω}-Nitro-D-arginine methyl ester hydrochloride (D-NAME), and ODQ were purchased from Sigma-Aldrich Chemicals Co. (Missouri, USA). Betaxolol, noradrenaline and tetrodotoxin (TTX) were obtained from Cayman Chemicals (Michigan, USA). 6-Nitrodopamine and 6-nitrodopamine-d_4 were acquired from Toronto Research Chemicals (Ontario, CA). Dopamine-d_3 hydrochloride, DL-noradrenaline-d_6 hydrochloride and adrenaline-d_6 hydrochloride were acquired from CDN Isotopes (Quebec, CA). Strata™-X 33 mm Polymeric Reversed SPE cartridges were bought from Phenomenex (California, USA) and GIST-HP C_{18} columns were obtained from Shimadzu (Duisburg, Germany). Calcium chloride ($CaCl_2$), dextrose, magnesium sulfate ($MgSO_4$), potassium chloride (KCl), sodium bicarbonate

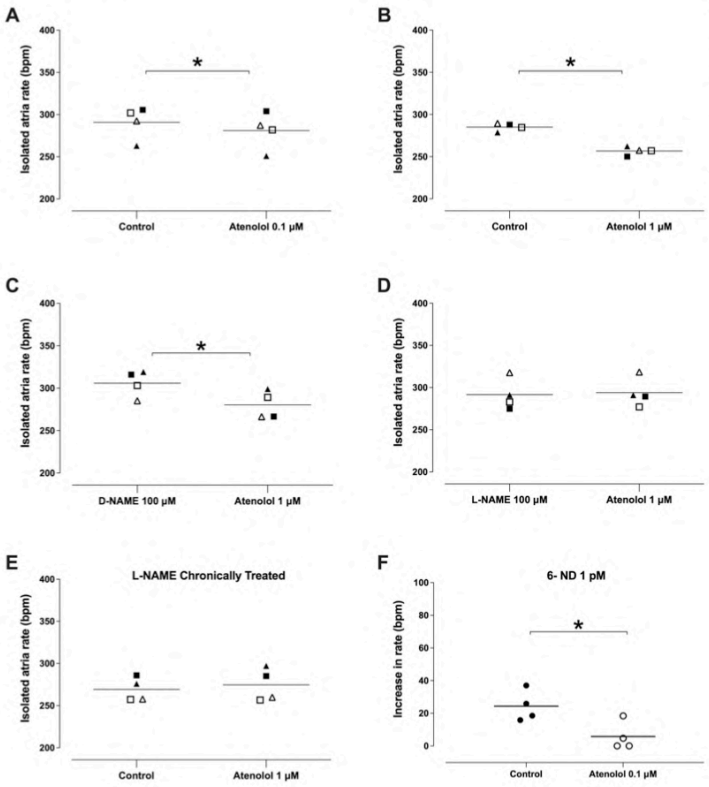


Fig. 4. Antagonism of the positive chronotropic responses of 6-nitrodopamine (6-ND) by the β_1-adrenergic receptor antagonist atenolol. Atenolol (0.1 μM; A) and (1 μM; B) caused significant reductions of the rat isolated atrial rate. In D-NAME (100 μM, 30 min)-treated preparations, atenolol (1 μM, 30 min) also caused a significant reduction of atrial rate (C), which was not observed in atria pre-treated with L-NAME (100 μM, 30 min; D) or in atria obtained from animals chronically treated with L-NAME (E). At 0.1 μM, atenolol (30 min) significantly reduced the increase in atrial rate induced by 6-ND (F). *$P < 0.05$.

($NaHCO_3$), potassium phosphate monobasic (KH_2PO_4) and sodium chloride (NaCl), were bought from Merck KGaA (Hesse, Germany). Acetonitrile and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA) and formic acid from Mallinckrodt (St Louis, Missouri, USA). The composition of the KHS was in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and dextrose 5.6.

3. Results

3.1. Release of 6-ND, dopamine, noradrenaline and adrenaline from rat isolated right atria by LC-MS-MS

The right atria (Fig. 1A and B) exhibited a basal release of 6-ND, as detected in the KHS by LC-MS/MS. The release was significantly reduced when the atria (Fig. 1A, n = 10) were pre-treated (20 min) with L-NAME (100 μM) *in vitro*. The release of 6-ND was also decreased in the atria (Fig. 1B, n = 8) obtained from rats chronically treated with L-NAME.

Dopamine was detected only in 15/36 samples (1.21 ± 0.47 ng/mL) and noradrenaline in 11/36 samples (0.27 ± 0.05 ng/mL) of atrial KHS. Adrenaline levels were below 0.1 ng/mL (LOQ) in all samples.

3.2. Effect of catecholamines in rat isolated right atrium frequency

6-ND at 1 pM induced a significant increase in the frequency of rat isolated right atrial (Fig. 2A). The minimum concentration for noradrenaline (Fig. 2B) and adrenaline (Fig. 2C) to increase the atrial frequency was 100 pM whereas for dopamine was 10 nM (Fig. 2D). Dopamine, noradrenaline, and adrenaline displayed concentration-dependent responses, as opposed to 6-ND. Another difference from the other catecholamines is the duration of the response induced by 6-ND. The increase in the atrial rate induced by 6-ND was maintained for at least 30 min after the 6-ND washout (Fig. 2E), whereas the increase in atrial rate induced by the other catecholamines returned to baseline levels within 5 min from the washout (Fig. 2E).

3.3. Effect of intravenous bolus injections of catecholamines on heart rate and blood pressure of the anaesthetized rat

The intravenous administration of 6-ND at 1 pmol/kg caused a significant and long-lasting increase in the heart rate, which was maintained for at least 1 h (Fig. 2F). At the same dose (1 pmol/kg), dopamine, noradrenaline and adrenaline did not alter HR (Fig. 2F). At 1 pmol/kg, the catecholamines tested did not cause changes in the MABP (Fig. S1A-D).

3.4. Effect of the nitric oxide synthase and soluble guanylate cyclase inhibitions on the atrial rate

Incubation of the atria for 30 min with L-NAME (100 μM; Fig. 3A; $n = 6$), but not with its inactive enantiomer D-NAME (100 μM; Fig. 3B; $n = 4$), caused a significant fall in the atrial rate. Atria collected from animals chronically treated with L-NAME also presented a significant decrease of atrial frequency compared to control animals (Fig. 3C; $n = 8$).

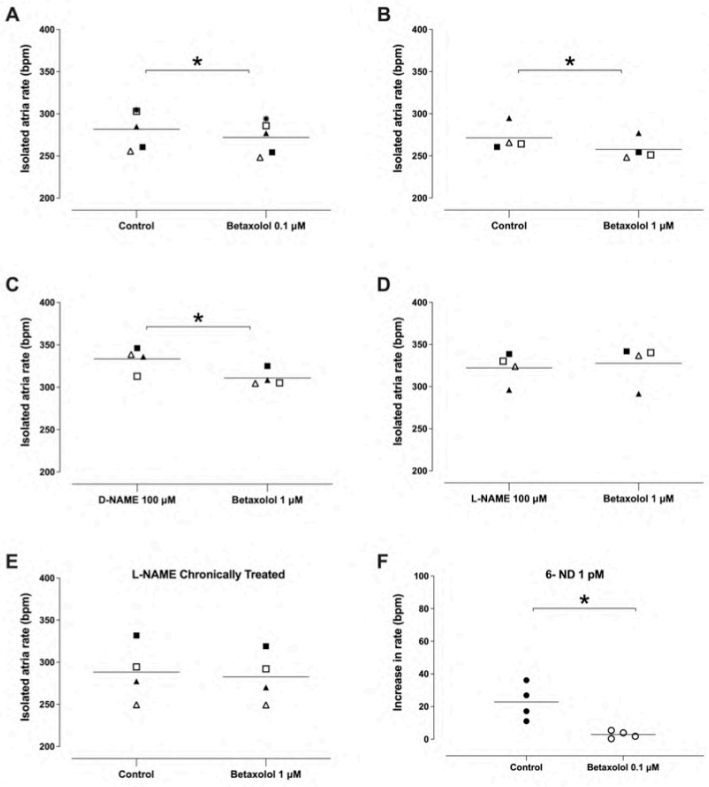


Fig. 5. Antagonism of the positive chronotropic responses of 6-nitrodopamine (6-ND) by the β_1-adrenergic receptor antagonist betaxolol. Betaxolol (0.1 μM; A) and (1 μM; B) caused significant reductions of the rat isolated atrial rate. In D-NAME (100 μM, 30 min)-treated preparations, betaxolol (1 μM, 30 min) also caused a significant reduction of atrial rate (C), which was not observed in atria pre-treated with L-NAME (100 μM, 30 min; D) or in atria obtained from animals chronically treated with L-NAME (E). At 0.1 μM, betaxolol (30 min) significantly reduced the increase in atrial rate induced by 6-ND (F). *$P < 0.05$.

The heart rate of the rats chronically treated with L-NAME was significantly lower compared to vehicle treated rats (Fig. 3D; n = 6) whereas the systolic blood pressure of L-NAME-treated rats was significantly increased (173 ± 4 and 135 ± 4 mmHg, for L-NAME-treated and untreated rats, respectively; $P < 0.001$, n = 8). Pre-treatment (30 min) with ODQ (100 μM) did not affect the atrial rate (Fig. 3E; n = 6). Atrial pre-treatment (30 min) with ODQ (100 μM) did not prevent the fall in the atrial rate induced by L-NAME (100 mM; Fig. 3F; $n = 4$).

3.5. *Effect of β_1-adrenoceptor selective antagonists on the rat isolated right atria rate*

Atenolol at 0.1 μM (30 min; Fig. 4A; $n = 4$) and 1 μM (30 min; Fig. 4B; $n = 4$) caused concentration-dependent reductions in the atrial frequency. Atenolol at 1 μM reduced the atrial rate of D-NAME (100 μM) pre-treated atria (Fig. 4C; $n = 4$), but it had no effect on L-NAME (100 μM) pre-treated atria (Fig. 4D; $n = 4$), or in atria harvested from animals chronically treated with L-NAME (Fig. 4E; n = 4). At 0.1 μM, atenolol (30 min) significantly reduced the increase in atrial rate induced by 6-ND (1 pM; Fig. 4F; $n = 4$), but it did not affect the increased atrial rate induced by noradrenaline (100 pM; 11 ± 3 and 13 ± 3 bpm for control and atenolol, respectively; $n = 4$), dopamine (10 nM; 16 ± 3 and 14 ± 4 bpm for control and atenolol, respectively; n = 4) and adrenaline (100 pM; 17 ± 3 and 14 ± 3 bpm for control and atenolol, respectively; $n = 4$).

Betaxolol at 0.1 μM (30 min; Fig. 5A; $n = 5$) and 1 μM (30 min; Fig. 5B; $n = 4$) caused concentration-dependent reductions in the atrial frequency. Betaxolol at 1 μM reduced the atrial rate of D-NAME (100 μM) pre-treated atria (Fig. 5C; $n = 4$), but it had no effect on L-NAME (100 μM) pre-treated atria (Fig. 5D; $n = 4$), or in atria harvested from animals chronically treated with L-NAME (Fig. 5E; $n = 4$). At 0.1 μM, betaxolol (30 min) significantly reduced the increase in atrial rate induced by 6-ND (1 pM; Fig. 5F; n = 4), but it did not affect the increases in atrial rate induced by noradrenaline (100 pM; 9 ± 1 and 11 ± 2 for control and betaxolol, respectively; n = 4), dopamine (10 nM; 9 ± 2 and 9 ± 1 for control and betaxolol, respectively; $n = 4$) and adrenaline (100 pM; 9 ± 2 and 8 ± 2 for control and betaxolol, respectively; $n = 4$).

Metoprolol at 0.1 μM (30 min; Fig. 6A; n = 4) and 1 μM (30 min; Fig. 6B; n = 4) caused concentration-dependent reductions in the atrial frequency. Metoprolol at 1 μM reduced the atrial rate of D-NAME (100 μM) pre-treated atria (Fig. 6C; $n = 4$), but it had no effect on L-NAME (100 μM) pre-treated atria (Fig. 6D; $n = 4$), or in atria harvested from animals chronically treated with L-NAME (Fig. 6E; $n = 4$). At 0.1 μM, metoprolol (30 min) significantly reduced the increase in atrial rate induced by 6-ND (1 pM; Fig. 6F; $n = 4$), but it did not affect the increased atrial rate induced by noradrenaline (100 pM;14 ± 3 and 14 ± 4 for control and metoprolol, respectively; $n = 4$), dopamine (10 nM; 13 ± 3 and 14 ± 4 for control and metoprolol, respectively; n = 4) and adrenaline (100 pM; 16 ± 3 and 17 ± 4 for control and metoprolol, respectively; n = 4).

3.6. *Effect of tetrodotoxin (TTX) on the rat isolated right atria rate*

In order to evaluate a potential neurogenic origin of 6-ND and/or

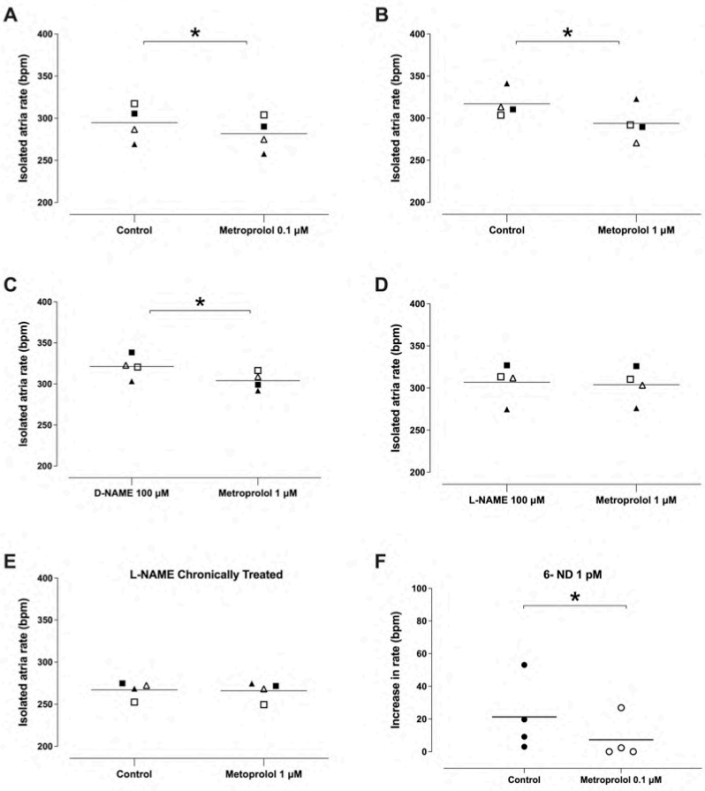


Fig. 6. Antagonism of the positive chronotropic responses of 6-nitrodopamine (6-ND) by the β_1-adrenergic receptor antagonist metoprolol. Metoprolol (0.1 μM; A) and (1 μM; B) caused significant reductions of the rat isolated atrial rate. In D-NAME (100 μM, 30 min)-treated preparations, metoprolol (1 μM, 30 min) also caused a significant reduction of atrial rate (C), which was not observed in atria pre-treated with L-NAME (100 μM, 30 min; D) or in atria obtained from animals chronically treated with L-NAME (E). At 0.1 μM, metoprolol (30 min) significantly reduced the increase in atrial rate induced by 6-ND (F). *$P < 0.05$.

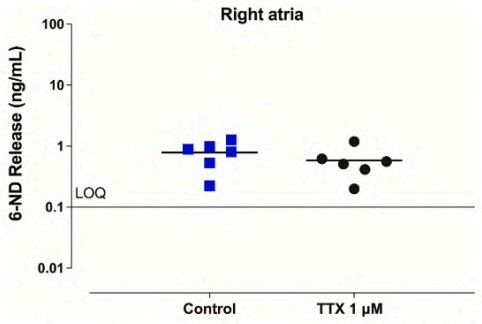


Fig. 7. Basal release of 6-ND in isolated right atria treated with tetrodotoxin. Incubation of both rat isolated atria with tetrodotoxin (30 min, 1 μM) had no effect on the basal release of 6-ND.

neurogenic component on its action, the sodium channel blocker tetrodotoxin was employed. Incubation of rat isolated atria (Fig. 7; $n = 6$) with TTX (30 min, 1 μM) did not affect the basal release of 6-ND. Incubation of the atria with TTX (1 μM, 30 min) caused a significant fall in the frequency of control atria (Fig. 8A; n = 4) and in L-NAME (100 μM) pre-treated (30 min) atria (Fig. 8B; n = 4). L-NAME (100 μM, 30 min) caused a significant fall in the atrial frequency of TTX (1 μM) pre-treated (30 min) atria (Fig. 8C; $n = 5$). In TTX (1 μM) pre-treated (30 min) atria, the β_1-AR antagonists atenolol (1 μM, 30 min; Fig. 8D; n = 4), betaxolol (1 μM, 30 min; Fig. 8E; n = 4) and metoprolol (1 μM, 30 min; Fig. 8F; n = 4) all caused significant reductions in the atrial rate. Atropine (100 nM; 30 min) caused a significant increase in atrial frequency (Fig. 8G; n = 4), which was not observed when the atria were pre-treated with TTX (30 min, 1 μM; Fig. 8H; n = 4). Pre-treatment of the atria with TTX (30 min, 1 μM) did not prevent the increase in atrial rate induced by 6-ND (1 pM; Fig. 8I; n = 4). Pre-treatment of the atria with atropine (100 nM; 30 min) did not affect the reduced atrial frequency induced by L-NAME (100 μM; Fig. S2; n = 4).

4. Discussion

The results clearly demonstrate that 6-ND is the major catecholamine released from rat isolated right atria. The findings that the basal 6-ND release was inhibited by incubation with the NO synthase inhibitor L-NAME or animals chronically treated with L-NAME release smaller amounts of 6-ND indicate that the synthesis of 6-ND is coupled to NO synthesis. Inhibition of 6-ND release by L-NAME was also observed in HUCV [3] and in rat and human isolated vas deferens [5,6]. In rats chronically treated with L-NAME, a 50 % reduction in the levels of the 6-nitronoradrenaline extracted from the rat brain was found [21].

Although NO can nitrate catecholamines *in vitro* [22], it is not yet established whether the nitrosation/nitration of 6-ND *in vivo* is due to a chemical or enzymatic process. As for the source of 6-ND, the finding that pre-incubation of the atrial with tetrodotoxin did not affect its

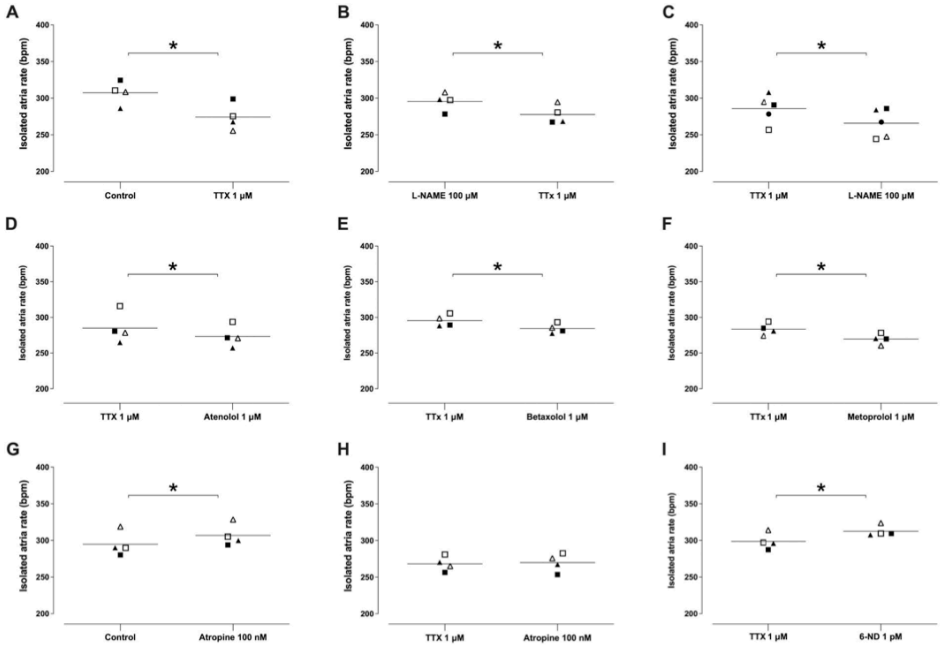


Fig. 8. Effect of tetrodotoxin on rat isolated right atrial rate. Tetrodotoxin (TTX; 1 mM, 30 min) caused significant reductions of atrial rate in both control (A) and L-NAME (100 µM) pre-treated (30 min) atria (B). L-NAME (100 µM, 30 min) caused a significant fall in atrial rate of TTX (1 µM) pre-treated atria (C). In TTX (1 µM) pre-treated atria, atenolol (1 µM, D), betaxolol (1 µM, E) and metoprolol (1 µM, F) all caused significant reductions of the atrial rate. Atropine (100 nM, 30 min) increased the atrial rate (H), which was blocked by TTX (1 µM) pretreatment (H). The increased atrial rate induced by 6-ND (1 pM) remained elevated in TTX-pre-treated (1 µM) preparations (I). *$P < 0.05$.

release indicates that the origin of the heart 6-ND is unlikely to be neurogenic. This is confirmed by the finding that tetrodotoxin blocked the nerve transmission in the heart, since TTX at the same concentration (1 µM), prevented the increased heart rate induced by atropine. Thus, it is likely that in the heart 6-ND comes from either the endocardium and/or the myocardium. In this context, it is worth noting that intrinsic cardiac adrenergic cells have been identified in the neonatal rat heart, using immunofluorescent histochemical staining techniques with antibodies that specifically recognize the major enzymes in the catecholamine biosynthetic pathway [23].

The findings that 6-ND is released from rat atria *in vitro* and that it has a positive chronotropic effect one hundred times more potent than noradrenaline and adrenaline do not demonstrate that 6-ND is an endogenous mediator of heart chronotropism. However, the reduction of 6-ND synthesis/release may be the mechanism by which acute [3,24,25] and chronic [26] NO synthesis inhibition reduces the heart rate. For instance, pre-treatment with the soluble guanylate cyclase inhibitor ODQ did not affect the atrial rate and did not prevent the fall in atrial rate induced by L-NAME, indicating that the mechanism by which L-NAME treatment causes fall of atrial rate is not due to the NO-sGC-cGMP pathway inhibition. This concept is further supported by the evidence that ODQ administration to rats affected neither MABP nor heart rate, although *ex-vivo* inhibition of sGC was confirmed [27]. It is important to note that the fall in atrial rate induced by L-NAME is not due to cholinergic stimulation, since it occurs in atropine treated atria. Similar results were obtained *in vivo*, that is, in conscious rats pre-treated with atropine, acute L-NAME administration causes a significant reduction in heart rate [28].

Since 6-ND also causes potent relaxations of HUCV pre-contracted with U-46619, which are insensitive to L-NAME pre-treatment, it is also very likely that the increases in blood pressure caused by acute and chronic NO inhibition are due to 6-ND inhibition. Indeed, in different hypertension models such as spontaneously hypertensive rats [29], two-kidney one-clip [30], one-kidney one-clip [31] and deoxycorticosterone acetate salt [32], the heart rate was either increased or unaffected whereas hypertension induced by acute or chronic L-NAME administration was accompanied by a significant reduction in heart rate [26,33].

Another evidence for the endogenous role of 6-ND as the major mediator of heart chronotropism comes from the results obtained with the β_1-AR selective antagonists atenolol, betaxolol and metoprolol. The fall in atrial rate induced by these compounds was observed at concentrations that selectively block the increase in atrial rate induced by 6-ND but did not affect the increase in atrial rate induced by noradrenaline, adrenaline and dopamine. In addition, the reductions in atrial rate induced by the β_1-AR selective antagonists were neither observed in atrial pre-treated with L-NAME nor in atria obtained from animals chronically treated with L-NAME, implying that receptor blockade of 6-ND is the main mechanism of action of this class of compounds. The finding that the effects of β_1-AR antagonists in the heart are due to the blockade of 6-ND action offers an interesting explanation for some clinical observations in heart and heart-lung transplanted patients. For instance, patients subjected to heart transplantation present extrinsic heart denervation caused by axonal Wallerian degeneration due to surgical interruption of the parasympathetic vagal neurons and the intrinsic post-ganglionic sympathetic nerve fibers travelling from the stellate ganglia to the myocardium [34]. In these patients, the donor's heart is surgically denervated, yet the heart rate increases in response to

exercise [35] and this peak response is supposed to be due to an increase in circulating levels of catecholamines released by the adrenal glands [36]. Treatment of these patients with atenolol causes a significant reduction of both resting and maximum heart rates [37].

In conclusion 6-ND is an endogenous modulator of the rat heart chronotropism. The positive chronotropic effect of 6-ND has two distinct characteristics compared to that induced by the classical catecholamines; it has a flat concentration-response curve, and the effect is long lasting, both *in vitro* and *in vivo*. β_1-Adrenoceptor stimulation activates adenylate cyclase increases the cyclic adenosine monophosphate (cAMP), which is responsible for increasing both heart rate and contractility [38]. Since the signaling of β_1-AR is rapidly terminated by cAMP degradation by cAMP phosphodiesterases [39], it is possible that the transduction mechanism following 6-ND receptor activation is not coupled to adenylate cyclase stimulation. Nevertheless, the finding that β_1-AR antagonists act in the heart as potent 6-ND antagonists should cause a careful reevaluation of the physiological role of the adrenergic nervous system in the heart. Activating the sympathetic nervous system is responsible for the body's "Fight or Flight" reaction [40]. Possibly due to 6-ND, one may not need to fight or fly constantly.

5. Conclusion

The results clearly establish 6-ND as a novel non-neurogenic mediator of heart chronotropism and offer a novel mechanism of action of NO as a modulator of both heart rate and blood pressure. Measurement of circulating levels in clinical scenarios where patients present high heart rate and low blood pressure, as in septic shock, may clarify the pathophysiological role of 6-ND.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2022.120879.

CRediT authorship contribution statement

José Britto-Júnior: Methodology, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Mariana Gonçalves de Oliveira:** Methodology, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Carolina dos Reis Gati:** Methodology, Investigation, Visualization. **Rafael Campos:** Methodology. **Manoel Odorico Moraes:** Methodology. **Maria Elisabete A. Moraes:** Methodology. **Fabíola Z. Mónica:** Methodology, Visualization, Supervision. **Edson Antunes:** Conceptualization, Methodology, Visualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Gilberto De Nucci:** Conceptualization, Methodology, Visualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Data availability

No data was used for the research described in the article.

Acknowledgments

Funding

Coordination for the Improvement of Higher Education Personnel (CAPES) grants 001 (J.B.J); 23067.050073/2018-19 (R. C).

Brazilian Ministry of Health grant 23067.050073/2018-19.

State of São Paulo Research Foundation (FAPESP) grants 2018/09765-3 (M.G.O); 2017/15175-1 (E.A.); 2019/16805-4 (G.D.N).

National Council for Scientific and Technological Development (CNPq) grant 303839/2019-8 (G.D.N.)

Fund to Support Education, Research and Extension (FAEPEX) grant 2380/22 (G.D.N).

Declaration of competing interest

Authors declare that they have no competing interests.

References

[1] M. Tsunoda, E. Uchino, K. Imai, K. Hayakawa, T. Funatsu, Determination of nitrocatecholamines in rat brain using high-performance liquid chromatography-peroxyoxalate chemiluminescence reaction detection, J. Chromatogr. A 1164 (1-2) (2007) 162–166, https://doi.org/10.1016/j.chroma.2007.07.005, 14.

[2] X.H. Chiari, Z. Li, H.L. Xu, J.C. Pan, Eisenach, formation of 6-nitro-norepinephrine from nitric oxide and norepinephrine in the spinal cord and its role in spinal analgesia, Neuroscience 101 (2000) 189–196, https://doi.org/10.1016/s0306-4522(00)00328-6.

[3] J. Britto-Júnior, W.C. Coelho-Silva, G.F. Murari, C.E. Nachi, F.Z. Mónica, E. Antunes, G. De Nucci, 6-Nitrodopamine is released by human umbilical cord vessels and modulates vascular reactivity, Life Sci. 276 (2021), 119425, https://doi.org/10.1016/j.lfs.2021.119425.

[4] R. Campos, D.H.A. Pinheiro, J. Britto-Júnior, H.A. Castro, G.D. Mendes, M. O. Moraes, M.E.A. Moraes, R.A.B. Lopes-Martins, N.J. Antunes, G. De Nucci, Quantification of 6-nitrodopamine in Krebs-Henseleit's solution by LC-MS/MS for the assessment of its basal release from Chelonoidis carbonaria aortae in vitro, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 22 (2021) 1173, https://doi.org/10.1016/j.jchromb.2021.122668, 122668.

[5] J. Britto-Júnior, L. Ximenes, A. Ribeiro, A. Fregonesi, R. Campos, L.R.A. Kiguti, F. Z. Mónica, E. Antunes, G. De Nucci, 6-Nitrodopamine is an endogenous mediator of rat isolated epididymal vas deferens contractions induced by electric-field stimulation, Eur. J. Pharmacol. 15 (2021) 911, https://doi.org/10.1016/j.ejphar.2021.174544, 174544.

[6] J. Britto-Júnior, W.P. Silva-Filho, A.C. Amorim, R. Campos, M.O. Moraes, M.E. A. Moraes, A. Fregonesi, F.Z. Mónica, E. Antunes, G.De Nucci, 6-Nitrodopamine is a major endogenous modulator of human vas deferens contractility, Andrology 10 (2022) 13263, https://doi.org/10.1111/andr.13263.

[7] J. Britto-Júnior, A. Ribeiro, L. Ximenes, A.T. Lima, F.F. Jacintho, A. Fregonesi, F. Z. Mónica, E. Antunes, G. De Nucci, Alpha1-adrenergic antagonists block 6-nitrodopamine contractions on the rat isolated epididymal vas deferens, Eur. J. Pharmacol. 15 (2022) 915, https://doi.org/10.1016/j.ejphar.2021.174716, 174716.

[8] A.T. Lima, A.C. Amorim, J. Britto-Júnior, R.R. Campitelli, F.Z. Mónica, E. Antunes, G.De Nucci, A. Fregonesi, β1- and β1/β2-Adrenergic receptor antagonists block 6-nitrodopamine-induced contractions of the rat isolated epididymal vas deferens Naunyn-Schmiedeb, Arch. Pharmacol. (2022), https://doi.org/10.1007/s00210-022-02268-6.

[9] D. Annane, L. Ouanes-Besbes, D. de Backer, B. Du, A.C. Gordon, G. Hernández, K. M. Olsen, T.M. Osborn, S. Peake, J.A. Russell, S.Z. Cavazzoni, A global perspective on vasoactive agents in shock, Intensive Care Med. 44 (2018) 833–846, https://doi.org/10.1007/s00134-018-5242-5.

[10] J.C. Kleinjans, J.F. Smits, C.M. Kasbergen, H.T. Vervoort-Peters, H.A. Struyker Boudier, Blood pressure response to chronic low-dose intrarenal noradrenaline infusion in conscious rats, Clin. Sci. (Lond.) 65 (1983) 111–116, https://doi.org/10.1042/cs0650111.

[11] S.A. Doggrell, Characterization of the contractile responses to noradrenaline and adrenaline of aorta from normotensive and hypertensive rats, Gen. Pharmacol. 25 (1994) 1001–1008, https://doi.org/10.1016/0306-3623(94)90110-4.

[12] G.L. Stiles, S. Taylor, R.J. Lefkowitz, Human cardiac beta-adrenergic receptors: subtype heterogeneity delineated by direct radioligand binding, Life Sci. 33 (1983) 467–473, https://doi.org/10.1016/0024-3205(83)90796-8.

[13] M.R. Bristow, R. Ginsburg, V. Umans, M. Fowler, W. Minobe, R. Rasmussen, P. Zera, R. Menlove, P. Shah, S. Jamieson, et al., Beta 1- and beta 2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective beta 1-receptor down-regulation in heart failure, Circ. Res. 59 (1986) 297–309, https://doi.org/10.1161/01.res.59.3.297.

[14] R.J. Lefkowitz, H.A. Rockman, W.J. Koch, Catecholamines, cardiac beta-adrenergic receptors, and heart failure, Circulation 101 (14) (2000) 1634–1637, https://doi.org/10.1161/01.cir.101.14.1634, 11.

[15] M.L. Andersen, Guia brasileiro de produção, manutenção ou utilização de animais em atividade de ensino ou pesquisa científica, Conselho nacional de controle de experimentação animal, Ministério da Ciência, Tecnologia e Inovação, Brasília, 2016.

[16] N. Percie du Sert, V. Hurst, A. Ahluwalia, S. Alam, M.T. Avey, M. Baker, W. J. Browne, A. Clark, I.C. Cuthill, U. Dirnagl, M. Emerson, P. Garner, S.T. Holgate, D.W. Howells, N.A. Karp, S.E. Lazic, K. Lidster, C.J. MacCallum, M. Macleod, E. J. Pearl, O.H. Petersen, F. Rawle, P. Reynolds, K. Rooney, E.S. Sena, S. D. Silberberg, T. Steckler, H. Würbel, The ARRIVE guidelines 2.0: updated guidelines for reporting animal research, PLoS Biol. 14 (18(7)) (2020), e3000410, https://doi.org/10.1371/journal.pbio.3000410.

[17] M.O. Ribeiro, E. Antunes, G. De Nucci, S.M. Lovisolo, R. Zatz, Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension, Hypertension 20 (1992) 298–303, https://doi.org/10.1161/01.hyp.20.3.298.

[18] S.R. Riado, A. Zanesco, L.A. Barker, I.M. De Luca, E. Antunes, G. De Nucci, Long-term nitric oxide inhibition and chronotropic responses in rat isolated right atria, Hypertension 34 (1999) 802–807, https://doi.org/10.1161/01.hyp.34.4.802.

[19] J. Britto-Júnior, N.J. Antunes, R. Campos, M. Sucupira, G.D. Mendes, F. Fernandes, M.O. Moraes, M.E.A. Moraes, G. De Nucci, Determination of dopamine, noradrenaline, and adrenaline in Krebs-Henseleit solution by liquid chromatography coupled with tandem mass spectrometry and measurement of their basal release from Chelonoidis carbonaria aortae in vitro, Biomed. Chromatogr. 35 (2021), e4978, https://doi.org/10.1002/bmc.4978.

[20] R. Meesters, S. Voswinkel, Bioanalytical method development and validation: from the USFDA 2001 to the USFDA 2018 guidance for industry, J. Appl. Bioanal. 4 (3) (2018) 67–73.

[21] F. Shintani, T. Kinoshita, S. Kanba, T. Ishikawa, E. Suzuki, N. Sasakawa, R. Kato, M. Asai, T. Nakaki, Bioactive 6-nitronorepinephrine identified in mammalian brain, J. Biol. Chem. 23 (1996) 13561–13565, https://doi.org/10.1074/jbc.271.23.13561.

[22] M. d'Ischia, C. Costantini, Nitric oxide-induced nitration of catecholamine neurotransmitters: a key to neuronal degeneration? Bioorg. Med. Chem. 7 (1995) 923–927, https://doi.org/10.1016/0968-0896(95)00083-s.

[23] A.R. Natarajan, Q. Rong, A.N. Katchman, Ebert, Intrinsic cardiac catecholamines help maintain beating activeity in neonatal rat cardiomyocyte cultures, Pediatr Res. 56 (2004) 411–417, https://doi.org/10.1203/01.PDR.0000136279.80897.4C.

[24] D.D. Rees, R.M. Palme, S. Moncada, Role of endothelium-derived nitric oxide in the regulation of blood pressure, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 3375–3378, https://doi.org/10.1073/pnas.86.9.3375.

[25] R. Pabla, M.J. Curtis, Endogenous protection against reperfusion-induced ventricular fibrillation: role of neuronal versus non-neuronal sources of nitric oxide and species dependence in the rat versus rabbit isolated heart, J. Mol. Cell. Cardiol. 28 (1996) 2097–2110, https://doi.org/10.1006/jmcc.1996.0202.

[26] K.E. Scrogin, D.C. Hatton, Y. Chi, F.C. Luft, Chronic nitric oxide inhibition with L-NAME: effects on autonomic control of the cardiovascular system, Am. J. Phys. 274 (1998) R367–R374, https://doi.org/10.1152/ajpregu.1998.274.2.R367.

[27] S. Cechova, T.N. Pajewski, The soluble guanylyl cyclase inhibitor ODQ, 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one, dose-dependently reduces the threshold for isoflurane anesthesia in rats, Anesth. Analg. 99 (2004) 752–757, https://doi.org/10.1213/01.ANE.0000132695.77191.F3.

[28] R.E. Widdop, S.M. Gardiner, P.A. Kemp, T. Bennett, The influence of atropine and atenolol on the cardiac haemodynamic effects of NG-nitro-L-arginine methyl ester in conscious, Long Evans rats, Br. J. Pharmacol. 105 (1992) 653–656, https://doi.org/10.1111/j.1476-5381.1992.tb09034.x.

[29] G.A. Head, M.A. Adams, Characterization of the baroreceptor heart rate reflex during development in spontaneously hypertensive rats, Clin. Exp. Pharmacol. Physiol. 19 (1992) 587–597, https://doi.org/10.1111/j.1440-1681.1992.tb00509.x.

[30] J.Y. Jung, J.U. Lee, W.J. Kim, Enhance activity of central adrenergic neurons in the two-kidney one-clip hypertension in Sprague-Dawley rats, Neurosci. Lett. 369 (2004) 14–18, https://doi.org/j.neylet.2004.07.029.

[31] B.H. Machado, H.C. Salgado, E.M. Krieger, Tachycardic response during development of renal hypertension, Hypertension 5 (1983) 122–127, https://doi.org/10.1161/01.hyp.5.6_pt_3.v122.

[32] M. Kubacka, M. Zadrożna, B. Nowak, M. Kotańska, B. Filipek, A.M. Waszkielewicz, H. Marona, S. Mogilski, Reversal of cardiac, vascular, and renal dysfunction by non-quinazoline α1-adrenolytics in DOCA-salt hypertensive rats: a comparison with prazosin, a quinazoline-based α1-adrenoceptor antagonist, Hypertens. Res. 42 (2019) 1125–1141, https://doi.org/10.1038/s41440-019-0239-1.

[33] A.L. Fellet, C. Di Verniero, P. Arza, A. Tomat, A. Varela, C. Arranz, A. M. Balaszczuk, Effect of acute nitric oxide synthase inhibition in the modulation of heart rate in rats, Braz. J. Med. Biol. Res. 36 (2003) 669–676, https://doi.org/10.1590/s0100-879x2003000500015.

[34] M. Awad, L.S. Czer, M. Hou, S.S. Golshani, M.De Robertis, M. Goltche, M. Kittleson, J. Patel, B. Azarbal, E. Kransdorf, F. Esmailian, A. Trento, J.A. Kobashigawa, Early denervation and later reinnervation of the heart following cardiac transplantation: a review, J. Am. Heart Assoc. 5 (2016), e004070, https://doi.org/10.1161/JAHA.116.004070.

[35] R.J. Quigg, M.B. Rocc, D.F. Gauthier, M.A. Creager, L.H. Hartley, W.S. Colucci, Mechanism of the attenuated peak heart rate response to exercise after orthotopic cardiac transplantation, J. Am. Coll. Cardiol. 14 (1989) 338–344, https://doi.org/10.1016/0735-1097(89)90183-6. PMID: 2666478.

[36] G. Ferretti, C. Marconi, G. Achilli, E. Caspani, R. Fiocchi, F. Mamprin, A. Gamba, P. Ferrazzi, P. Cerretelli, The heart rate response to exercise and circulating catecholamines in heart transplant recipients, Pflugers Arch. 443 (2002) 370–376, https://doi.org/10.1007/s004240100701.

[37] F. Buendía-Fuentes, L. Almenar-Bonet, L. Martínez-Dolz, M.J. Carranza, J. Moro, J. López, J. Ramón-Llin, I. Sánchez-Lázaro, J.M. Sánchez-Gómez, R.R. Raso, J. N. Manchón, A.S. Sanz, Exercise tolerance after beta blockade in recent cardiac transplant recipients, Transplant Proc. 42 (2009) 2250–2252, https://doi.org/10.1016/j.transproceed.2009.06.007.

[38] S. Alhayek, C.V. Preuss, Beta 1 receptors, in: StatPearls [Internet], StatPearls Publishing, Treasure Island (FL), 2022.

[39] A.T. Bender, J.A. Beavo, Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use, Pharmacol. Rev. 58 (2006) 488–520, https://doi.org/10.1124/pr.58.3.5.

[40] W.B. Cannon, The Wisdom of the Body, WW Norton and Company, NY, 1932.

Anexo 12

Artigo 14 – Release of 6-nitrodopamine modulates vascular reactivity of *Pantherophis guttatus* aortic rings.

Autores Lima AT, Dos Santos EX, Britto-Júnior J, de Souza VB, Schenka AA, Campos R, Moraes MO, Moraes MEA, Antunes E, De Nucci G.

Revista: Comparative Biochemistry and Physiology, Part C

Situação: Aceito a publicação em 08 de agosto. Publicado on-line em 10 de agosto de 2022

Comparative Biochemistry and Physiology, Part C 262 (2022) 109471

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Release of 6-nitrodopamine modulates vascular reactivity of *Pantherophis guttatus* aortic rings

Antonio Tiago Lima [a], Eric Xavier dos Santos [a], José Britto-Júnior [a,*], Valéria B. de Souza [a], André A. Schenka [a], Rafael Campos [b,c], Manoel Odorico Moraes [c], Maria Elisabete A. Moraes [c], Edson Antunes [a], Gilberto De Nucci [a,c,d]

[a] Faculty of Medical Sciences, Department of Pharmacology, University of Campinas (UNICAMP), Campinas, Brazil
[b] Superior Institute of Biomedical Sciences, Ceará State University (UECE), Fortaleza, Brazil
[c] Clinical Pharmacology Unit, Drug Research and Development Center, Federal University of Ceará (UFC), Fortaleza, CE, Brazil
[d] Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo (USP), São Paulo, Brazil

ARTICLE INFO

Edited by Martin Grosell

Keywords:
Dopamine
Nitric oxide
L-761,626
ODQ
LC-MS/MS

ABSTRACT

6-Nitrodopamine (6-ND) is a novel catecholamine that is released from human umbilical cord vessels and *Chelonoidis carbonaria* aortic rings. The synthesis/release of 6-ND is inhibited by either pre-incubation of the vessels with the nitric oxide (NO) synthase inhibitor L-NAME or by mechanical removal of the endothelium. 6-ND causes powerful vasorelaxation, acting as a potent and selective dopamine D_2-like receptor antagonist.

Basal release of 6-ND from *Panterophis guttatus* endothelium intact and denuded aortic rings was quantified by LC-MS/MS. In order to evaluate the interaction of 6-ND with other catecholamines, aortic rings were suspended vertically between two metal hooks in 10-mL organ baths containing Krebs-Henseleit's solution and attached to isometric transducers.

Endothelium intact aortic rings presented basal release of 6-ND, which was significantly reduced by previous incubation with L-NAME (100 μM). In endothelin-1 (3 nM) pre-contracted endothelium intact aortic rings, 6-ND (10pM-1 μM) and the dopamine D_2-receptor antagonist L-761,626 (10 pM–1 μM) induced concentration-dependent relaxations, which were not affected by incubation with L-NAME but greatly reduced in endothelium-removed aortic rings.

6-ND (0.1–1 μM) produced significant rightward shifts of the concentration-response curves to dopamine in L-NAME pre-treated endothelium-intact (pA_2 7.01) rings. Contractions induced by noradrenaline and adrenaline were not affected by pre-incubation with 6-ND (1 μM). The EFS-induced contractions of L-NAME pre-treated endothelium-intact aortic rings were significantly inhibited by incubation with 6-ND (1 μM).

The results indicate that 6-ND released from *Pantherophis guttatus* aortic rings is coupled to NO release and represents a new mechanism by which NO can modulate vascular reactivity independently of cGMP production.

1. Introduction

Electrical field stimulation (EFS) produces frequency-dependent contractions in *Pantherophis guttatus* aortic rings, which are abolished by the mechanical removal of the endothelium (Campos et al., 2018a). Since the EFS-induced contractions are antagonized by the α_1 and α_2-adrenergic antagonist phentolamine (Gould and Reddy, 1976) and are insensitive to pre-treatment with the sodium channel blocker tetrodotoxin (Narahashi et al., 1967), these results indicated that endothelium-derived catecholamines are responsible for the EFS-induced contractions. Similar results were obtained with the aorta from the venomous snakes *Crotalus durissus terrificus* and *Bothrops jararaca* (Campos et al., 2018b).

Human umbilical arteries and vein release both catecholamines dopamine (Britto-Júnior et al., 2020) and 6-nitrodopamine (6-ND; Britto-Júnior et al., 2021a), which are also dependent of the integrity of the endothelium. Similar results were reported for aortic rings obtained from the tortoise *Chelonoidis carbonarius*, where the release of 6-ND was

* Corresponding author at: University of Campinas (Unicamp), Faculty of Medical Sciences, Department of Pharmacology, Rua Tessália Vieira de Camargo, 126, Cidade Universitária, 13083-887 Campinas, São Paulo, Brazil.
E-mail address: josebrittojr@dac.unicamp.br (J. Britto-Júnior).

https://doi.org/10.1016/j.cbpc.2022.109471
Received 26 June 2022; Received in revised form 10 September 2022; Accepted 15 September 2022
Available online 17 September 2022
1532-0456/© 2022 Elsevier Inc. All rights reserved.

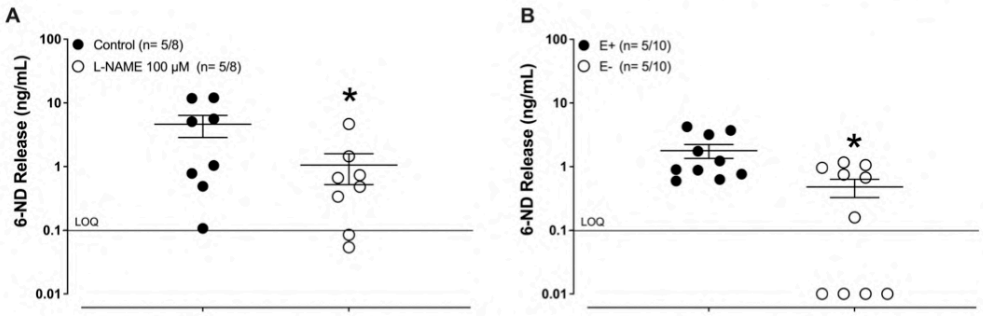


Fig. 1. Basal release of 6-nitrodopamine (6-ND) from aortic rings of *Pantherophis guttatus*. The basal release of 6-ND was measured by LC-MS/MS following a 30 min-period incubation in Krebs-Henseleit's solution. Treatment with L-NAME (100 μM; panel A) or mechanical removal of endothelium (E−; panel B) reduced the 6-ND basal release from the aortic rings. Data are expressed as mean ± S.E.M. * indicates $p < 0.05$. In panel A, it was employed 5 aortas and 16 rings and in panel B, 5 aortas and 20 rings. Unpaired *t*-test was applied in Panels A and B.

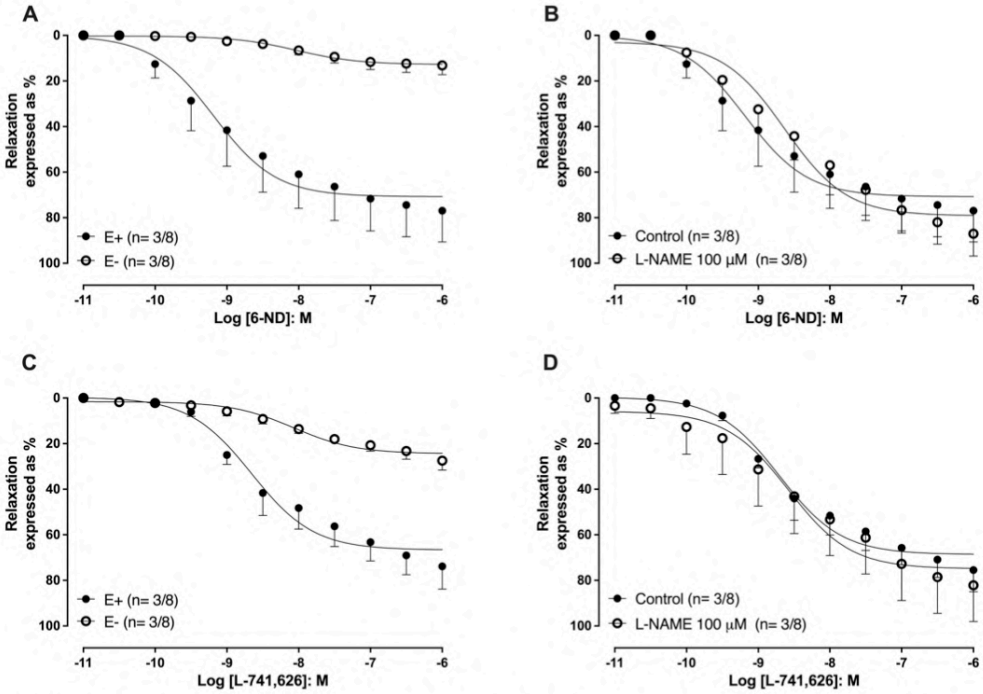


Fig. 2. Relaxations induced by 6-ND and L-741,626. In endotelin-1 pre-contracted (3 nM) *Pantherophis guttatus* aortic rings 6-nitrodopamine (6-ND, panel A–B) and L-742,626 (panel C–D) caused concentration-dependent relaxations of *Pantherophis guttatus* aortic rings. The relaxations were abolished by endothelium removal (E-; panel A–C) but not affected by incubation with L-NAME (panel B–D). Data are expressed as mean ± S.E.M. In panel A, it was employed 3 aortas and 16 rings; in panel B, 3 aortas and 16 rings; in panel C, 3 aortas and 16 rings and in panel D, 3 aortas and 16 rings. Unpaired t-test was applied in Panels A-D.

decreased by either mechanical endothelium removal or pre-treatment with the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; Britto-Júnior et al., 2022). In both human and tortoise vessels, this novel catecholamine 6-ND causes a powerful vasorelaxation, acting as a potent and selective dopamine D_2-like receptor antagonist (Britto-Júnior et al., 2021a, Britto-Júnior et al., 2022).

The above findings that the vasculature endothelium releases both dopamine and 6-ND, and that they have antagonistic action in the smooth muscle tonus, open the exciting possibility that this dynamic balance may be a crucial mechanism for local blood flow control (Quigg et al., 1989). Although the sympathetic nervous system has been implicated in the control of the microcirculation resistance (Awad et al., 2016), there is hardly any morphological evidence of the presence of sympathetic nerve terminals in the microcirculation.

Considering all the vertebrates, snakes show remarkable adaptations involving the cardiovascular system and gravity (Lillywhite et al.,

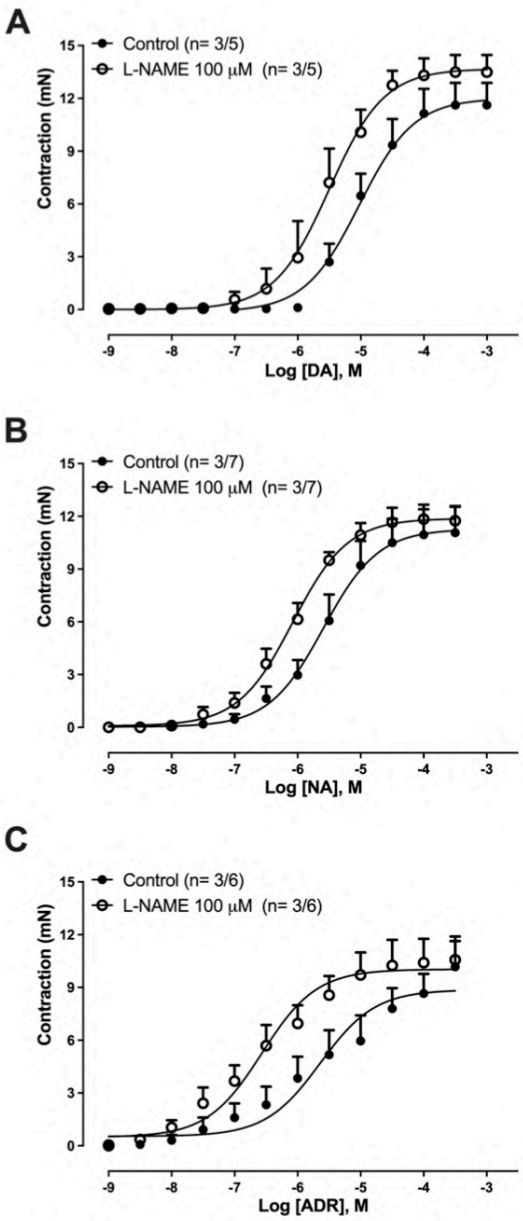


Fig. 3. Effect of L-NAME on the contractions induced by dopamine, noradrenaline and adrenaline. Pre-incubation with the NO synthesis inhibitor L-NAME (100 µM) caused significant leftward shifts of the concentration-response curves dopamine (DA, panel A), noradrenaline (NA, panel B) and adrenaline (ADR, panel C). Data are expressed as mean ± S.E.M. In panel A, it was employed 3 aortas and 10 rings; in panel B, 3 aortas and 14 rings and in panel C, 3 aortas and 12 rings. Unpaired *t*-test was applied in Panels A–C.

2012); a corn snake may crawl straight up the trunk of a tree in search of eggs (Lillywhite, 1988). Since *Pantherophis guttatus* aorta releases classical catecholamines, it was investigated here whether 6-ND is also released and its potential interactions with dopamine, noradrenaline and adrenaline in this tissue.

2. Material and methods

2.1. Animals

All experimental procedures using *Pantherophis guttatus* of either sex (weight varied from 400 to 700 g) was approved by the Institutional Animal Care and Use Committee (CEUA/UNICAMP: 5266-1/2019) and followed the ARRIVE guidelines (Percie du Sert et al., 2020). The animals were obtained from Parque Ecológico do Tietê (São Paulo, SP, Brazil) and Centro de Reabilitação de Animais Silvestres (CRAS-Univap; São José dos Campos, SP, Brazil).

2.2. Basal release of 6-nitrodopamine, dopamine, noradrenaline and adrenaline from Pantherophis guttatus aortic rings

The snakes were anesthetized with ketamine (70 mg/kg, IM) after sedation with midazolam (2 mg/kg; IM), after which the animals were euthanized by exsanguination. A segment of aorta was removed and immediately placed in oxygenated (95 % O_2/5 % CO_2) Krebs-Henseleit's (KHS; pH 7.4) at 27 °C (Campos et al., 2018b). Subsequently, two aortic rings per animal (15-mm diameter) with intact endothelium and another two rings with denuded endothelium from the same snake were suspended in 5-mL organ bath containing oxygenated KHS and ascorbic acid (3 mM) at 27 °C for 30 min. The removal of endothelial cells was done mechanically by gently rubbing the vessels with forceps. The endothelium-intact aortic rings were incubated in the absence and in the presence of the NO synthesis inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 µM). A 2 mL KHS aliquot was transferred to an Eppendorf tube and stored at −20 °C until analysis.

2.3. Determination of catecholamine concentrations in KHS by tandem mass spectrometry (LC-MS/MS)

The LC-MS/MS method for quantification of 6-ND (Campos et al., 2020) was modified to allow the measurement of the four catecholamines (6-ND, dopamine, noradrenaline and adrenaline) in a single chromatographic run (Britto-Júnior et al., 2021b). The extraction of the catecholamines from 1 mL of KHS was performed by solid phase extraction. Briefly, to 1 mL of KHS was added 50 µL of the internal standards (100 ng/mL of 6-nitrodopamine-d_4, dopamine-d_3, noradrenaline-d_6, and adrenaline-d_6). The samples were homogenized for 10 s. The Strata™-X 33 mm Polymeric Reversed Solid Phase Extraction (SPE) cartridges were pre-conditioned with 1 mL of methanol and then balanced with 2 mL of deionized water. The samples were injected into the cartridge, and the cartridge was subsequently washed 3 times with deionized water. The samples were then eluted with 0.9 mL methanol/water (90/10, v/v) with 0.1 % formic acid. The eluate was evaporated under N_2 flow at 50 °C. The residue was dissolved with 100 µL of acetonitrile/water (50/50, v/v) with 0.1 % formic acid and transferred to vials ready for injection. The LC–MS/MS system consisted of LC ADVp Liquid Chromatograph Shimadzu System (Shimadzu Corporation, Kyoto, Japan) coupled to an 8060 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan) operating in electrospray positive-ionization mode. Samples were injected into the system by means of a SIL-30AC autoinjector, at a temperature of 8 °C. The chromatography separation was performed at room temperature using a GIST-HP C_{18} column (150 mm × 3.0 mm, 3 mm) column (Shimadzu, Duisburg, Germany). A 75 % mobile phase A consisting of deionized water with 0.1 % formic acid (v/v) and 25 % mobile phase B consisting of acetonitrile/water (90/10, v/v) with 0.1 % formic acid at a flow rate

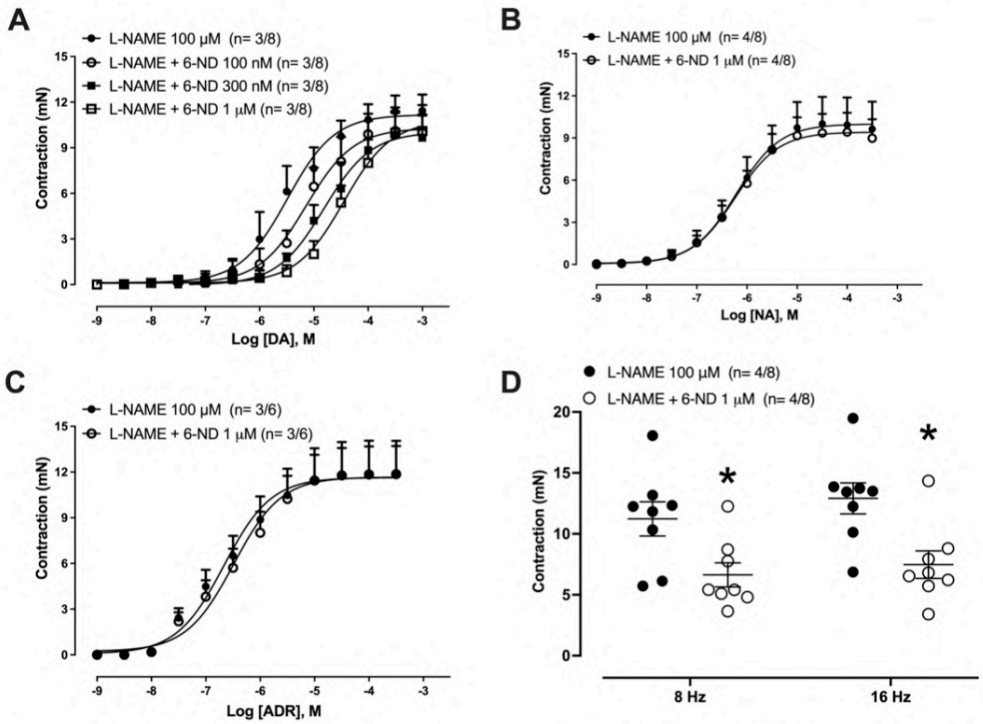


Fig. 4. Effect of 6-ND on the contractions induced by dopamine, noradrenaline, adrenaline and EFS. Endothelium-intact aortic rings were initially pre-treated with L-NAME (100 μM). Pre-incubation with 6-ND (0.1 to 1 μM) caused significant rightward shifts in the concentration-response to dopamine (DA, panel A), without modifying the responses to noradrenaline (NA, panel B) and adrenaline (Panel C). The EFS (8 and 16 Hz)-induced contractions were significantly reduced by pre-incubation with 6-ND (panel D). Data are expressed as mean ± S.E.M. * indicates $p < 0.05$. In panel A, it was employed 3 aortas and 32 rings; in panel B, 4 aortas and 16 rings; in panel C, 3 aortas and 12 rings and in panel D, 4 aortas and 16 rings. ANOVA followed by the Newman–Keuls post-test was applied in Panel A whereas the unpaired t-test was applied in Panels B–D.

of 0.35 mL/min were used. The electrospray was configured for multiple reaction monitoring (MRM) to monitor the transitions 199.10 > 181.95 for 6-nitrodopamine, 203.10 > 186.00 for 6-nitrodopamine-d_4, 154.00 > 91.15 for dopamine, 157.00 > 93.00 for dopamine-d_3, 170.10 > 107.10 for noradrenaline, 176.10 > 158.10 for noradrenaline-d_6, 184.20 > 107.00 for adrenaline, and 190.00 > 171.95 for adrenaline-d_6. The injection volume was 3 μL of each sample. The total run-time was 3.5 min. The method validation was carried out according to the United States Food and Drug Administration (FDA, 2001) bioanalytical method validation guidelines.

2.4. Aortic ring preparations and isometric tension recordings

The aortic rings (3 mm) were suspended vertically between two metal hooks in 10-mL organ baths containing KHS, gassed with a mixture of 95 % O_2: 5 % CO_2 (pH 7.4) at 27 °C. Isometric force was recorded using a PowerLab 400TM data acquisition system (Software Chart, version 7.0, AD Instrument, MA, USA). The tissues were allowed to equilibrate for 1 h before starting the experiments, after which the rings were pre-contracted with potassium chloride (KCl, 80 mM). The integrity of the endothelium in the aortic rings was evaluated through acetylcholine-induced relaxation (ACh, 1 μM; Mónica et al., 2012; Filogonio et al., 2020).

2.5. Concentration-response curves to dopamine, noradrenaline and adrenaline

Cumulative concentration-response curves to dopamine (1 nM-1 mM), noradrenaline (1 nM-300 μM) and adrenaline (1 nM-300 μM) were performed in endothelium-intact aortic rings in the absence and the presence of L-NAME (100 μM, 30 min). In the L-NAME pre-treated preparations, the cumulative concentration-response curves to dopamine (1 nM-1 mM) were carried out in the absence and the presence of either 6-ND (0.1, 0.3 and 1 μM) or the selective α_1-adrenergic receptor antagonist prazosin (10, 30 and 100 nM). Cumulative concentration-response curves to noradrenaline (1 nM-300 μM) and adrenaline (1 nM-300 μM) were performed in endothelium-intact aortic rings treated with L-NAME (100 μM, 30 min) in the absence and the presence of either 6-ND (1 μM) or prazosin (10, 30 and 100 nM).

2.6. Electrical-field stimulation (EFS)-induced aorta contractions

Endothelium-intact aortic rings were submitted to EFS at 60 V for 30 s, at 8–16 Hz in square-wave pulses (0.3 ms pulse width, 0.1 ms delay), using a Grass S88 stimulator (Astro-Medical, RI, USA). The EFS-induced contractions of aortic rings were performed in the presence or not of either L-NAME (100 μM) or the soluble guanylate cyclase inhibitor ODQ (100 μM).

Aortic rings pretreated with L-NAME (100 μM) were also submitted

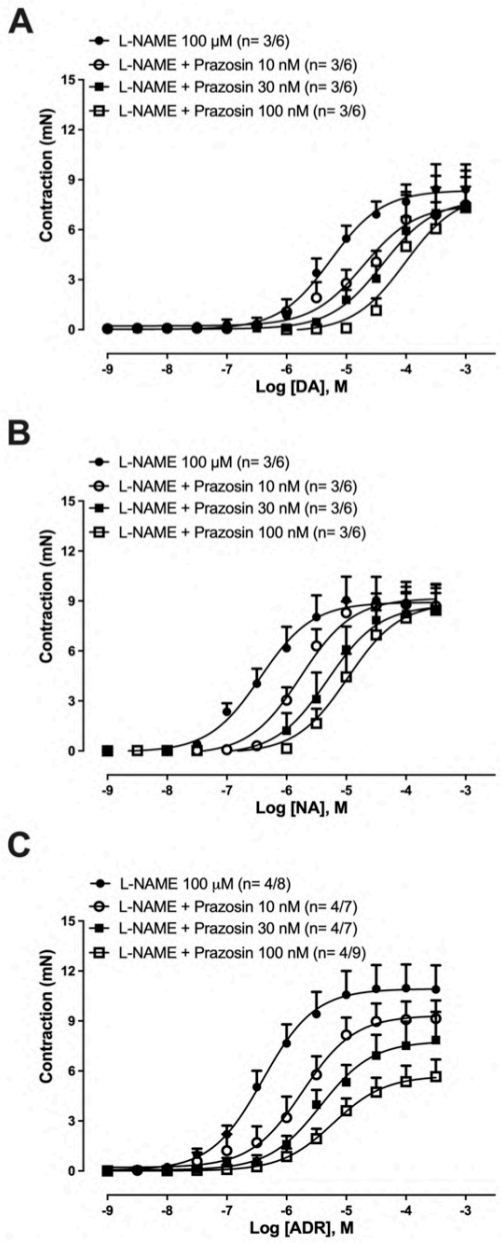


Fig. 5. Effect of prazosin on the contractions induced by dopamine, noradrenaline and adrenaline. Endothelium-intact aortic rings were initially pre-treated with L-NAME (100 μM). The selective α_1-adrenergic antagonist prazosin (10, 30 and 100 nM) produced concentration-dependent rightward shifts on the concentration-response curves to dopamine (DA, panel A), noradrenaline (NA, panel B) and adrenaline (ADR, panel C). Data are expressed as mean ± S.E.M. In panel A, it was employed 3 aortas and 24 rings; in panel B, 3 aortas and 24 rings and in panel C, 4 aortas and 31 rings. ANOVA followed by the Newman–Keuls post-test was applied in Panels A–C.

to EFS in the presence of 6-ND (1 μM). Potassium chloride (KCl, 80 mM) was added at the beginning and at the end of the experimental protocols to evaluate the tissue reactivity after EFS (Campos et al., 2020; Britto-Júnior et al., 2021a).

2.7. Effect of 6-ND in pre-contracted aortic rings

In aortic rings with intact and denuded endothelium and in endothelium-intact pre-treated with L-NAME (100 μM), the preparations were pre-contracted with endothelin-1 (ET-1; 3 nM). After a sustained contraction was obtained, cumulative concentration-response curves to either 6-ND (10 pM–1 μM) or the selective dopamine D_2-receptor antagonist L-741,626 (10 pM–1 μM; Bowery et al., 1996) were performed.

2.8. Immunohistochemistry for S-100 protein and calretinin

Following euthanasia, samples of the *Pantherophis guttatus* intact aorta (n = 3) were collected, fixed in 10 % neutral buffered formalin for 24 h at 24 °C, dehydrated, embedded in paraffin wax and sectioned at 4–5 μm. Subsequently, these sections were stained for S-100 protein (a neural tissue marker; Bao et al., 2011) or calretinin (a neural/neuronal marker; Morona et al., 2011) to investigate the presence of nerve fibers within the aortic walls using the following primary antibodies: (1) anti-S-100 (rabbit monoclonal antibody, Cat.# MAB0791, at 1:200, which reacts with bovine, human, rat and mouse S100 protein; Millipore, USA) and (2) anti-calretinin (mouse monoclonal antibody; Cat.# IS627; at 1:200, which reacts with human calretinin; DAKO/Agilent, USA). Immunohistochemistry was performed manually. Briefly, the sections were deparaffinized in xylene and rehydrated in a series of ethanol baths of increasing concentration. They were then incubated in citrate buffer at pH 6.0 in a steamer set for 20 min (at approximately 95 °C). The sections were then incubated for 2 h at room temperature (25 °C) with the above-mentioned primary antibodies. Subsequently, these sections were incubated with the NovoLink Max Polymer Detection System (Novocastra/Leica Biosystems), following the manufacturer's instructions, and using diaminobenzidine (liquid DAB, DakoCytomation, Carpenteria, USA) as a chromogen (which renders a brown precipitate at the antibody binding site). Finally, the sections were counter-stained with Harris' hematoxylin and cover-slipped in Entellan. Negative controls consisted of omission of the primary antibody and incubation with the primary antibody diluents, as well as with the detection system. This was performed for all the immunohistochemical assays to identify any background staining. Formalin-fixed, paraffin-embedded *Pantherophis guttatus* brain sections (n = 3) were used as positive controls for the presence of both antigens (*i.e.*, S-100 protein and calretinin). Furthermore, formalin-fixed, paraffin-embedded sections of a human pilocytic astrocytoma and a normal human colon containing myenteric ganglia and nerve fibers were used as additional positive controls for the presence of S-100 and calretinin, respectively. All slides were examined and photomicrographed using an Olympus CX43 trinocular microscope (Olympus, USA) coupled to a Nikon Coolpix 3.3MP CCD digital camera (Nikon, Japan). Positivity was assessed by an experienced MD, PhD pathologist (AAS), who was blind to the presence/absence of the primary antibody on the sample under examination (the observer did not know whether a test sample or an omission control was being assessed). Blinding was achieved by covering the slide labels with a removable occluding sticker.

2.9. Drugs and solutions

Dopamine, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), ascorbic acid and prazosin were obtained from Sigma-Aldrich Chemicals Co. (St Louis, Missouri, USA). Adrenaline, endothelin-1 (human, porcine), L-741,626, noradrenaline and 1H-[1,2,4]oxadiazolo [4,3-*a*]quinoxalin-1-one (ODQ) were purchased from Cayman Chemical

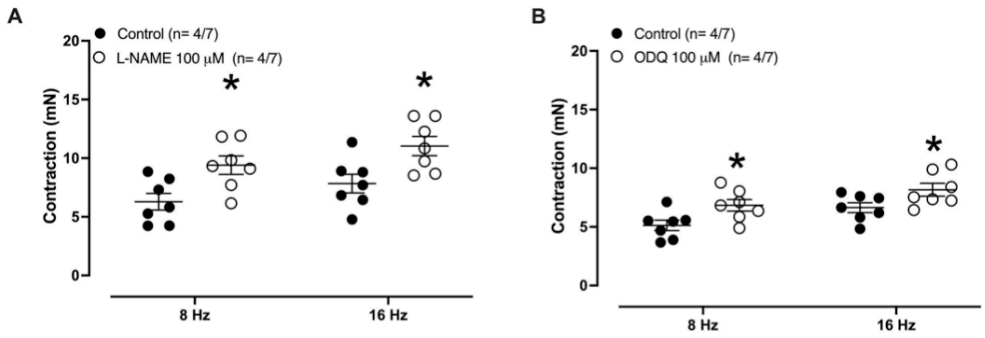


Fig. 6. Effects of L-NAME and ODQ on EFS-induced contractions. The EFS (8 and 16 Hz)-induced contractions were significantly increased by pre-incubation with L-NAME (100 μM; Panel A) or ODQ (100 μM, panel B). Data are expressed as mean ± S.E.M. * indicates $p < 0.05$. In panel A, it was employed 4 aortas and 14 rings and in panel B, 4 aortas and 14 rings. Unpaired t-test was applied in Panels A and B.

Co (Michigan, USA). 6-Nitrodopamine and 6-nitrodopamine-d_4 were acquired from Toronto Research Chemicals (Ontario, CA). Dopamine-d_3 hydrochloride, DL-noradrenaline-d_6 hydrochloride and adrenaline-d_6 hydrochloride were acquired from CDN Isotopes (Quebec, CA). Strata™-X 33 mm Polymeric Reversed SPE cartridges were bought from Phenomenex (California, USA) and GIST-HP C_{18} columns were obtained from Shimadzu (Duisburg, Germany). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$), potassium phosphate monobasic (KH_2PO_4) and glucose were acquired from Merck KGaA (Darmstadt, Germany). The composition of the KHS was in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and dextrose 5.6.

2.10. Data analysis

Nonlinear regression analysis to determine the pEC_{50} was carried out using GraphPad Prism (GraphPad Software, version 9.4, San Diego, CA, USA) with the constraint that $F = 0$. All concentration–response data were evaluated for a fit to a logistics function in the form: $E = E_{max} / ([1 + (10c / 10\times)n] + F$, where E represents the increase in response contractile induced by the agonist, E_{max} is the effect agonist maximum, c is the logarithm of concentration of the agonist that produces 50 % of E_{max}, x is the logarithm of the concentration of the drug; the exponential term, n, is a curve fitting parameter that defines the slope of the concentration–response line, and F is the response observed in the absence of added drug. The pA_2 values were calculated from the intercept on the concentration axis and by application of the equation; $pA_2 = \log$ (antagonist concentration) − log (CR-1) − log (antagonist concentration) (Arunlakshana and Schild, 1959). In addition, standard ANOVA, followed by the Newman–Keuls post-test, were used when more than two groups were involved. A p value of <0.05 was considered statistically significant. The values of pEC_{50} data represent the mean ± standard error of the mean (S.E.M.) of *n* experiments. Values of E_{max} were expressed in milli-Newtons (mN).

In the pharmacological experiments, the number of experiments in expressed as x/y, where x represents the number of aortas (animals) and y the number of rings employed in the experiment. One ring was used as the control response and the other ring was incubated with an antagonist/inhibitor. Student's two-tail unpaired *t*-test was employed and the differences between groups and $p < 0.05$ were considered significant. For E_{max} and pEC_{50} analysis, unpaired Student's t-test was used.

3. Results

3.1. Basal release of 6-ND from Pantherophis guttatus aortic rings

Levels of 6-ND were quantified by LC-MS/MS in the KHS bathing *Pantherophis guttatus* aortic rings (Fig. 1). Pre-treatment of the rings with L-NAME (100 μM, 30 min) reduced by 77 % ($p < 0.05$) the 6-ND release (4.61 ± 1.75 and 1.06 ± 0.54 ng/mL, for control and L-NAME aortic rings, respectively, $p = 0.0365$; Fig. 1A). The 6-ND release was also significantly decreased by mechanical removal of the endothelium (1.79 ± 0.43 and 0.48 ± 0.15 ng/mL for endothelium-intact and denuded aortic rings, respectively, $p = 0.0058$; Fig. 1B). The basal release of dopamine, noradrenaline, and adrenaline was below the limit of quantitation (LOQ; 0.1 ng/mL, data not shown).

3.2. Relaxing effects of 6-ND and L-741,626 on pre-contracted Pantherophis guttatus aortic rings

In endothelin-1 (3 nM) pre-contracted aortic rings, incubation with 6-ND (10 pM–1 μM) produced concentration-dependent relaxations with pEC_{50} and Emax values of 9.18 ± 0.25 and 76.91 ± 13.75 %, respectively ($n = 3/8$; Fig. 2A and B). The relaxations were nearly absent in endothelium-denuded aortic rings (Fig. 2A), but unaffected by pre-incubation with L-NAME (Fig. 2B).

In another set of endothelin-1 (3 nM) pre-contracted aortic rings, incubation with the selective dopamine D_2-receptor antagonist L-741,626 (10 pM–1 μM) produced concentration-dependent relaxations (pEC_{50} 8.66 ± 0.15; Emax 73.87 ± 10.03 %; $n = 3/8$; Fig. 2C and D). Similar to 6-ND, the relaxations induced by L-741,626 were greatly reduced by endothelium removal (Fig. 2C), but unaffected by incubation with L-NAME (Fig. 2D).

3.3. Contractile effects of 6-ND, dopamine, noradrenaline, and adrenaline on Pantherophis guttatus aortic rings

Incubation with 6-nitrodopamine (1 nM–1 μM) caused no contractions in *Pantherophis guttatus* aortic rings (data not shown; $n = 5/10$). Dopamine (1 nM–1 mM), noradrenaline (1 nM–300 μM) and adrenaline (1 nM–300 μM) caused concentration-dependent aortic contractions (Fig. 3A–C). Pre-incubation with L-NAME (100 μM) provoked significant leftward shifts ($p < 0.05$) of the concentration-response curves to dopamine (pEC_{50} 5.02 ± 0.09 and 5.50 ± 0.10 for control and L-NAME, respectively, $n = 3/5$; Fig. 3A), noradrenaline (pEC_{50} 5.59 ± 0.13 and 6.08 ± 0.08 for control and L-NAME, respectively, $n = 3/7$; Fig. 3B) and adrenaline (pEC_{50} 5.67 ± 0.17 and 6.59 ± 0.14 for control and L-NAME, respectively, $n = 3/6$; Fig. 3C). Incubation with 6-nitrodopamine (1

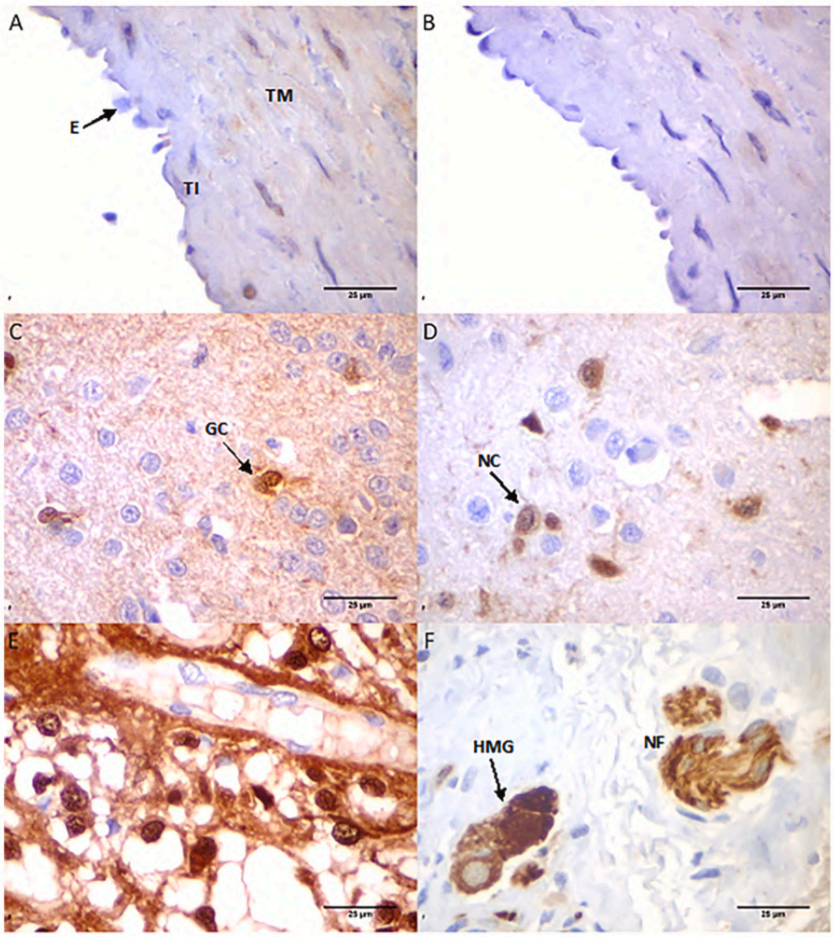


Fig. 7. Detection of S-100 protein and calretinin (neural/neuronal markers) by immunohistochemistry: (panel A) absence of S-100 protein in *Pantherophis guttatus* aorta (both tunica intima (TI) and tunica media (TM) are devoid of any positivity); (panel B) absence of calretinin in *Pantherophis guttatus* aorta (both in tunica intima (TI) and tunica media (TM) are negative); (panel C) S-100 protein positivity in *Pantherophis guttatus* central nervous system glial cells and neuropil (positive control); (panel D) calretinin positivity in *Pantherophis guttatus* central nervous system neurons (positive control); (panel E) S-100 protein positivity in a human pilocytic astrocytoma (positive control); (panel F) calretinin positivity in normal human myenteric ganglia (HMG) and nerve fibers (NF) (large bowel) – positive control. Immunoperoxidase, 400× (original magnification): scale bars = 25 μm. E: endothelial cells; GC: glial cells; NC: neuron cells.

nM–1 μM) caused no contractions in *Pantherophis guttatus* aortic rings pre-treated with L-NAME (data not shown; n = 5/10).

3.4. Inhibitory effects of 6-ND on the contractions induced by dopamine and EFS in Pantherophis guttatus aortic rings

In L-NAME pre-treated aortic rings, 6-ND (0.1–1 μM, 30 min) produced a significant rightward shift of the dopamine concentration-response curves (pA_2 7.01 ± 0.57, n = 3/8, p = 0.0121; Fig. 4A). In contrast to dopamine, 6-ND (1 μM, 30 min) had no significant effect on noradrenaline- (pEC_{50} 6.21 ± 0.18 and 6.23 ± 0.15 for control and 6-ND respectively; n = 4/8, p = 0.4798; Fig. 4B) and adrenaline-induced contractions (pEC_{50} 6.67 ± 0.17 and 6.50 ± 0.15 for control and 6-ND respectively; n = 3/6, p = 0.2230; Fig. 4C). In L-NAME pre-treated aortic rings, the EFS (8 and 16 Hz)-induced contractions were significantly inhibited by pre-incubation (30 min) with 6-ND (1 μM; 11.23 ± 1.40 and 6.64 ± 0.99 mN for 8 Hz and 12.90 ± 1.27 and 7.47 ± 1.13 mN for 16 Hz for control and 6-ND, respectively; n = 4/8; Fig. 4D).

3.5. Effect of prazosin on the contractions induced by dopamine, noradrenaline, and adrenaline in Pantherophis guttatus aortic rings

In endothelium-intact aortic rings pre-treated with L-NAME, prior incubation (30 min) with the selective α_1-adrenergic antagonist prazosin (10, 30 and 100 nM) produced significant concentration-dependent rightward shifts of the concentration-response curves to dopamine (pA_2 8.66 ± 0.31, n = 3/5, p = 0.0019; Fig. 5A), noradrenaline (pA_2 8.65 ± 0.24, n = 3/6; p = 0.0001; Fig. 5B) and adrenaline (pA_2 8.69 ± 0.23, n = 4/7, p = 0.0003; Fig. 5C).

3.6. EFS-induced contractions of Pantherophis guttatus aortic rings: effects of pre-incubation with L-NAME and ODQ

Electric-field stimulation (EFS) at 8 Hz and 16 Hz caused frequency-dependent contractions of the *Pantherophis guttatus* aortic rings. Pre-treatment with L-NAME (100 μM; 30 min) significantly augmented the EFS-induced aortic contractions (6.28 ± 0.71 and 9.41 ± 0.79 mN for 8 Hz; and 7.83 ± 0.79 and 11.08 ± 0.82 16 Hz; $p < 0.05$; for control and L-NAME, respectively; n = 4/7; Fig. 6A). Pre-treatment with ODQ (100 μM; 30 min) also significantly increased the EFS-induced aortic contractions (5.13 ± 0.44 and 6.83 ± 0.49 mN for 8 Hz; and 6.63 ± 0.42 and 8.16 ± 0.55 mN for 16 Hz; for control and ODQ, respectively; $p < 0.05$; n = 4/7; Fig. 6B). It is interesting that the increase caused by L-NAME was significantly ($p < 0.05$) higher (3.31 ± 0.74 mN) than that caused by ODQ pre-treatment (1.67 ± 0.50 mN).

3.7. Immunohistochemistry

Immunoreactivity for both S-100 protein and calretinin were consistently negative in *Pantherophis guttatus* aorta samples (n = 3; Fig. 7A and B). As positive controls, immunoreactivity for both S-100 protein and calretinin was detected in sections of central nervous system of *Pantherophis guttatus* (Fig. 7C and D), human pilocytic astrocytoma (Fig. 7E) and human myenteric ganglia/nerve fibers (Fig. 7F).

4. Discussion

Our results clearly indicate that 6-ND is the major catecholamine released by endothelium of *Pantherophis guttatus* aortic rings. Incubation with L-NAME and mechanical endothelium removal substantially reduced the 6-ND release (73 %–77 %) in a similar way to human umbilical vessels (75 %–90 %; Britto-Júnior et al., 2021a) and *Chelonoidis carbonarius* aorta (55 %–65 %; Campos et al., 2021; Britto-Júnior et al., 2022). However, L-NAME and endothelium removal did not abolish the 6-ND release, suggesting an extra-endothelium source for 6-ND synthesis/release. This secondary source of 6-ND is not composed of nerve terminals since human umbilical cord vessels (Reilly and Russell, 1997; Britto-Júnior et al., 2020), *Chelonoidis carbonarius* aorta (Campos et al., 2020) and *Pantherophis guttatus* aorta do not contain nerve terminals, as demonstrated here by the absence of immunoreactivity for the neuronal markers S-100 (Bao et al., 2011) and calretinin (Morona et al., 2011).

The finding that both 6-ND and L-741,626 (selective dopamine D_2-receptor antagonist) are potent vasodilators in *Pantherophis guttatus* aortic rings independent of NO release, but sensitive to endothelium removal, further supports the concept of endothelium-derived dopamine as one of the major modulators of vascular reactivity (Britto-Júnior et al., 2020). Although there is no evidence yet that dopamine receptors are expressed in *Panterophis guttatus* aorta, dopaminergic receptors in mammalian vascular beds have been characterized *in vitro* by radioligand-receptor binding and autoradiographic techniques. The localization of dopamine D_1 and D_2 receptors have been demonstrated in the smooth muscle of rat cerebral, mesenteric and renal arteries (Amenta et al., 1990). Oral administration of the non-ergot selective dopamine D_2 receptor agonist pramipexole (Schneider and Mierau, 1987) to healthy male volunteers caused significant increases in both blood pressure and heart rate (Farha et al., 2014). As observed in both human umbilical arteries and vein, 6-ND act as a very potent and highly selective dopamine D_2-like receptor antagonist (Britto-Júnior et al., 2021a) since in contrast to other alpha-adrenergic antagonists such as prazosin (Bogeso et al., 1988) and phentolamine (Owens et al., 1989), 6-ND only blocks dopamine-induced vasoconstriction.

The significant reduction of EFS-induced contractions by 6-ND confirms that endothelium-derived dopamine is a major mediator responsible for the contraction. Thus, the balance between dopamine and 6-ND constitutes an important mechanism for the control of local blood flow. Another important finding is that the increase in EFS-induced contractions caused by L-NAME is significantly higher than that caused by ODQ (Schrammel et al., 1996). The vasodilation caused by NO is supposed to be due to stimulation of soluble guanylate cyclase (Arnold et al., 1977) and increase in cGMP production (Murad et al., 1986). This increased potentiation of EFS-induced contractions caused by L-NAME indicates that inhibition of 6-ND synthesis/release rather than inhibition of soluble guanylate cyclase constitutes the major mechanism by which NO causes vascular smooth muscle relaxation. This novel concept is further supported by the evidence that ODQ administration to rats affected neither mean arterial blood pressure nor heart rate, although *ex-vivo* inhibition of soluble guanylate cyclase was confirmed (Cechova and Pajewski, 2004).

In summary, our data show that 6-ND is the major catecholamine released by endothelium of *Pantherophis guttatus* aortic rings and exerts potent vasorelaxant activity by acting as a selective dopamine D_2-like receptor antagonist. Synthesis of 6-ND by the endothelium could be the major mechanism by which NO controls vascular smooth muscle reactivity.

Declaration of competing interest

The authors declare no competing or financial interests.

Data availability

No data was used for the research described in the article.

Acknowledgment

ATL thanks FAPESP for PhD fellowship (2021/13593-6). JBJ thanks CAPES for PhD fellowship (001). VBS thanks FAPESP for post-doctoral fellowship (2021/13726-6). RC thanks CAPES for post-doctoral fellowship (88887.358153/2019-00). EA thanks FAPES(2017/15175-1). GDN thanks FAPESP (2019/16805-4) and CNPq (303839/2019-8).

References

Amenta, F., Collier, W.L., Ricci, A., 1990. Autoradiographic localization of vascular dopamine receptors. Am. J. Hypertens. 3 (6 Pt 2), 34S–36S. https://doi.org/10.1093/ajh/3.6.34s. PMID: 2200435.

Arnold, W.P., Mittal, C.K., Katsuki, S., Murad, F., 1977. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. Proc. Natl. Acad. Sci. U. S. A. 74 (8), 3203–3207. https://doi.org/10.1073/pnas.74.8.3203.

Arunlakshana, O., Schild, H.O., 1959. Some quantitative uses of drug antagonists. Br. J. Pharmacol. Chemother. 14 (1), 48–58. https://doi.org/10.1111/j.1476-5381.1959.tb00928.x.

Awad, M., Czer, L.S., Hou, M., Golshani, S.S., Goltche, M., De Robertis, M., Kittleson, M., Patel, J., Azarbal, B., Kransdorf, E., Esmailian, F., Trento, A., Kobashigawa, J.A., 2016. Early denervation and later reinnervation of the heart following cardiac transplantation: a review. J. Am. Heart Assoc. 1 (5), e004070 https://doi.org/10.1161/JAHA.116.004070, 11.

Bao, H.J., Liu, Y., Qin, J.H., Xu, C.S., Hei, N.N., Jaber, J.R., Chen, Q.S., 2011. An immunohistochemical study of S-100 protein in the intestinal tract of chinese soft-shelled turtle, Pelodiscus sinensis. Res. Vet. Sci. 91, e16–e24. https://doi.org/10.1016/j.rvsc.2011.02.015.

Bogeso, K.P., Arnt, J., Boeck, V., Christensen, A.V., Hyttel, J., Jensen, K.G., 1988. Antihypertensive activity in a series of 1-piperazino-3-phenylindans with potent 5-HT2-antagonistic activity, 1988 Dec J. Med. Chem. 31 (12), 2247–2256. https://doi.org/10.1021/jm00120a003. PMID: 2903929.

Bowery, B.J., Razzaque, Z., Emms, F., Patel, S., Freedman, S., Bristow, L., Kulagowski, J., Seabrook, G.R., 1996. Antagonism of the effects of (+)-PD 128907 on midbrain dopamine neurones in rat brain slices by a selective D2 receptor antagonist L-741,626. Br. J. Pharmacol. 119 (7), 1491–1497. https://doi.org/10.1111/j.1476-5381.1996.tb16063.x.

Britto-Júnior, J., Campos, R., Peixoto, M., Lima, A.T., Jacintho, F.F., Mónica, F.Z., Antunes, E., De Nucci, G., 2022. 6-nitrodopamine acts as an endogenous selective dopamine receptor antagonist in Chelonoidis carbonaria aorta. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 1532-0456. https://doi.org/10.1016/j.cbpc.2022.109403.

Britto-Júnior, J., Coelho-Silva, W.C., Murari, G.F., Serpellone Nash, C.E., Mónica, F.Z., Antunes, E., De Nucci, G., Murari, G.F., 2021a. 6-Nitrodopamine is released by human umbilical cord vessels and modulates vascular reactivity. Life Sci. 276, 119425 https://doi.org/10.1016/j.lfs.2021.119425. PMID: 33781827.

Britto-Júnior, J., Pinheiro, D.H.A., Justo, A.F.O., Figueiredo Murari, G.M., Campos, R., Mariano, F.V., de Souza, V.B., Schenka, A.A., Mónica, F.Z., Antunes, E., De Nucci, G., 2020. Endothelium-derived dopamine modulates EFS-induced contractions of human umbilical vessels. Pharmacol. Res. Perspect. 8 (4), e00612 https://doi.org/10.1002/prp2.612.

Britto-Júnior, J., Ximenes, L., Ribeiro, A., Fregonesi, A., Campos, R., Kiguti, L., Mónica, F. Z., Antunes, E., De Nucci, G., 2021b. 6-nitrodopamine is an endogenous mediator of rat isolated epididymal vas deferens contractions induced by electric-field stimulation. Eur. J. Pharmacol. 15 (911), 174544 https://doi.org/10.1016/j.ejphar.2021.174544. Indicar a letra para 2021.

Campos, R., Jacintho, F.F., Britto-Júnior, J., Mónica, F.Z., Justo, A.F.O., Pupo, A.S., Moreno, R.A., Souza, V.B., Schenka, A.A., Antunes, E., De Nucci, G., 2020. Endothelium modulates electrical field stimulation-induced contractions of chelonoidis carbonaria aortic rings. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 233, 108763 https://doi.org/10.1016/j.cbpc.2020.108763.

Campos, R., Justo, A.F.O., Mónica, F.Z., Cogo, J.C., Moreno, R.A., de Souza, V.B., Schenka, A.A., De Nucci, G., 2018b. Electrical field-induced contractions on Crotalus durissus terrificus and Bothrops jararaca aortae are caused by endothelium-derived catecholamine. PLoS One 10 (13), e0203573. https://doi.org/10.1371/journal.pone.0203573.

Campos, R., Mónica, F.Z., Justo, A.F.O., Cogo, J.C., Oliveira, E.T., Moreno, R.A., Antunes, E., De Nucci, G., 2018. Electrical field stimulation-induced contractions on *Pantherophis guttatus* corpora cavernosa and aortae. PLoS One 13 (4), e0196123. https://doi.org/10.1371/journal.pone.0196123. PMID: 29672643; PMCID: PMC5908133.

Campos, R., Pinheiro, D.H.A., Britto-Júnior, J., Castro, H.A., Mendes, G.D., Moraes, M.O., Moraes, M.E.A., Lopes-Martins, R.Á.B., Antunes, N.J., De Nucci, G., 2021. Quantification of 6-nitrodopamine in Krebs-Henseleit's solution by LC-MS/MS for the assessment of its basal release from chelonoidis carbonaria aortae in vitro. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 22 (1173), 122668 https://doi.org/10.1016/j.jchromb.2021.122668.

Cechova, S., Pajewski, T.N., 2004. The soluble guanylyl cyclase inhibitor ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, dose-dependently reduces the threshold for isoflurane anesthesia in rats. Anesth. Analg. 99 (3), 752–757. https://doi.org/10.1213/01.ANE.0000132695.77191.F3.

Farha, K., Baljé-Volkers, C., Tamminga, W., Den Daas, I., Van Os, S., 2014. Dopamine D2R agonist-induced cardiovascular effects in healthy male subjects: potential implications in clinical settings. ISRN Neurol. 22 (2014), 956353 https://doi.org/10.1155/2014/956353.

Filogonio, R., Sartori, M.R., Morgensen, S., Tavares, D., Campos, R., Abe, A.S., Taylor, E. W., Rodrigues, G.J., De Nucci, G., Simonsen, U., Leite, C.A.C., Wang, T., 2020. Cholinergic regulation along the pulmonary arterial tree of the south american rattlesnake: vascular reactivity, muscarinic receptors, and vagal innervation. Am. J. Phys. Regul. Integr. Comp. Phys. 1 (319), 2. https://doi.org/10.1152/ajpregu.00310.2019. R156–R170.

Gould, L., Reddy, C.V., 1976. Phentolamine. Am. Heart J. 92 (3), 397–402. https://doi.org/10.1016/s0002-8703(76)80121-4.

Lillywhite, H.B., Albert, J.S., Sheehy, C.M., Seymour, R.S., 2012. Gravity and the evolution of cardiopulmonary morphology in snakes. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 161 (2), 230–242. https://doi.org/10.1016/j.cbpa.2011.10.029.

Lillywhite, H.B., 1988. Snakes, blood circulation and gravity. Sci. Am. 259 (6), 92–99.

Morona, R., López, J.M., González, A., 2011. Localization of calbindin-d28k and calretinin in the brain of dermophis mexicanus (amphibia: gymnophiona) and its bearing on the interpretation of newly recognized neuroanatomical regions. Brain Behav. Evol. 77 (4), 231–269. https://doi.org/10.1159/000329521.

Narahashi, T., Moore, J.W., Poston, R.N., 1967. Tetrodotoxin derivatives: chemical structure and blockage of nerve membrane conductance. Science 19 (156), 3777. https://doi.org/10.1126/science.156.3777.976, 976–9.

Schrammel, A., Behrends, S., Schmidt, K., Koesling, D., Mayer, B., 1996. Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. Mol. Pharmacol. 50 (1), 1–5.

Reilly, F.D., Russell, P.T., 1997. Neurohistochemical evidence supporting an absence of adrenergic and cholinergic innervation in the human placenta and umbilical cord. Anat. Rec. 188 (3), 277–286. https://doi.org/10.1002/ar.1091880302.

Schneider, C.S., Mierau, J., 1987. Dopamine autoreceptor agonists: resolution and pharmacological activity of 2,6-diaminotetrahydrobenzothiazole and an aminothiazole analogue of apomorphine. J. Med. Chem. 30 (3), 494–498. https://doi.org/10.1021/jm00386a009.

Mónica, F.Z., Rojas-Moscoso, J., Porto, M., Schenka, A.A., Antunes, E., Cogo, J.C., De Nucci, G., 2012. Immunohistochemical and functional characterization of nitric oxide signaling pathway in isolated aorta from Crotalus durissus terrificus. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 155 (3), 433–439. https://doi.org/10.1016/j.cbpc.2011.11.003.

Murad, F., Waldman, S.A., Fiscus, R.R., Rapoport, R.M., 1986. Regulation of cyclic GMP synthesis and the interactions with calcium. J. Cardiovasc. Pharmacol. 8 (S8) https://doi.org/10.1097/00005344-198600088-00012. S57–60.

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W. J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E.J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E.S., Silberberg, S.D., Steckler, T., Würbel, H., 2020. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. PLoS Biol. 14 (18), e3000410 https://doi.org/10.1371/journal.pbio.3000410, 7.

Owens, M.J., Morgan, W.N., Plott, S.J., Nemeroff, C.B., 1989. Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites, 1997 Dec J. Pharmacol. Exp. Ther. 283 (3), 1305–1322. PMID: 9400006.

Quigg, R.J., Rocco, M.B., Gauthier, D.F., Creager, M.A., Hartley, L.H., Colucci, W.S., 1989. Mechanism of the attenuated peak heart rate response to exercise after orthotopic cardiac transplantation. J. Am. Coll. Cardiol. 14 (2), 338–344. https://doi.org/10.1016/0735-1097(89)90183-6.

United States Food and Drug Administration (FDA), 2001. Bioanalytical Method Validation Guidelines.

Anexo 13

Entrevista: First person – José Britto-Júnior
Revista: Biology Open
Data da publicação 20 janeiro 2021
Na coleção: BiO: First Person interviews

© 2021. Published by The Company of Biologists Ltd | Biology Open (2021) 9, bio058305. doi:10.1242/bio.058305

FIRST PERSON

First person – José Britto-Júnior

First Person is a series of interviews with the first authors of a selection of papers published in Biology Open, helping early-career researchers promote themselves alongside their papers. José Britto-Júnior is first author on 'The basal release of endothelium-derived catecholamines regulates the contractions of Chelonoidis carbonaria aorta caused by electrical-field stimulation', published in BiO. José conducted the research described in this article while a master's student in Professor Matheus L. Rocha's laboratory at Faculty of Pharmacy, University of Goias, and is now a PhD student in the Department of Pharmacology at the University of Campinas, Brasil, investigating basic cardiovascular pharmacology, endothelium, endothelial catecholamines and comparative physiology.

What is your scientific background and the general focus of your lab?
Basic vascular pharmacology. The lab is focused on endothelium-derived catecholamines.

How would you explain the main findings of your paper to non-scientific family and friends?
We have demonstrated that tortoise vascular tissue presents basal release of catecholamines. These substances are important vasoactive mediators and until now thought to be released only from nerve terminals and adrenal glands.

"We have demonstrated that tortoise vascular tissue presents basal release of catecholamines."

What are the potential implications of these results for your field of research?
These endothelium-derived mediators may constitute important therapeutic targets for treatment of vascular diseases such as hypertension.

What has surprised you the most while conducting your research?
In this era of molecular biology, how interesting are bioassay experiments.

José Britto-Júnior's contact details: Rua Tessália Vieira de Camargo,126 Cidade Universitária Zeferino Vaz. CEP 13083-887, SP, Brasil.
E-mail: josebrittojr@gmail.com

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

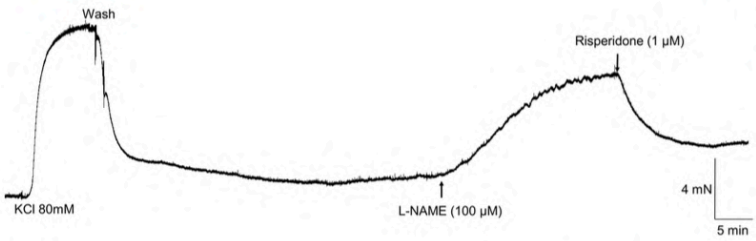
José Britto-Júnior

What, in your opinion, are some of the greatest achievements in your field and how has this influenced your research?
The discovery of nitric oxide and endothelin release by endothelial cells.

What changes do you think could improve the professional lives of early-career scientists?
Increase availability of fellowships for post-graduate education.

What's next for you?
To do a post-doc after finishing my PhD.

Biology Open (2021) 9, bio058305. doi:10.1242/bio.058305



Representative tracing showing the reversal by risperidone (1 μM; n = 5/5) of the elevated tonus induced by L-NAME (100 μM) in aortic rings of *Chelonoidis carbonaria*.

Reference
Britto-Júnior, J., Jacintho, F. F., Campos, R., Araújo Pinheiro, D. H., Murari, G. M. F., de Souza, V. B., Schenka, A. A., Mónica, F. Z. and Moreno, R. A. (2021). The basal release of endothelium-derived catecholamines regulates the contractions of Chelonoidis carbonaria aorta caused by electrical-field stimulation. *Biology Open* **9**, 057042. doi:10.1242/bio.057042

Anexo 14 - PARECER DO COMITE DE ÉTICA

USP - INSTITUTO DE CIÊNCIAS BIOMÉDICAS DA UNIVERSIDADE DE SÃO PAULO - ICB/USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: GDN 030/18 - Avaliação dos vasos do cordão umbilical humano como fonte de catecolaminas produzidas pelo endotélio

Pesquisador: GILBERTO DE NUCCI

Área Temática:

Versão: 3

CAAE: 06379418.0.0000.5467

Instituição Proponente: Instituto de Ciências Biomédicas da Universidade de São Paulo - ICB/USP

Patrocinador Principal: Financiamento Próprio

DADOS DA NOTIFICAÇÃO

Tipo de Notificação: Outros

Detalhe: Aumento do número de cordões

Justificativa: Justificativa para aumento do número de cordões umbilicais.

Data do Envio: 01/08/2019

Situação da Notificação: Parecer Consubstanciado Emitido

DADOS DO PARECER

Número do Parecer: 3.588.498

Apresentação da Notificação:

O Pesquisador principal apresenta dados até então obtidos, inéditos, e artigo enviado para publicação.

Objetivo da Notificação:

Autorização para aumentar o número de cordões umbilicais em função dos resultados até então obtidos.

Avaliação dos Riscos e Benefícios:

Não pertinente

Comentários e Considerações sobre a Notificação:

Os resultados obtidos em cordões umbilicais são muito interessantes e até com ineditismo. Com isso o grupo pretende expandir os experimentos. Para tanto há necessidade de aumentar o

Endereço: Av. Profº Lineu Prestes, 2415
Bairro: Cidade Universitária **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-7733 **E-mail:** cep@icb.usp.br

número de cordões umbilicais a serem coletados.

Considerações sobre os Termos de apresentação obrigatória:

Sem alteração em razão de presente notificação.

Recomendações:

Sem recomendações.

Conclusões ou Pendências e Lista de Inadequações:

Conclui-se pela aprovação por não conter inadequações ou pendências.

Considerações Finais a critério do CEP:

O Colegiado acata o parecer do Relator.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Outros	03018_notificacao_CEP.pdf	01/08/2019 11:17:40	GILBERTO DE NUCCI	Postado

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SAO PAULO, 20 de Setembro de 2019

Assinado por:
Regina Scivoletto
(Coordenador(a))

Endereço: Av. Profº Lineu Prestes, 2415
Bairro: Cidade Universitária **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-7733 **E-mail:** cep@icb.usp.br

USP - INSTITUTO DE CIÊNCIAS BIOMÉDICAS DA UNIVERSIDADE DE SÃO PAULO - ICB/USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: GDN 030/18 - Avaliação dos vasos do cordão umbilical humano como fonte de catecolaminas produzidas pelo endotélio

Pesquisador: GILBERTO DE NUCCI

Área Temática:

Versão: 3

CAAE: 06379418.0.0000.5467

Instituição Proponente: Instituto de Ciências Biomédicas da Universidade de São Paulo - ICB/USP

Patrocinador Principal: Financiamento Próprio

DADOS DA NOTIFICAÇÃO

Tipo de Notificação: Outros

Detalhe: Aumento do número de cordões

Justificativa: Justificativa para aumento do número de cordões umbilicais.

Data do Envio: 01/08/2019

Situação da Notificação: Parecer Consubstanciado Emitido

DADOS DO PARECER

Número do Parecer: 3.588.498

Apresentação da Notificação:

O Pesquisador principal apresenta dados até então obtidos, inéditos, e artigo enviado para publicação.

Objetivo da Notificação:

Autorização para aumentar o número de cordões umbilicais em função dos resultados até então obtidos.

Avaliação dos Riscos e Benefícios:

Não pertinente

Comentários e Considerações sobre a Notificação:

Os resultados obtidos em cordões umbilicais são muito interessantes e até com ineditismo. Com isso o grupo pretende expandir os experimentos. Para tanto há necessidade de aumentar o

Endereço: Av. Profº Lineu Prestes, 2415
Bairro: Cidade Universitária **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-7733 **E-mail:** cep@icb.usp.br

USP - INSTITUTO DE CIÊNCIAS BIOMÉDICAS DA UNIVERSIDADE DE SÃO PAULO - ICB/USP

Continuação do Parecer: 3.588.498

número de cordões umbilicais a serem coletados.

Considerações sobre os Termos de apresentação obrigatória:

Sem alteração em razão de presente notificação.

Recomendações:

Sem recomendações.

Conclusões ou Pendências e Lista de Inadequações:

Conclui-se pela aprovação por não conter inadequações ou pendências.

Considerações Finais a critério do CEP:

O Colegiado acata o parecer do Relator.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Outros	03018_notificacao_CEP.pdf	01/08/2019 11:17:40	GILBERTO DE NUCCI	Postado

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SAO PAULO, 20 de Setembro de 2019

Assinado por:
Regina Scivoletto
(Coordenador(a))

Endereço: Av. Profº Lineu Prestes, 2415
Bairro: Cidade Universitária **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-7733 **E-mail:** cep@icb.usp.br

UNICAMP - CAMPUS CAMPINAS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: GDN 018/20 - PAPEL FISIOPATOLÓGICO DAS CATECOLAMINAS ENDOTELIAIS DO CORDÃO UMBILICAL HUMANO EM GESTAÇÕES DE RISCO

Pesquisador: GILBERTO DE NUCCI

Área Temática:

Versão: 1

CAAE: 40561020.6.0000.5404

Instituição Proponente: Hospital da Mulher Prof. Dr. José Aristodemo Pinotti - CAISM

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.469.687

Apresentação do Projeto:

As informações contidas nos campos "Apresentação do Projeto", "Objetivo da Pesquisa" e "Avaliação dos Riscos e Benefícios" foram obtidas dos documentos apresentados para apreciação ética e das informações inseridas pelo Pesquisador Responsável do estudo na Plataforma Brasil.

As catecolaminas como dopamina, epinefrina, norepinefrina, L-DOPA são uma classe de metabólitos de monoamina que se diferem pelo seu radical na cadeia de compostos orgânicos; catecol - anel benzênico com dois grupos hidroxil adjacentes nas posições 3' e 4' uma amina ligada a um grupamento etil, derivados do aminoácido tirosina. Os procedimentos experimentais com cordões umbilicais podem ampliar o conhecimento sobre a fisiopatologia de diversas condições e tem potencial de identificar possíveis alvos terapêuticos futuros para diversas situações derivadas do conhecimento adquirido pela pesquisa básica

Hipótese: O endotélio presente nos vasos umbilicais estariam habilitados a participar do controle do tônus local por produzirem e secretarem catecolaminas.

Metodologia Proposta: Os cordões umbilicais serão provenientes de parte do descarte após o parto. Fragmento com aproximadamente 10 (dez) centímetros serão fornecidos pelo médico ou enfermagem e imediatamente lavado com o tampão adequado (Krebs-Henseleit) para manter a viabilidade do tecido. Serão coletadas apenas as porções destinadas ao descarte biológico. Desta forma, a coleta do cordão não acarretará nenhum dano nem riscos à saúde das parturientes ou dos lactentes.O isolamento dos vasos (artérias e veia) do cordão umbilical humano (HUCV) será

Endereço: Rua Tessália Vieira de Camargo, 126
Bairro: Barão Geraldo **CEP:** 13.083-887
UF: SP **Município:** CAMPINAS
Telefone: (19)3521-8936 **Fax:** (19)3521-7187 **E-mail:** cep@fcm.unicamp.br

Comentários e Considerações sobre a Pesquisa:

1) Descrição do estudo: estudo laboratorial com amostras de tecidos humanos obtidos de descarte cirúrgico;
2) Desenho: estudo laboratorial;
3) Medicamento em teste: nenhum;
4) Tratamento: nenhum;
5) Número de participantes: 70 participantes
6) Patrocinador: financiamento próprio
7) Centro coordenador: CAISM - UNICAMP
8) Centros participantes: centro único;
9) Países participantes: Brasil;
10) Uso de placebo: não se aplica;
11) suspenção do tratamento em uso (wash-out): não se aplica;
12) Acesso ao medicamento após o término do estudo: não se aplica;
13) Utilização de amostras biológicas: não está previsto armazenamento;
14) Cronograma: inclusão de participantes de 04/01/2021 a 28/02/2021;
15) Orçamento: R$ 2.200,00

Considerações sobre os Termos de apresentação obrigatória:

Foram analisados sete documentos listados no quadro "documentos postados":
-PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1629648.pdf 11/11/2020
-Funcional.jpg 11/11/2020
-Livre_Docente.jpg 11/11/2020
-Parecer_CAISM.pdf 13/10/2020
-01820_TCLE.pdf 13/10/2020
-01820_CordaoUmbilical_Protocolo.pdf 13/10/2020
-Folha_Rosto.pdf 13/10/2020
Ver o campo Conclusões ou Pendências e Lista de Inadequações.

Recomendações:

Ver o campo Conclusões ou Pendências e Lista de Inadequações.

Conclusões ou Pendências e Lista de Inadequações:

Não foram encontrados óbices éticos no projeto proposto.

realizado após a retirada da geléia de Wharton. numa placa de Petri contento a solução de Krebs para transporte, mantida em baixas temperaturas. Após este procedimento os HUCV serão dissecados e livres de todo tecido conectivo e adiposo. Em sequência, os HUCV serão seccionados em anéis de 3-5 mm de comprimento. Critério de Inclusão: 1. Gestantes com idade superior a 18 anos completos e inferior a 50 anos completos;2. Gestantes saudáveis mães de conceptos saudáveis comporão o grupo controle deste estudo;3. Gestantes com doenças dos seguintes grupos: obesidade, diabetes, diabetes gestacional, síndromes hipertensivas na gestação (eclampsia, pré-eclâmpsia, hipertensão arterial sistêmica, etc), hipertensão arterial sistêmica ou mãe de concepto portador de malformação congênita Critério de Exclusão: 1. Gestante com idade inferior a 18 anos completos; 2. Gestante com idade superior a 50 anos completos;3. Gestante com doença outra patologia que não as descritas nos critérios de inclusão;

Objetivo da Pesquisa:

Objetivo Primário: Comparar a reatividade vascular de artérias e veia de cordão umbilical humano e a produção de mediadores vasculares endoteliais entre gestantes saudáveis e gestantes portadoras de patologias, como Síndromes Hipertensivas, Distúrbios Metabólicos e em caso de partos de conceptos com Malformações Congênitas Objetivo Secundário: Caracterizar o papel adrenérgico na contração induzida por EFS em músculo liso vascular de cordão umbilical humano; Identificar e localizar enzimas envolvidas na síntese de catecolaminas (tirosina hidroxilase, dopamina beta-hidroxilase, dopa descarboxilase) em vasos isolados do cordão.

Avaliação dos Riscos e Benefícios:

Riscos: Como a coleta será de um material destinado ao descarte, não haverá riscos para a participante nem para o bebê, uma vez que a equipe de pesquisa não terá contato direto com eles (apenas para assinatura do TCLE). Pode haver risco de exposição da identidade da participante, porém esforços serão feitos para evitar, monitorando toda a equipe de pesquisa Benefícios: Esse estudo não oferecerá nenhum tipo de benefício direto ao participante. Benefícios futuros podem ocorrer após as análises dos resultados sobre a função das catecolaminas nesse aspecto.

Endereço: Rua Tessália Vieira de Camargo, 126
Bairro: Barão Geraldo **CEP:** 13.083-887
UF: SP **Município:** CAMPINAS
Telefone: (19)3521-8936 **Fax:** (19)3521-7187 **E-mail:** cep@fcm.unicamp.br

Diante do exposto e à luz da Resolução CNS 466/2012 e da NO 001/2013, tendo em vista ainda as aprovações prévias do CEP do Centro Coordenador (Hospital de Clínicas da Universidade Federal do Rio Grande do Sul) e da CONEP, o projeto de pesquisa e o Termo de Consentimento Livre e Esclarecido, assim como os demais documentos, podem ser enquadrados na categoria APROVADO.

Considerações Finais a critério do CEP:

- O participante da pesquisa deve receber uma via do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (quando aplicável).

- O participante da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (quando aplicável).

- O pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado. Se o pesquisador considerar a descontinuação do estudo, esta deve ser justificada e somente ser realizada após análise das razões da descontinuidade pelo CEP que o aprovou. O pesquisador deve aguardar o parecer do CEP quanto à descontinuação, exceto quando perceber risco ou dano não previsto ao participante ou quando constatar a superioridade de uma estratégia diagnóstica ou terapêutica oferecida a um dos grupos da pesquisa, isto é, somente em caso de necessidade de ação imediata com intuito de proteger os participantes.

- O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo. É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

- Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas e aguardando a aprovação do CEP para continuidade da pesquisa. Em caso de projetos do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma, junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial.

- Relatórios parciais e final devem ser apresentados ao CEP, inicialmente seis meses após a data deste parecer de aprovação e ao término do estudo.

-Lembramos que segundo a Resolução 466/2012 , item XI.2 letra e, "cabe ao pesquisador apresentar dados solicitados pelo CEP ou pela CONEP a qualquer momento".

-O pesquisador deve manter os dados da pesquisa em arquivo, físico ou digital, sob sua guarda e responsabilidade, por um período de 5 anos após o término da pesquisa.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1629648.pdf	11/11/2020 14:43:25		Aceito
Outros	Funcional.jpg	11/11/2020 14:43:13	GILBERTO DE NUCCI	Aceito
Outros	Livre_Docente.jpg	11/11/2020 14:42:51	GILBERTO DE NUCCI	Aceito
Parecer Anterior	Parecer_CAISM.pdf	13/10/2020 16:57:37	GILBERTO DE NUCCI	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	01820_TCLE.pdf	13/10/2020 16:57:07	GILBERTO DE NUCCI	Aceito
Projeto Detalhado / Brochura Investigador	01820_CordaoUmbilical_Protocolo.pdf	13/10/2020 16:56:51	GILBERTO DE NUCCI	Aceito
Folha de Rosto	Folha_Rosto.pdf	13/10/2020 16:51:44	GILBERTO DE NUCCI	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CAMPINAS, 16 de Dezembro de 2020

Assinado por:
Alessandro Rozim Zorzi
(Coordenador(a))

Endereço: Rua Tessália Vieira de Camargo, 126
Bairro: Barão Geraldo **CEP:** 13.083-887
UF: SP **Município:** CAMPINAS
Telefone: (19)3521-8936 **Fax:** (19)3521-7187 **E-mail:** cep@fcm.unicamp.br

Anexo 14 – Autorização da revista

Terms and Conditions of Sale and Purchase

These Terms and Conditions of Sale and Purchase ("Terms") set forth the terms and conditions governing the purchase of products and services from Elsevier B.V. and/or its affiliated Elsevier company Elsevier Ltd ("Elsevier") through its web site at https://authors.elsevier.com ("Site"). By submitting an order for such products or services, you agree to be bound by these Purchase Terms.

Address

Elsevier B.V. Radarweg 29, 1043 NX Amsterdam, The Netherlands, registered at the Chamber of Commerce Amsterdam, nr. 33158992

Contract Formation

If you wish to purchase any of the products or services offered on this Site, you must submit a completed order form. You will be required to select your products, services or digital content for purchase and complete your delivery and payment details. Once these have been completed, you will have a further chance to check (and correct if necessary) these details prior to submission of your order, and you should print out this page and a copy of these Terms for your future reference.

If we accept your order, you will be sent a confirmation email giving you an order reference number ("Email Confirmation"). Once we have sent this E-mail Confirmation, you will not be able to amend or cancel your order (subject to any right to cancel as described below).

We may correct any error in any unconfirmed order, marketing literature or other document or information issued by us or placed on this Site without any liability.

Right to Cancel

Save as set out below, consumers in the European Union have the right to cancel the purchase until fourteen (14) days after date of delivery of the product or date the service was purchased by sending written notice of cancellation by email to the Site's customer service department. A model cancellation form is available below for your use but you are not obliged to use this method of cancellation.

This right of cancellation applies to all products and services offered on the Site except:

(a) audio, video, and software products that have been unsealed from their packaging;

(b) digital content not on a tangible medium (e.g. eBooks) once the item has been downloaded;

(c) newspapers, periodicals and magazines which are not supplied under subscription contracts;

(d) services for which performance has completed; and

(e) offprints, colour figures, print on demand services and other products made or services provided to your specification or clearly bespoke to you.

If you cancel the purchase on this basis, we will reimburse to you all payments received from you, including the costs of delivery (except for the additional costs arising if you choose a type of delivery other than the least expensive type of standard delivery offered by us). We may make a deduction from the reimbursement for loss in value of any goods supplied, if the loss is the result of unnecessary handling by you.

Returns

You must return the goods without undue delay and in any event no later than 14 days from the date of notification of cancellation. We will then make the reimbursement without undue delay, and no later than-

(a) 14 days after the day we receive back from you any goods supplied, or

(b) (if earlier) 14 days after the day you provide evidence that you have returned the goods, or

(c) If there were no (physical) goods supplied, 14 days after the day on which we are informed about your decision to cancel the purchase.

We will make the reimbursement using the same means of payment as you used for the initial transaction, unless you have expressly agreed otherwise; in any event, you will not incur any fees as a result of the reimbursement.

Whether these rights of cancellation apply or not, you will be responsible for and will pay the cost of returning the items to us by the same method as it was delivered. The items will be returned at your risk.

Prices (including taxes and delivery) and Payment

The currency and price of products and services offered on this Site shall be that quoted on the Site on the date you place your order. While we make every effort to ensure that prices quoted on the Site are accurate, if there has been an inadvertent error, the correct price will be stated in the E-mail Confirmation and you will have the opportunity to withdraw from the order at that stage if the correct price is higher than that quoted and you are not happy with that higher price.

All prices are exclusive of the cost of postage, packaging and delivery and any applicable Value Added Tax or other sales tax, which will be separately stated on the Site and/or the Email Confirmation.

If your order is for Gold Open Access publication of your article in a hybrid journal (that publishes both subscription and Gold Open Access content), you acknowledge that open access publication of your article is conditional on the timely payment of the article publication charge (APC) required by the journal. If the APC is not paid within the invoice deadline, we reserve the right to remove the Creative Commons license from the article, and to publish and distribute the article as we see fit, including offering the article for sale on a subscription basis. We will also require you to remove any copies of the article that you have posted on any online platform(s).

Delivery/Performance

Where applicable, products ordered will be delivered to the shipping address stated in your order, subject to any geographical restrictions stated on the Site. However, the time and

method of delivery of products or performance of services will be at our discretion, unless otherwise specified and accepted as part of the order. Time for delivery or performance shall not be of the essence.

If the items delivered to you do not include all of the items that you ordered in any particular order or include incorrect items, you must inform us in writing as soon as possible. In any event, our liability is limited to the price of the items not delivered or the cost of replacing such items.

Consumer Protection

If you are a consumer, your statutory rights as a consumer are not affected by these Purchase Terms, and nothing in these Purchase Terms shall exclude or limit our liability arising by virtue of consumer protection legislation, or our liability for fraud or for death or personal injury caused by our negligence, or any other liability to the extent that we cannot so exclude or limit such liability as a matter of law.

Complaints

If you have any questions, comments or complaints regarding these Purchase Terms or your order, please contact our Customer Services Department which is located at:

Author Support
Elsevier Ltd
The Boulevard
Langford Lane
Kidlington
Oxford
OX5 1GB
United Kingdom

Contact us via our Support Center
https://service.elsevier.com
Phone: +44 1865 84 3577

Model Cancellation Form

To: Author Support
Subject: Cancellation of order for [article_reference].

Or, when sending this by post:

Author Support
Elsevier Ltd
The Boulevard, Langford Lane
Kidlington
Oxford
OX5 1GB
United Kingdom

Dear Sir, Madam,

I hereby give notice that I cancel my contract [DELETE AS APPLICABLE: [of sale of the

following goods/for the supply of the following service].

Goods/Service:

Ordered on:
Received on:
Name of consumer:
Address of consumer:

Signature of consumer:

Date:

General
In the event of any inconsistency between these Terms and the Terms and Conditions of the Site, these Terms shall prevail.

Last revised: 21st October 2019

Copyright © 2019 Elsevier B.V. All rights reserved. Privacy Policy Terms & Conditions Help

Pharmacology Research & Perspectives

Published by Wiley and British Pharmacological Society and American Society for Pharmacology and Experimental Therapeutics (together the "Owner")

LICENSE AGREEMENT FOR PUBLISHING CC-BY

Date: May 21, 2020

Contributor name: José Britto-Júnior

Contributor address:

Manuscript number: PRP2-2020-03-0042.R1

Re: Manuscript entitled Endothelium-derived dopamine modulates EFS-induced contractions of human umbilical vessels (the "Contribution")

for publication in Pharmacology Research & Perspectives (the "Journal")

published by John Wiley & Sons Ltd ("Wiley")

Dear Contributor(s):

Thank you for submitting your Contribution for publication. In order to expedite the editing and publishing process and enable Wiley to disseminate your Contribution to the fullest extent, we need to have this Agreement executed. If the Contribution is not accepted for publication, or if the Contribution is subsequently rejected, this Agreement will be null and void.
Publication cannot proceed without a signed copy of this Agreement and payment of the appropriate article publication charge.

A. TERMS OF USE

1. The Contribution will be made Open Access under the terms of the Creative Commons Attribution License which permits use, distribution and reproduction in any medium, provided that the Contribution is properly cited.
2. For an understanding of what is meant by the terms of the Creative Commons License, please refer to Wiley's Open Access Terms and Conditions (http://www.wileyauthors.com/OAA).
3. The Owner (and Wiley, where Wiley is not the Owner) reserves the right to require changes to the Contribution, including changes to the length of the Contribution, as a condition of acceptance. The Owner (and Wiley, where Wiley is not the Owner) reserves the right, notwithstanding acceptance, not to publish the Contribution if for any reason such publication would in the reasonable judgment of the Owner (and Wiley, where Wiley is not the Owner), result in legal liability or violation of journal ethical practices. If the Owner (or Wiley, where Wiley is not the Owner) decides not to publish the Contribution, no Article Processing Charge or any other fee shall be charged. The Contributor is free to submit the Contribution to any other journal from any other publisher.

B. RETAINED RIGHTS

The Contributor or, if applicable, the Contributor's employer, retains all proprietary rights in addition to copyright, such as patent rights in any process, procedure or article of manufacture described in the Contribution.

C. LICENSE

Owner, Wiley (where Wiley is not the Owner), and users have non-exclusive rights under the terms of the Creative Commons Attribution License. For the avoidance of doubt, "Contribution" is defined to only include the article submitted by the Contributor for publication in the Journal (including any embedded rich media) and does not extend to any supporting information submitted with or referred to in the Contribution ("Supporting Information"). To the extent that any Supporting Information is submitted to the Journal, the Owner is granted a perpetual, non-exclusive license to publish, republish, transmit, sell, distribute and otherwise use this Supporting Information in whole or in part in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so.

D. CONTRIBUTIONS OWNED BY EMPLOYER

If the Contribution was written by the Contributor in the course of the Contributor's employment as a "work-made-for-hire" and the employer owns the copyright in the Contribution, the employer company/institution agrees to the terms of use set forth in paragraph A above and must execute this Agreement (in addition to the Contributor) in the space provided below.

E. COPYRIGHT NOTICE

Owner (and Wiley, where Wiley is not the Owner), the Contributor, and the company/institution agree that any and all copies of the Contribution or any part thereof distributed or posted by them in print or electronic format will include the notice of copyright as stipulated in the Journal and a full citation to the final published version of the Contribution in the Journal as published by Wiley.

F. CONTRIBUTOR'S REPRESENTATIONS

The Contributor represents that: (i) the Contribution is the Contributor's original work, all individuals identified as Contributors actually contributed to the Contribution, and all individuals who contributed are included; (ii) if the Contribution was prepared jointly, the Contributor has informed the co-Contributors of the terms of this Agreement and has obtained their signed written permission to execute this Agreement on their behalf as their agent; (iii) the Contribution is submitted only to this Journal and has not been published before, has not been included in another manuscript, and is not currently under consideration or accepted for publication elsewhere; (iv) if excerpts from copyrighted works owned by third parties are included, the Contributor shall obtain written permission from the copyright owners for all uses as set forth in the standard permissions form or the Journal's Author Guidelines, and show credit to the sources in the Contribution; (v) the Contribution and any submitted Supporting Information contain no libelous or unlawful statements, do not infringe upon the rights (including without limitation the copyright, patent or trademark rights) or the privacy of others, do not breach any confidentiality obligation, do not violate a contract or any law, or do not contain material or instructions that might cause harm or injury, and only utilize data that has been obtained in accordance with applicable legal requirements and Journal policies; (vi) there are no conflicts of interest relating to the Contribution, except as disclosed. Accordingly, the Contributor represents that the following information shall be clearly identified on the title page of the Contribution: (1) all financial and material support for the research and work; (2) any financial interests the Contributor or any co-Contributors may have in companies or other entities that have an interest in the information in the Contribution or any submitted Supporting Information (e.g., grants, advisory boards, employment, consultancies, contracts, honoraria, royalties, expert testimony, partnerships, or stock ownership); and (3) indication of no such financial interests if appropriate.

Wiley reserves the right, notwithstanding acceptance, to require changes to the Contribution, including changes to the length of the Contribution, and the right not to publish the Contribution if for any reason such publication would in the reasonable judgment of Wiley, result in legal liability or violation of journal ethical practices.

G. USE OF INFORMATION

The Contributor acknowledges that, during the term of this Agreement and thereafter (for as long as necessary), the Owner (and Wiley, where Wiley is not the owner) may process the Contributor's personal data, including storing or transferring data outside of the country of the Contributor's residence, in order to process transactions related to this Agreement and to communicate with the Contributor and that the Publisher has a legitimate interest in processing the Contributor's personal data. By entering into this Agreement, the Contributor agrees to the processing of the Contributor's personal data (and, where

applicable, confirms that the Contributor has obtained the permission from all other contributors to process their personal data). Wiley shall comply with all applicable laws, statutes and regulations relating to data protection and privacy and shall process such personal data in accordance with Wiley's Privacy Policy located at: https://www.wiley.com/en-us/privacy.

[X] I agree to the OPEN ACCESS AGREEMENT as shown above, consent to execution and delivery of the Open Access Agreement electronically and agree that an electronic signature shall be given the same legal force as a handwritten signature, and have obtained written permission from all other contributors to execute this Agreement on their behalf.

Contributor's signature (type name here): José Britto Júnior

Date: May 21, 2020

SELECT FROM OPTIONS BELOW:

[X] Contributor-owned work

[ ] U.S. Government work

Note to U.S. Government Employees

*A contribution prepared by a U.S. federal government employee as part of the employee's official duties, or which is an official U.S. Government publication, is called a "U.S. Government work", and is in the public domain in the United States. If the Contribution was not prepared as part of the employee's duties or is not an official U.S. government publication, or if at least one author is **not** a U.S. government employee, it is not a U.S. Government work. If at least one author is **not** a U.S. government employee, then the non-government author should also sign the form, selecting the appropriate additional ownership selection option. If more than one author is not a U.S. government employee, one may sign on behalf of the others.*

[ ] U.K. Government work (Crown Copyright)

The rights in a contribution prepared by an employee of a UK government department, agency or other Crown body as part of his/her official duties, or which is an official government publication, belong to the Crown and must be made available under the terms of the Open Government License. Contributors must ensure they comply with departmental regulations and submit the appropriate authorisation to publish. If your status as a government employee legally prevents you from signing this Agreement, please contact the Journal production editor. If this selection does not apply to at least one author in the group, this author should also sign the form, indicating agreement to publication on CC-BY basis and selecting the appropriate additional ownership selection option. If this applies to more than one author, one may sign on behalf of the others.

[ ] Other

Including Other Government work or Non-Governmental Organisation work

Note to Non-U.S., Non-U.K. Government Employees or Non-Governmental Organisation Employees

If you are employed by the World Health Organization or UNU-WIDER, please download a copy of the license agreement from http://www.wileyauthors.com/licensingFAQ and upload the form to the Wiley Author Services Dashboard. If your status as a government or non-governmental organisation employee legally prevents you from signing this Agreement, please contact the Journal production editor.

Name of Government/Non-Governmental Organisation:

_____

[ ] Company/institution owned work (made for hire in the course of employment)

If this selection does not apply to at least one author in the group, this author should also sign the form, indicating agreement to publication on CC-BY basis and selecting the appropriate additional ownership selection option. If this applies to more than one author, one may sign on behalf of the others.

Name of Company/Institution:

_____

Authorized Signature of Employer: _____

Date: _____

Signature of Employee: _____

Date: _____

Biomedical Chromatography

Published by Wiley (the "Owner")

COPYRIGHT TRANSFER AGREEMENT

Date: September 02, 2020

Contributor name: Natalícia Antunes

Contributor address:

Manuscript number: BMC-20-0240.R2

Re: Manuscript entitled Determination of dopamine, noradrenaline and adrenaline in Krebs-Henseleit's solution by liquid chromatography coupled tandem mass spectrometry. Measurement of basal release from Chelonoidis carbonaria aortae in vitro (the "Contribution")

for publication in Biomedical Chromatography (the "Journal")

published by John Wiley & Sons Ltd ("Wiley")

Dear Contributor(s):

Thank you for submitting your Contribution for publication. In order to expedite the editing and publishing process and enable the Owner to disseminate your Contribution to the fullest extent, we need to have this Copyright Transfer Agreement executed. If the Contribution is not accepted for publication, or if the Contribution is subsequently rejected, this Agreement shall be null and void.
Publication cannot proceed without a signed copy of this Agreement.

A. COPYRIGHT

1. The Contributor assigns to the Owner, during the full term of copyright and any extensions or renewals, all copyright in and to the Contribution, and all rights therein, including but not limited to the right to publish, republish, transmit, sell, distribute and otherwise use the Contribution in whole or in part in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so. For the avoidance of doubt, "Contribution" is defined to only include the article submitted by the Contributor for publication in the Journal (including any embedded rich media) and does not extend to any supporting information submitted with or referred to in the Contribution ("Supporting Information"). To the extent that any Supporting Information is submitted to the Journal, the Owner is granted a perpetual, non-exclusive license to publish, republish, transmit, sell, distribute and otherwise use this Supporting Information in whole or in part in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so.

2. Reproduction, posting, transmission or other distribution or use of the final Contribution in whole or in part in any medium by the Contributor as permitted by this Agreement requires a citation to the Journal suitable in form and content as follows: (Title of Article, Contributor, Journal Title and Volume/Issue, Copyright © [year], copyright owner as specified in the Journal, Publisher). Links to the final article on the publisher website are encouraged where appropriate.

B. RETAINED RIGHTS

Notwithstanding the above, the Contributor or, if applicable, the Contributor's employer, retains all proprietary rights other than copyright, such as patent rights, in any process, procedure or article of manufacture described in the Contribution.

C. PERMITTED USES BY CONTRIBUTOR

1. Submitted Version. The Owner licenses back the following rights to the Contributor in the version of the Contribution as originally submitted for publication (the "Submitted Version"):

> **a.** The right to self-archive the Submitted Version on: the Contributor's personal website; a not for profit subject-based preprint server or repository; a Scholarly Collaboration Network (SCN) which has signed up to the STM article sharing principles [http://www.stm-assoc.org/stm-consultations/scn-consultation-2015/] ("Compliant SCNs"); or the Contributor's company/ institutional repository or archive. This right extends to both intranets and the Internet. The Contributor may replace the Submitted Version with the Accepted Version, after any relevant embargo period as set out in paragraph C.2(a) below has elapsed. The Contributor may wish to add a note about acceptance by the Journal and upon publication it is recommended that Contributors add a Digital Object Identifier (DOI) link back to the Final Published Version.
>
> **b.** The right to transmit, print and share copies of the Submitted Version with colleagues, including via Compliant SCNs, provided that there is no systematic distribution of the Submitted Version, e.g. posting on a listserve, network (including SCNs which have not signed up to the STM sharing principles) or automated delivery.

2. Accepted Version. The Owner licenses back the following rights to the Contributor in the version of the Contribution that has been peer-reviewed and accepted for publication, but not final (the "Accepted Version"):

> **a.** The right to self-archive the Accepted Version on: the Contributor's personal website; the Contributor's company/institutional repository or archive; Compliant SCNs; and not for profit subject-based repositories such as PubMed Central, all subject to an embargo period of 12 months for scientific, technical and medical (STM) journals and 24 months for social science and humanities (SSH) journals following publication of the Final Published Version. There are separate arrangements with certain funding agencies governing reuse of the Accepted Version as set forth at the following website: http://www.wileyauthors.com/funderagreements. The Contributor may not update the Accepted Version or replace it with the Final Published Version. The Accepted Version posted must contain a legend as follows: This is the accepted version of the following article: FULL CITE, which has been published in final form at [Link to final article]. This article may be used for non-commercial purposes in accordance with the Wiley Self-Archiving Policy [http://www.wileyauthors.com/self-archiving].
>
> **b.** The right to transmit, print and share copies of the Accepted Version with colleagues, including via Compliant SCNs (in private research groups only before the embargo and publicly after), provided that there is no systematic distribution of the Accepted Version, e.g. posting on a listserve, network (including SCNs which have not signed up to the STM sharing principles) or automated delivery.

3. Final Published Version. The Owner hereby licenses back to the Contributor the following rights with respect to the final published version of the Contribution (the "Final Published Version"):

> **a.** Copies for colleagues. The personal right of the Contributor only to send or transmit individual copies of the Final Published Version in any format to colleagues upon their specific request, and to share copies in private

sharing groups in Compliant SCNs, provided no fee is charged, and further provided that there is no systematic external or public distribution of the Final Published Version, e.g. posting on a listserve, network or automated delivery.

b. Re-use in other publications. The right to re-use the Final Published Version or parts thereof for any publication authored or edited by the Contributor (excluding journal articles) where such re-used material constitutes less than half of the total material in such publication. In such case, any modifications must be accurately noted.

c. Teaching duties. The right to include the Final Published Version in teaching or training duties at the Contributor's institution/place of employment including in course packs, e-reserves, presentation at professional conferences, in-house training, or distance learning. The Final Published Version may not be used in seminars outside of normal teaching obligations (e.g. commercial seminars). Electronic posting of the Final Published Version in connection with teaching/training at the Contributor's company/institution is permitted subject to the implementation of reasonable access control mechanisms, such as user name and password. Posting the Final Published Version on the open Internet is not permitted.

d. Oral presentations. The right to make oral presentations based on the Final Published Version.

4. Article Abstracts, Figures, Tables, Artwork and Selected Text (up to 250 words).

a. Contributors may re-use unmodified abstracts for any non-commercial purpose. For online uses of the abstracts, the Owner encourages but does not require linking back to the Final Published Version.

b. Contributors may re-use figures, tables, artwork, and selected text up to 250 words from their Contributions, provided the following conditions are met:

(i) Full and accurate credit must be given to the Final Published Version.

(ii) Modifications to the figures and tables must be noted. Otherwise, no changes may be made.

(iii) The re-use may not be made for direct commercial purposes, or for financial consideration to the Contributor.

(iv) Nothing herein will permit dual publication in violation of journal ethical practices.

D. CONTRIBUTIONS OWNED BY EMPLOYER

1. If the Contribution was written by the Contributor in the course of the Contributor's employment as a "work-made-for-hire" in the course of employment, the Contribution is owned by the company/institution which must execute this Agreement (in addition to the Contributor's signature). In such case, the company/institution hereby agrees to the terms of use set forth in paragraph A above and assigns to the Owner, during the full term of copyright, all copyright in and to the Contribution for the full term of copyright throughout the world as specified in paragraph A above.

2. In addition to the rights specified as retained in paragraph B above and the rights granted back to the Contributor pursuant to paragraph C above, the Owner hereby grants back, without charge, to such company/institution, its subsidiaries and divisions, the right to make copies of and distribute the Final Published Version internally in print format or electronically on the Company's internal network. Copies so used may not be resold or distributed externally. However, the company/institution may include information and text from the Final Published Version as part of an information package included with software or other products offered for sale or license or included in patent applications. Posting of the Final Published Version by the company/institution on a public access website may only be done with written permission, and payment of any applicable fee(s). Also, upon payment of the applicable reprint fee, the company/institution may distribute print copies of the Final Published Version externally.

E. GOVERNMENT CONTRACTS

In the case of a Contribution prepared under U.S. Government contract or grant, the U.S. Government may reproduce, without charge, all or portions of the Contribution and may authorize others to do so, for official U.S. Government purposes only, if the U.S. Government contract or grant so requires. (U.S. Government, U.K. Government, and other government employees: see notes at end.)

F. COPYRIGHT NOTICE

The Contributor and the company/institution agree that any and all copies of the Final Published Version or any part thereof distributed or posted by them in print or electronic format as permitted herein will include the notice of copyright as stipulated in the Journal and a full citation to the Journal.

G. CONTRIBUTOR'S REPRESENTATIONS

The Contributor represents that: (i) the Contribution is the Contributor's original work, all individuals identified as Contributors actually contributed to the Contribution, and all individuals who contributed are included; (ii) if the Contribution was prepared jointly, the Contributor has informed the co-Contributors of the terms of this Agreement and has obtained their signed written permission to execute this Agreement on their behalf; (iii) the Contribution is submitted only to this Journal and has not been published before, has not been included in another manuscript, and is not currently under consideration or accepted for publication elsewhere; (iv) if excerpts from copyrighted works owned by third parties are included, the Contributor shall obtain written permission from the copyright owners for all uses as set forth in the standard permissions form and the Journal's Author Guidelines, and show credit to the sources in the Contribution; (v) the Contribution and any submitted Supporting Information contain no libelous or unlawful statements, do not infringe upon the rights (including without limitation the copyright, patent or trademark rights) or the privacy of others, do not breach any confidentiality obligation, do not violate a contract or any law, do not contain material or instructions that might cause harm or injury, and only utilize data that has been obtained in accordance with applicable legal requirements and Journal policies; (vi) there are no conflicts of interest relating to the Contribution, except as disclosed. Accordingly, the Contributor represents that the following information shall be clearly identified on the title page of the Contribution: (1) all financial and material support for the research and work; (2) any financial interests the Contributor or any co-Contributors may have in companies or other entities that have an interest in the information in the Contribution or any submitted Supporting Information (e.g., grants, advisory boards, employment, consultancies, contracts, honoraria, royalties, expert testimony, partnerships, or stock ownership); and (3) indication of no such financial interests if appropriate.

Wiley reserves the right, notwithstanding acceptance, to require changes to the Contribution, including changes to the length of the Contribution, and the right not to publish the Contribution if for any reason such publication would in the reasonable judgment of Wiley, result in legal liability or violation of journal ethical practices.

H. USE OF INFORMATION

The Contributor acknowledges that, during the term of this Agreement and thereafter, the Owner (and Wiley where Wiley is not the Owner) may process the Contributor's personal data, including storing or transferring data outside of the country of the Contributor's residence, in order to process transactions related to this Agreement and to communicate with the Contributor, and that the Publisher has a legitimate interest in processing the Contributor's personal data. By entering into this Agreement, the Contributor agrees to the processing of the Contributor's personal data (and, where applicable, confirms that the Contributor has obtained the permission from all other contributors to process their personal data). Wiley shall comply with all applicable laws, statutes and regulations relating to data protection and privacy and shall process such personal data in accordance with Wiley's Privacy Policy located at: https://www.wiley.com/en-us/privacy.

[X] I agree to the COPYRIGHT TRANSFER AGREEMENT as shown above, consent to execution and delivery of the Copyright Transfer Agreement electronically and agree that an electronic signature shall be given the same legal force as a handwritten signature, and have obtained written permission from all other contributors to execute this Agreement on their behalf.

Contributor's signature (type name here): Natalicia de Jesus Antunes

Date: September 02, 2020

SELECT FROM OPTIONS BELOW:

[X] Contributor-owned work

[ ] U.S. Government work

Note to U.S. Government Employees

A contribution prepared by a U.S. federal government employee as part of the employee's official duties, or which is an official U.S. government publication, is called a "U.S. government work", and is in the public domain in the United States. If the Contribution was not prepared as part of the employee's duties, is not an official U.S. government publication, or if at least one author is ***not*** *a U.S. government employee, it is not a U.S. government work. If at least one author is* ***not*** *a U.S. government employee, then the non-government author should also sign the form, selecting the appropriate ownership option. If more than one author is not a U.S. government employee, one may sign on behalf of the others.*

[ ] U.K. Government work (Crown Copyright)

Note to U.K. Government Employees

For Crown Copyright this form should be signed in the Contributor's signatures section above by the appropriately authorised individual and uploaded to the Wiley Author Services Dashboard. For production editor contact details please visit the Journal's online author guidelines. *The rights in a contribution prepared by an employee of a UK government department, agency or other Crown body as part of his/her official duties, or which is an official government publication, belong to the Crown and must be made available under the terms of the Open Government Licence. Contributors must ensure they comply with departmental regulations and submit the appropriate authorisation to publish. If your status as a government employee legally prevents you from signing this Agreement, please contact the Journal production editor. If this selection does not apply to at least one author in the group, this author should also sign the form, indicating transfer of those rights which that author has and selecting the appropriate additional ownership selection option. If this applies to more than one author, one may sign on behalf of the others.*

[ ] Other

Including Other Government work or Non-Governmental Organisation work

Note to Non-U.S., Non-U.K. Government Employees or Non-Governmental Organisation Employees

For Other Government or Non-Governmental Organisation work this form should be signed in the Contributor's signatures section above by the appropriately authorised individual and uploaded to the Wiley Author Services Dashboard. For production editor contact details please visit the Journal's online author guidelines. *If you are employed by the Australian Government, the World Bank, the World Health Organization, the International Monetary Fund, the European Atomic Energy Community, the Jet Propulsion Laboratory at California Institute of Technology, the Asian Development Bank, the Bank of International Settlements, or are a Canadian Government civil servant, please download a copy of the license agreement from http://www.wileyauthors.com /licensingFAQ and upload the form to the Wiley Author Services Dashboard. If your status as a government or non-*

governmental organisation employee legally prevents you from signing this Agreement, please contact the Journal production editor.

Name of Government/Non-Governmental Organisation:

_____

[ ] Company/institution owned work (made for hire in the course of employment)

For "work made for hire" this form should be signed and uploaded to the Wiley Author Services Dashboard.

For production editor contact details please visit the Journal's online author guidelines. *If you are an employee of Amgen, please download a copy of the company addendum from http://www.wileyauthors.com/licensingFAQ and return your signed license agreement along with the addendum. If this selection does not apply to at least one author in the group, this author should also sign the form, indicating transfer of those rights which that author has and selecting the appropriate additional ownership selection option. If this applies to more than one author, one may sign on behalf of the others.*

Name of Company/Institution:

_____

Authorized Signature of Employer: _____

Date: _____

Signature of Employee: _____

Date: _____

Terms and Conditions of Sale and Purchase

These Terms and Conditions of Sale and Purchase ("Terms") set forth the terms and conditions governing the purchase of products and services from Elsevier B.V. and/or its affiliated Elsevier company Elsevier Ltd ("Elsevier") through its web site at https://authors.elsevier.com ("Site"). By submitting an order for such products or services, you agree to be bound by these Purchase Terms.

Address

Elsevier B.V. Radarweg 29, 1043 NX Amsterdam, The Netherlands, registered at the Chamber of Commerce Amsterdam, nr. 33158992

Contract Formation

If you wish to purchase any of the products or services offered on this Site, you must submit a completed order form. You will be required to select your products, services or digital content for purchase and complete your delivery and payment details. Once these have been completed, you will have a further chance to check (and correct if necessary) these details prior to submission of your order, and you should print out this page and a copy of these Terms for your future reference.

If we accept your order, you will be sent a confirmation email giving you an order reference number ("Email Confirmation"). Once we have sent this E-mail Confirmation, you will not be able to amend or cancel your order (subject to any right to cancel as described below).

We may correct any error in any unconfirmed order, marketing literature or other document or information issued by us or placed on this Site without any liability.

Right to Cancel

Save as set out below, consumers in the European Union have the right to cancel the purchase until fourteen (14) days after date of delivery of the product or date the service was purchased by sending written notice of cancellation by email to the Site's customer service department. A model cancellation form is available below for your use but you are not obliged to use this method of cancellation.

This right of cancellation applies to all products and services offered on the Site except:

(a) audio, video, and software products that have been unsealed from their packaging;

(b) digital content not on a tangible medium (e.g. eBooks) once the item has been downloaded;

(c) newspapers, periodicals and magazines which are not supplied under subscription contracts;

(d) services for which performance has completed; and

(e) offprints, colour figures, print on demand services and other products made or services provided to your specification or clearly bespoke to you.

If you cancel the purchase on this basis, we will reimburse to you all payments received from you, including the costs of delivery (except for the additional costs arising if you choose a type of delivery other than the least expensive type of standard delivery offered by us). We may make a deduction from the reimbursement for loss in value of any goods supplied, if the loss is the result of unnecessary handling by you.

Returns

You must return the goods without undue delay and in any event no later than 14 days from the date of notification of cancellation. We will then make the reimbursement without undue delay, and no later than-

(a) 14 days after the day we receive back from you any goods supplied, or

(b) (if earlier) 14 days after the day you provide evidence that you have returned the goods, or

(c) If there were no (physical) goods supplied, 14 days after the day on which we are informed about your decision to cancel the purchase.

We will make the reimbursement using the same means of payment as you used for the initial transaction, unless you have expressly agreed otherwise; in any event, you will not incur any fees as a result of the reimbursement.

Whether these rights of cancellation apply or not, you will be responsible for and will pay the cost of returning the items to us by the same method as it was delivered. The items will be returned at your risk.

Prices (including taxes and delivery) and Payment

The currency and price of products and services offered on this Site shall be that quoted on the Site on the date you place your order. While we make every effort to ensure that prices quoted on the Site are accurate, if there has been an inadvertent error, the correct price will be stated in the E-mail Confirmation and you will have the opportunity to withdraw from the order at that stage if the correct price is higher than that quoted and you are not happy with that higher price.

All prices are exclusive of the cost of postage, packaging and delivery and any applicable Value Added Tax or other sales tax, which will be separately stated on the Site and/or the Email Confirmation.

Delivery/Performance

Where applicable, products ordered will be delivered to the shipping address stated in your order, subject to any geographical restrictions stated on the Site. However, the time and method of delivery of products or performance of services will be at our discretion, unless otherwise specified and accepted as part of the order. Time for delivery or performance shall not be of the essence.

If the items delivered to you do not include all of the items that you ordered in any particular order or include incorrect items, you must inform us in writing as soon as possible. In any event, our liability is limited to the price of the items not delivered or the cost of replacing such items.

Consumer Protection

If you are a consumer, your statutory rights as a consumer are not affected by these Purchase Terms, and nothing in these Purchase Terms shall exclude or limit our liability arising by virtue of consumer protection legislation, or our liability for fraud or for death or personal injury caused by our negligence, or any other liability to the extent that we cannot so exclude or limit such liability as a matter of law.

Complaints

If you have any questions, comments or complaints regarding these Purchase Terms or your order, please contact our Customer Services Department which is located at:

Author Support
Elsevier Ltd
The Boulevard
Langford Lane
Kidlington
Oxford
OX5 1GB
United Kingdom

Contact us via our Support Center
https://service.elsevier.com
Phone: +44 1865 84 3577

Model Cancellation Form

To: Author Support
Subject: Cancellation of order for [article_reference].

Or, when sending this by post:

Author Support
Elsevier Ltd
The Boulevard, Langford Lane
Kidlington
Oxford
OX5 1GB
United Kingdom

Dear Sir, Madam,

I hereby give notice that I cancel my contract [DELETE AS APPLICABLE: [of sale of the following goods/for the supply of the following service].

Goods/Service:

Ordered on:
Received on:
Name of consumer:
Address of consumer:

Signature of consumer:

Date:

General
In the event of any inconsistency between these Terms and the Terms and Conditions of the Site, these Terms shall prevail.

Last revised: 19th January 2022

Copyright © 2022 Elsevier B.V. All rights reserved. Privacy Policy Terms & Conditions Help

Terms and Conditions of Sale and Purchase

These Terms and Conditions of Sale and Purchase ("Terms") set forth the terms and conditions governing the purchase of products and services from Elsevier B.V. and/or its affiliated Elsevier company Elsevier Ltd ("Elsevier") through its web site at https://authors.elsevier.com ("Site"). By submitting an order for such products or services, you agree to be bound by these Purchase Terms.

Address

Elsevier B.V. Radarweg 29, 1043 NX Amsterdam, The Netherlands, registered at the Chamber of Commerce Amsterdam, nr. 33158992

Contract Formation

If you wish to purchase any of the products or services offered on this Site, you must submit a completed order form. You will be required to select your products, services or digital content for purchase and complete your delivery and payment details. Once these have been completed, you will have a further chance to check (and correct if necessary) these details prior to submission of your order, and you should print out this page and a copy of these Terms for your future reference.

If we accept your order, you will be sent a confirmation email giving you an order reference number ("Email Confirmation"). Once we have sent this E-mail Confirmation, you will not be able to amend or cancel your order (subject to any right to cancel as described below).

We may correct any error in any unconfirmed order, marketing literature or other document or information issued by us or placed on this Site without any liability.

Right to Cancel

Save as set out below, consumers in the European Union have the right to cancel the purchase until fourteen (14) days after date of delivery of the product or date the service was purchased by sending written notice of cancellation by email to the Site's customer service department. A model cancellation form is available below for your use but you are not obliged to use this method of cancellation.

This right of cancellation applies to all products and services offered on the Site except:

(a) audio, video, and software products that have been unsealed from their packaging;

(b) digital content not on a tangible medium (e.g. eBooks) once the item has been downloaded;

(c) newspapers, periodicals and magazines which are not supplied under subscription contracts;

(d) services for which performance has completed; and

(e) offprints, colour figures, print on demand services and other products made or services provided to your specification or clearly bespoke to you.

If you cancel the purchase on this basis, we will reimburse to you all payments received from you, including the costs of delivery (except for the additional costs arising if you choose a type of delivery other than the least expensive type of standard delivery offered by us). We may make a deduction from the reimbursement for loss in value of any goods supplied, if the loss is the result of unnecessary handling by you.

Returns

You must return the goods without undue delay and in any event no later than 14 days from the date of notification of cancellation. We will then make the reimbursement without undue delay, and no later than-

(a) 14 days after the day we receive back from you any goods supplied, or

(b) (if earlier) 14 days after the day you provide evidence that you have returned the goods, or

(c) If there were no (physical) goods supplied, 14 days after the day on which we are informed about your decision to cancel the purchase.

We will make the reimbursement using the same means of payment as you used for the initial transaction, unless you have expressly agreed otherwise; in any event, you will not incur any fees as a result of the reimbursement.

Whether these rights of cancellation apply or not, you will be responsible for and will pay the cost of returning the items to us by the same method as it was delivered. The items will be returned at your risk.

Prices (including taxes and delivery) and Payment

The currency and price of products and services offered on this Site shall be that quoted on the Site on the date you place your order. While we make every effort to ensure that prices quoted on the Site are accurate, if there has been an inadvertent error, the correct price will be stated in the E-mail Confirmation and you will have the opportunity to withdraw from the order at that stage if the correct price is higher than that quoted and you are not happy with that higher price.

All prices are exclusive of the cost of postage, packaging and delivery and any applicable Value Added Tax or other sales tax, which will be separately stated on the Site and/or the Email Confirmation.

Delivery/Performance

Where applicable, products ordered will be delivered to the shipping address stated in your order, subject to any geographical restrictions stated on the Site. However, the time and method of delivery of products or performance of services will be at our discretion, unless otherwise specified and accepted as part of the order. Time for delivery or performance shall not be of the essence.

If the items delivered to you do not include all of the items that you ordered in any particular order or include incorrect items, you must inform us in writing as soon as possible. In any event, our liability is limited to the price of the items not delivered or the cost of replacing such items.

Consumer Protection

If you are a consumer, your statutory rights as a consumer are not affected by these Purchase Terms, and nothing in these Purchase Terms shall exclude or limit our liability arising by virtue of consumer protection legislation, or our liability for fraud or for death or personal injury caused by our negligence, or any other liability to the extent that we cannot so exclude or limit such liability as a matter of law.

Complaints

If you have any questions, comments or complaints regarding these Purchase Terms or your order, please contact our Customer Services Department which is located at:

Author Support
Elsevier Ltd
The Boulevard
Langford Lane
Kidlington
Oxford
OX5 1GB
United Kingdom

Contact us via our Support Center
https://service.elsevier.com
Phone: +44 1865 84 3577

Model Cancellation Form

To: Author Support
Subject: Cancellation of order for [article_reference].

Or, when sending this by post:

Author Support
Elsevier Ltd
The Boulevard, Langford Lane
Kidlington
Oxford
OX5 1GB
United Kingdom

Dear Sir, Madam,

I hereby give notice that I cancel my contract [DELETE AS APPLICABLE: [of sale of the following goods/for the supply of the following service].

Goods/Service:

Ordered on:
Received on:
Name of consumer:
Address of consumer:

Signature of consumer:

Date:

General
In the event of any inconsistency between these Terms and the Terms and Conditions of the Site, these Terms shall prevail.

Last revised: 19th January 2022

Copyright © 2022 Elsevier B.V. All rights reserved. Privacy Policy Terms & Conditions Help

Terms and Conditions of Sale and Purchase

These Terms and Conditions of Sale and Purchase (“Terms”) set forth the terms and conditions governing the purchase of products and services from Elsevier B.V. and/or its affiliated Elsevier company Elsevier Ltd ("Elsevier") through its web site at https://authors.elsevier.com ("Site"). By submitting an order for such products or services, you agree to be bound by these Purchase Terms.

Address

Elsevier B.V. Radarweg 29, 1043 NX Amsterdam, The Netherlands, registered at the Chamber of Commerce Amsterdam, nr. 33158992

Contract Formation

If you wish to purchase any of the products or services offered on this Site, you must submit a completed order form. You will be required to select your products, services or digital content for purchase and complete your delivery and payment details. Once these have been completed, you will have a further chance to check (and correct if necessary) these details prior to submission of your order, and you should print out this page and a copy of these Terms for your future reference.

If we accept your order, you will be sent a confirmation email giving you an order reference number (“Email Confirmation”). Once we have sent this E-mail Confirmation, you will not be able to amend or cancel your order (subject to any right to cancel as described below).

We may correct any error in any unconfirmed order, marketing literature or other document or information issued by us or placed on this Site without any liability.

Right to Cancel

Save as set out below, consumers in the European Union have the right to cancel the purchase until fourteen (14) days after date of delivery of the product or date the service was purchased by sending written notice of cancellation by email to the Site’s customer service department. A model cancellation form is available below for your use but you are not obliged to use this method of cancellation.

This right of cancellation applies to all products and services offered on the Site except:

(a) audio, video, and software products that have been unsealed from their packaging;

(b) digital content not on a tangible medium (e.g. eBooks) once the item has been downloaded;

(c) newspapers, periodicals and magazines which are not supplied under subscription contracts;

(d) services for which performance has completed; and

(e) offprints, colour figures, print on demand services and other products made or services provided to your specification or clearly bespoke to you.

If you cancel the purchase on this basis, we will reimburse to you all payments received from you, including the costs of delivery (except for the additional costs arising if you choose a type of delivery other than the least expensive type of standard delivery offered by us). We may make a deduction from the reimbursement for loss in value of any goods supplied, if the loss is the result of unnecessary handling by you.

Returns

You must return the goods without undue delay and in any event no later than 14 days from the date of notification of cancellation. We will then make the reimbursement without undue delay, and no later than-

(a) 14 days after the day we receive back from you any goods supplied, or

(b) (if earlier) 14 days after the day you provide evidence that you have returned the goods, or

(c) If there were no (physical) goods supplied, 14 days after the day on which we are informed about your decision to cancel the purchase.

We will make the reimbursement using the same means of payment as you used for the initial transaction, unless you have expressly agreed otherwise; in any event, you will not incur any fees as a result of the reimbursement.

Whether these rights of cancellation apply or not, you will be responsible for and will pay the cost of returning the items to us by the same method as it was delivered. The items will be returned at your risk.

Prices (including taxes and delivery) and Payment

The currency and price of products and services offered on this Site shall be that quoted on the Site on the date you place your order. While we make every effort to ensure that prices quoted on the Site are accurate, if there has been an inadvertent error, the correct price will be stated in the E-mail Confirmation and you will have the opportunity to withdraw from the order at that stage if the correct price is higher than that quoted and you are not happy with that higher price.

All prices are exclusive of the cost of postage, packaging and delivery and any applicable Value Added Tax or other sales tax, which will be separately stated on the Site and/or the Email Confirmation.

Delivery/Performance

Where applicable, products ordered will be delivered to the shipping address stated in your order, subject to any geographical restrictions stated on the Site. However, the time and method of delivery of products or performance of services will be at our discretion, unless otherwise specified and accepted as part of the order. Time for delivery or performance shall not be of the essence.

If the items delivered to you do not include all of the items that you ordered in any particular order or include incorrect items, you must inform us in writing as soon as possible. In any event, our liability is limited to the price of the items not delivered or the cost of replacing such items.

Consumer Protection

If you are a consumer, your statutory rights as a consumer are not affected by these Purchase Terms, and nothing in these Purchase Terms shall exclude or limit our liability arising by virtue of consumer protection legislation, or our liability for fraud or for death or personal injury caused by our negligence, or any other liability to the extent that we cannot so exclude or limit such liability as a matter of law.

Complaints

If you have any questions, comments or complaints regarding these Purchase Terms or your order, please contact our Customer Services Department which is located at:

Author Support
Elsevier Ltd
The Boulevard
Langford Lane
Kidlington
Oxford
OX5 1GB
United Kingdom

Contact us via our Support Center
https://service.elsevier.com
Phone: +44 1865 84 3577

Model Cancellation Form

To: Author Support
Subject: Cancellation of order for [article_reference].

Or, when sending this by post:

Author Support
Elsevier Ltd
The Boulevard, Langford Lane
Kidlington
Oxford
OX5 1GB
United Kingdom

Dear Sir, Madam,

I hereby give notice that I cancel my contract [DELETE AS APPLICABLE: [of sale of the following goods/for the supply of the following service].

Goods/Service:

Ordered on:
Received on:
Name of consumer:
Address of consumer:

Signature of consumer:

Date:

General
In the event of any inconsistency between these Terms and the Terms and Conditions of the Site, these Terms shall prevail.

Last revised: 19th January 2022

Copyright © 2022 Elsevier B.V. All rights reserved. Privacy Policy Terms & Conditions Help

Terms and Conditions of Sale and Purchase

These Terms and Conditions of Sale and Purchase (“Terms”) set forth the terms and conditions governing the purchase of products and services from Elsevier B.V. and/or its affiliated Elsevier company Elsevier Ltd ("Elsevier") through its web site at https://authors.elsevier.com ("Site"). By submitting an order for such products or services, you agree to be bound by these Purchase Terms.

Address

Elsevier B.V. Radarweg 29, 1043 NX Amsterdam, The Netherlands, registered at the Chamber of Commerce Amsterdam, nr. 33158992

Contract Formation

If you wish to purchase any of the products or services offered on this Site, you must submit a completed order form. You will be required to select your products, services or digital content for purchase and complete your delivery and payment details. Once these have been completed, you will have a further chance to check (and correct if necessary) these details prior to submission of your order, and you should print out this page and a copy of these Terms for your future reference.

If we accept your order, you will be sent a confirmation email giving you an order reference number (“Email Confirmation”). Once we have sent this E-mail Confirmation, you will not be able to amend or cancel your order (subject to any right to cancel as described below).

We may correct any error in any unconfirmed order, marketing literature or other document or information issued by us or placed on this Site without any liability.

Right to Cancel

Save as set out below, consumers in the European Union have the right to cancel the purchase until fourteen (14) days after date of delivery of the product or date the service was purchased by sending written notice of cancellation by email to the Site’s customer service department. A model cancellation form is available below for your use but you are not obliged to use this method of cancellation.

This right of cancellation applies to all products and services offered on the Site except:

(a) audio, video, and software products that have been unsealed from their packaging;

(b) digital content not on a tangible medium (e.g. eBooks) once the item has been downloaded;

(c) newspapers, periodicals and magazines which are not supplied under subscription contracts;

(d) services for which performance has completed; and

(e) offprints, colour figures, print on demand services and other products made or services provided to your specification or clearly bespoke to you.

If you cancel the purchase on this basis, we will reimburse to you all payments received from you, including the costs of delivery (except for the additional costs arising if you choose a type of delivery other than the least expensive type of standard delivery offered by us). We may make a deduction from the reimbursement for loss in value of any goods supplied, if the loss is the result of unnecessary handling by you.

Returns

You must return the goods without undue delay and in any event no later than 14 days from the date of notification of cancellation. We will then make the reimbursement without undue delay, and no later than-

(a) 14 days after the day we receive back from you any goods supplied, or

(b) (if earlier) 14 days after the day you provide evidence that you have returned the goods, or

(c) If there were no (physical) goods supplied, 14 days after the day on which we are informed about your decision to cancel the purchase.

We will make the reimbursement using the same means of payment as you used for the initial transaction, unless you have expressly agreed otherwise; in any event, you will not incur any fees as a result of the reimbursement.

Whether these rights of cancellation apply or not, you will be responsible for and will pay the cost of returning the items to us by the same method as it was delivered. The items will be returned at your risk.

Prices (including taxes and delivery) and Payment

The currency and price of products and services offered on this Site shall be that quoted on the Site on the date you place your order. While we make every effort to ensure that prices quoted on the Site are accurate, if there has been an inadvertent error, the correct price will be stated in the E-mail Confirmation and you will have the opportunity to withdraw from the order at that stage if the correct price is higher than that quoted and you are not happy with that higher price.

All prices are exclusive of the cost of postage, packaging and delivery and any applicable Value Added Tax or other sales tax, which will be separately stated on the Site and/or the Email Confirmation.

Delivery/Performance

Where applicable, products ordered will be delivered to the shipping address stated in your order, subject to any geographical restrictions stated on the Site. However, the time and method of delivery of products or performance of services will be at our discretion, unless otherwise specified and accepted as part of the order. Time for delivery or performance shall not be of the essence.

If the items delivered to you do not include all of the items that you ordered in any particular order or include incorrect items, you must inform us in writing as soon as possible. In any event, our liability is limited to the price of the items not delivered or the cost of replacing such items.

Consumer Protection

If you are a consumer, your statutory rights as a consumer are not affected by these Purchase Terms, and nothing in these Purchase Terms shall exclude or limit our liability arising by virtue of consumer protection legislation, or our liability for fraud or for death or personal injury caused by our negligence, or any other liability to the extent that we cannot so exclude or limit such liability as a matter of law.

Complaints

If you have any questions, comments or complaints regarding these Purchase Terms or your order, please contact our Customer Services Department which is located at:

Author Support
Elsevier Ltd
The Boulevard
Langford Lane
Kidlington
Oxford
OX5 1GB
United Kingdom

Contact us via our Support Center
https://service.elsevier.com
Phone: +44 1865 84 3577

Model Cancellation Form

To: Author Support
Subject: Cancellation of order for [article_reference].

Or, when sending this by post:

Author Support
Elsevier Ltd
The Boulevard, Langford Lane
Kidlington
Oxford
OX5 1GB
United Kingdom

Dear Sir, Madam,

I hereby give notice that I cancel my contract [DELETE AS APPLICABLE: [of sale of the following goods/for the supply of the following service].

Goods/Service:

Ordered on:
Received on:
Name of consumer:
Address of consumer:

Signature of consumer:

Date:

General
In the event of any inconsistency between these Terms and the Terms and Conditions of the Site, these Terms shall prevail.

Last revised: 19th January 2022

Copyright © 2022 Elsevier B.V. All rights reserved. Privacy Policy Terms & Conditions Help

Licence to Publish

SPRINGER NATURE

Licensee:	Springer-Verlag GmbH, DE	(the 'Licensee')
Journal Name:	Naunyn-Schmiedeberg's Archives of Pharmacology	(the 'Journal')
Manuscript Number:	d6f2ba3b-1d53-4984-a5d4-6913c8ce83ab	
Proposed Title of Article:	β1- and β1/β2-adrenergic receptor antagonists block 6-nitrodopamine-induced contractions of the rat isolated epididymal vas deferens	(the 'Article')
Author(s) [Please list all named Authors]:	José Britto-Júnior, Antonio Tiago Lima, Amanda Consulin Amorim, Raquel Rios Campitelli, Adriano Fregonesi, Fabíola Z. Mónica, Edson Antunes, Gilberto De Nucci	(the 'Author')
Corresponding Author Name:	José Britto-Júnior	

1 Grant of Rights

a) For good and valuable consideration, the Author hereby grants to the Licensee the perpetual, exclusive, world-wide, assignable, sublicensable and unlimited right to: publish, reproduce, copy, distribute, communicate, display publicly, sell, rent and/ or otherwise make available the article identified above, including any supplementary information and graphic elements therein (e.g. illustrations, charts, moving images) (the "Article") in any language, in any versions or editions in any and all forms and/or media of expression (including without limitation in connection with any and all end-user devices), whether now known or developed in the future. Without limitation, the above grant includes: (i) the right to edit, alter, adapt, adjust and prepare derivative works; (ii) all advertising and marketing rights including without limitation in relation to social media; (iii) rights for any training, educational and/or instructional purposes; (iv) the right to add and/or remove links or combinations with other media/works; and (v) the right to create, use and/or license and/or sublicense content data or metadata of any kind in relation to the Article (including abstracts and summaries) without restriction. The above rights are granted in relation to the Article as a whole or any part and with or in relation to any other works.

b) Without limiting the rights granted above, Licensee is granted the rights to use the Article for the purposes of analysis, testing, and development of publishing- and research-related workflows, systems, products, projects, and services; to confidentially share the Article with select third parties to do the same; and to retain and store the Article and any associated correspondence/files/forms to maintain the historical record, and to facilitate research integrity investigations. The grant of rights set forth in this clause (b) is irrevocable.

c) The Licensee will have the right, but not the obligation, to exercise any or all of the rights granted herein. If the Licensee elects not to publish the Article for any reason, all publishing rights under this Agreement as set forth in clause 1.a) above will revert to the Author.

2 Copyright

Ownership of copyright in the Article will be vested in the name of the Author. When reproducing the Article or extracts from it, the Author will acknowledge and reference first publication in the Journal.

3 Use of Article Versions

a) For purposes of this Agreement: (i) references to the "Article" include all versions of the Article; (ii) "Submitted Manuscript" means the version of the Article as first submitted by the Author; (iii) "Accepted Manuscript" means the version of the Article accepted for publication, but prior to copy-editing and typesetting; and (iv) "Version of Record" means the version of the Article published by the Licensee, after copy-editing and typesetting. Rights to all versions of the Manuscript are granted on an exclusive basis, except for the Submitted Manuscript, to which rights are granted on a non-exclusive basis.

b) The Author may make the Submitted Manuscript available at any time and under any terms (including, but not limited to, under a CC BY licence), at the Author's discretion. Once the Article has been published, the Author will include an acknowledgement and provide a link to the Version of Record on the publisher's website: "This preprint has not undergone peer review (when applicable) or any post-submission improvements or corrections. The Version of Record of this article is published in [insert journal title], and is available online at https://doi.org/[insert DOI]".

c) The Licensee grants to the Author (i) the right to make the Accepted Manuscript available on their own personal, self-maintained website immediately on acceptance, (ii) the right to make the Accepted Manuscript available for public release on any of the following twelve (12) months after

first publication (the "Embargo Period"): their employer's internal website; their institutional and/or funder repositories. Accepted Manuscripts may be deposited in such repositories immediately upon acceptance, provided they are not made publicly available until after the Embargo Period.

The rights granted to the Author with respect to the Accepted Manuscript are subject to the conditions that (i) the Accepted Manuscript is not enhanced or substantially reformatted by the Author or any third party, and (ii) the Author includes on the Accepted Manuscript an acknowledgement in the following form, together with a link to the published version on the publisher's website: "This version of the article has been accepted for publication, after peer review (when applicable) but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at: http://dx.doi.org/[insert DOI]. Use of this Accepted Version is subject to the publisher's Accepted Manuscript terms of use https://www.springernature.com/gp/open-research/policies/accepted-manuscript-terms". Under no circumstances may an Accepted Manuscript be shared or distributed under a Creative Commons or other form of open access licence.

d) The Licensee grants to the Author the following non-exclusive rights to the Version of Record, provided that, when reproducing the Version of Record or extracts from it, the Author acknowledges and references first publication in the Journal according to current citation standards. As a minimum, the acknowledgement must state: "First published in [Journal name, volume, page number, year] by Springer Nature".

   i. to reuse graphic elements created by the Author and contained in the Article, in presentations and other works created by them;

   ii. the Author and any academic institution where they work at the time may reproduce the Article for the purpose of course teaching (but not for inclusion in course pack material for onward sale by libraries and institutions);

   iii. to reuse the Version of Record or any part in a thesis written by the same Author, and to make a copy of that thesis available in a repository of the Author(s)' awarding academic institution, or other repository required by the awarding academic institution. An acknowledgement should be included in the citation: "Reproduced with permission from Springer Nature"; and

   iv. to reproduce, or to allow a third party to reproduce the Article, in whole or in part, in any other type of work (other than thesis) written by the Author for distribution by a publisher after an embargo period of 12 months.

4 Warranties & Representations

Author warrants and represents that:

a)

   i. the Author is the sole copyright owner or has been authorised by any additional copyright owner(s) to grant the rights defined in clause 1,

   ii. the Article does not infringe any intellectual property rights (including without limitation copyright, database rights or trade mark rights) or other third party rights and no licence from or payments to a third party are required to publish the Article,

   iii. the Article has not been previously published or licensed, nor has the Author committed to licensing any version of the Article under a licence inconsistent with the terms of this Agreement,

   iv. if the Article contains materials from other sources (e.g. illustrations, tables, text quotations), Author has obtained written permissions to the extent necessary from the copyright holder(s), to license to the Licensee the same rights as set out in clause 1 but on a non-exclusive basis and without the right to use any graphic elements on a stand-alone basis and has cited any such materials correctly;

b) all of the facts contained in the Article are according to the current body of research true and accurate;

c) nothing in the Article is obscene, defamatory, violates any right of privacy or publicity, infringes any other human, personal or other rights of any person or entity or is otherwise unlawful and that informed consent to publish has been obtained for any research participants;

d) nothing in the Article infringes any duty of confidentiality owed to any third party or violates any contract, express or implied, of the Author;

e) all institutional, governmental, and/or other approvals which may be required in connection with the research reflected in the Article have been obtained and continue in effect;

f) all statements and declarations made by the Author in connection with the Article are true and correct; and

g) the signatory who has signed this agreement has full right, power and authority to enter into this agreement on behalf of all of the Authors.

5 Cooperation

a) The Author will cooperate fully with the Licensee in relation to any legal action that might arise from the publication of the Article, and the Author will give the Licensee access at reasonable times to any relevant accounts, documents and records within the power or control of the Author. The Author agrees that any Licensee affiliate through which the Licensee exercises any rights or performs any obligations under this Agreement is intended to have the benefit of and will have the right to enforce the terms of this Agreement.

b) Author authorises the Licensee to take such steps as it considers necessary at its own expense in the Author's name(s) and on their behalf if the Licensee believes that a third party is infringing or is likely to infringe copyright in the Article including but not limited to initiating legal proceedings.

6 Author List

Changes of authorship, including, but not limited to, changes in the corresponding author or the sequence of authors, are not permitted after acceptance of a manuscript.

7 Post Publication Actions

The Author agrees that the Licensee may remove or retract the Article or publish a correction or other notice in relation to the Article if the Licensee determines that such actions are appropriate from an editorial, research integrity, or legal perspective.

8 Controlling Terms

The terms of this Agreement will supersede any other terms that the Author or any third party may assert apply to any version of the Article.

9 Governing Law

This Agreement will be governed by, and construed in accordance with, the laws of the Federal Republic of Germany. The courts of Berlin, Germany will have exclusive jurisdiction.

Signed for and on behalf of the Author(s)
Corresponding Author: José Britto-Júnior
Email: josebrittojr@gmail.com
IP Address: 177.9.52.23

Time Stamp: 2022-07-01 03:55:43

Andrology

Published by Wiley on behalf of American Society of Andrology and European Academy of Andrology (the "Owner")

COPYRIGHT TRANSFER AGREEMENT

Date: August 07, 2022

Contributor name: José Britto-Júnior

Contributor address:

Manuscript number: ANDR-2022-0336.R1

Re: Manuscript entitled 6-NITRODOPAMINE IS A MAJOR ENDOGENOUS MODULATOR OF HUMAN VAS DEFERENS CONTRACTILITY (the "Contribution")

for publication in Andrology (the "Journal")

published by Wiley Periodicals LLC ("Wiley")

Dear Contributor(s):

Thank you for submitting your Contribution for publication. In order to expedite the editing and publishing process and enable the Owner to disseminate your Contribution to the fullest extent, we need to have this Copyright Transfer Agreement executed. If the Contribution is not accepted for publication, or if the Contribution is subsequently rejected, this Agreement shall be null and void.
Publication cannot proceed without a signed copy of this Agreement.

A. COPYRIGHT

1. The Contributor assigns to the Owner, during the full term of copyright and any extensions or renewals, all copyright in and to the Contribution, and all rights therein, including but not limited to the right to publish, republish, transmit, sell, distribute and otherwise use the Contribution in whole or in part in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so. “Contribution” means the article submitted by the Contributor for publication in the Journal (including any embedded rich media) and all subsequent versions. The definition of Contribution does not extend to any supporting information submitted with or referred to in the Contribution (“Supporting Information”). To the extent that any Supporting Information is submitted to the Journal, the Owner is granted a perpetual, non-exclusive license to publish, republish, transmit, sell, distribute and otherwise use this Supporting Information in whole or in part in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so. If the Contribution was shared as a preprint, the Contributor grants to the Owner exclusivity as to any rights retained by the Contributor in the preprint.

2. Reproduction, posting, transmission or other distribution or use of the final Contribution in whole or in part in any medium by the Contributor as permitted by this Agreement requires a citation to the Journal suitable in form and content

as follows: (Title of Article, Contributor, Journal Title and Volume/Issue, Copyright © [year], copyright owner as specified in the Journal, Publisher). Links to the final article on the publisher website are encouraged where appropriate.

B. RETAINED RIGHTS

Notwithstanding the above, the Contributor or, if applicable, the Contributor's employer, retains all proprietary rights other than copyright, such as patent rights, in any process, procedure or article of manufacture described in the Contribution.

C. PERMITTED USES BY CONTRIBUTOR

1. Submitted Version. The Owner licenses back the following rights to the Contributor in the version of the Contribution as originally submitted for publication (the "Submitted Version"):

> **a.** The right to self-archive the Submitted Version on: the Contributor's personal website; a not for profit subject-based preprint server or repository; a Scholarly Collaboration Network (SCN) which has signed up to the STM article sharing principles [http://www.stm-assoc.org/stm-consultations/scn-consultation-2015/] ("Compliant SCNs"); or the Contributor's company/ institutional repository or archive. This right extends to both intranets and the Internet. The Contributor may replace the Submitted Version with the Accepted Version, after any relevant embargo period as set out in paragraph C.2(a) below has elapsed. The Contributor may wish to add a note about acceptance by the Journal and upon publication it is recommended that Contributors add a Digital Object Identifier (DOI) link back to the Final Published Version.
>
> **b.** The right to transmit, print and share copies of the Submitted Version with colleagues, including via Compliant SCNs, provided that there is no systematic distribution of the Submitted Version, e.g. posting on a listserve, network (including SCNs which have not signed up to the STM sharing principles) or automated delivery.

2. Accepted Version. The Owner licenses back the following rights to the Contributor in the version of the Contribution that has been peer-reviewed and accepted for publication, but not final (the "Accepted Version"):

> **a.** The right to self-archive the Accepted Version on: the Contributor's personal website; the Contributor's company/institutional repository or archive; Compliant SCNs; and not for profit subject-based repositories such as PubMed Central, all subject to an embargo period of 12 months for scientific, technical and medical (STM) journals and 24 months for social science and humanities (SSH) journals following publication of the Final Published Version. There are separate arrangements with certain funding agencies governing reuse of the Accepted Version as set forth at the following website: http://www.wileyauthors.com/funderagreements. The Contributor may not update the Accepted Version or replace it with the Final Published Version. The Accepted Version posted must contain a legend as follows: This is the accepted version of the following article: FULL CITE, which has been published in final form at [Link to final article]. This article may be used for non-commercial purposes in accordance with the Wiley Self-Archiving Policy [http://www.wileyauthors.com/self-archiving].
>
> **b.** The right to transmit, print and share copies of the Accepted Version with colleagues, including via Compliant SCNs (in private research groups only before the embargo and publicly after), provided that there is no systematic distribution of the Accepted Version, e.g. posting on a listserve, network (including SCNs which have not signed up to the STM sharing principles) or automated delivery.

3. Final Published Version. The Owner hereby licenses back to the Contributor the following rights with respect to the final published version of the Contribution (the "Final Published Version"):

a. Copies for colleagues. The personal right of the Contributor only to send or transmit individual copies of the Final Published Version in any format to colleagues upon their specific request, and to share copies in private sharing groups in Compliant SCNs, provided no fee is charged, and further provided that there is no systematic external or public distribution of the Final Published Version, e.g. posting on a listserve, network or automated delivery.

b. Re-use in other publications. The right to re-use the Final Published Version or parts thereof for any publication authored or edited by the Contributor (excluding journal articles) where such re-used material constitutes less than half of the total material in such publication. In such case, any modifications must be accurately noted.

c. Teaching duties. The right to include the Final Published Version in teaching or training duties at the Contributor's institution/place of employment including in course packs, e-reserves, presentation at professional conferences, in-house training, or distance learning. The Final Published Version may not be used in seminars outside of normal teaching obligations (e.g. commercial seminars). Electronic posting of the Final Published Version in connection with teaching/training at the Contributor's company/institution is permitted subject to the implementation of reasonable access control mechanisms, such as user name and password. Posting the Final Published Version on the open Internet is not permitted.

d. Oral presentations. The right to make oral presentations based on the Final Published Version.

4. Article Abstracts, Figures, Tables, Artwork and Selected Text (up to 250 words).

a. Contributors may re-use unmodified abstracts for any non-commercial purpose. For online uses of the abstracts, the Owner encourages but does not require linking back to the Final Published Version.

b. Contributors may re-use figures, tables, artwork, and selected text up to 250 words from their Contributions, provided the following conditions are met:

(i) Full and accurate credit must be given to the Final Published Version.

(ii) Modifications to the figures and tables must be noted. Otherwise, no changes may be made.

(iii) The re-use may not be made for direct commercial purposes, or for financial consideration to the Contributor.

(iv) Nothing herein will permit dual publication in violation of journal ethical practices.

D. CONTRIBUTIONS OWNED BY EMPLOYER

1. If the Contribution was written by the Contributor in the course of the Contributor's employment as a "work-made-for-hire" in the course of employment, the Contribution is owned by the company/institution which must execute this Agreement (in addition to the Contributor's signature). In such case, the company/institution hereby agrees to the terms of use set forth in paragraph A above and assigns to the Owner, during the full term of copyright, all copyright in and to the Contribution for the full term of copyright throughout the world as specified in paragraph A above.

2. In addition to the rights specified as retained in paragraph B above and the rights granted back to the Contributor pursuant to paragraph C above, the Owner hereby grants back, without charge, to such company/institution, its subsidiaries and divisions, the right to make copies of and distribute the Final Published Version internally in print format or electronically on the Company's internal network. Copies so used may not be resold or distributed externally. However, the company/institution may include information and text from the Final Published Version as part of an information package included with software or other products offered for sale or license or included in patent

applications. Posting of the Final Published Version by the company/institution on a public access website may only be done with written permission, and payment of any applicable fee(s). Also, upon payment of the applicable reprint fee, the company/institution may distribute print copies of the Final Published Version externally.

E. GOVERNMENT CONTRACTS

In the case of a Contribution prepared under U.S. Government contract or grant, the U.S. Government may reproduce, without charge, all or portions of the Contribution and may authorize others to do so, for official U.S. Government purposes only, if the U.S. Government contract or grant so requires. (U.S. Government, U.K. Government, and other government employees: see notes at end.)

F. COPYRIGHT NOTICE

The Contributor and the company/institution agree that any and all copies of the Final Published Version or any part thereof distributed or posted by them in print or electronic format as permitted herein will include the notice of copyright as stipulated in the Journal and a full citation to the Journal.

G. CONTRIBUTOR'S REPRESENTATIONS

The Contributor represents that: (i) the Contributor and all co-Contributors have the full power, authority and capability to enter into this Agreement, to grant the rights and license granted herein and to perform all obligations hereunder; (ii) neither the Contributor nor any co-Contributor has granted exclusive rights to, or transferred their copyright in, any version of the Contribution to any third party; (iii) the Contribution is the Contributor's original work, all individuals identified as Contributors actually contributed to the Contribution, and all individuals who contributed are included; (iv) if the Contribution was prepared jointly, the Contributor has informed the co-Contributors of the terms of this Agreement and has obtained their signed written permission to execute this Agreement on their behalf; (v) the Contribution is submitted only to this Journal and has not been published before, has not been included in another manuscript, and is not currently under consideration or accepted for publication elsewhere; (vi) if excerpts from copyrighted works owned by third parties are included, the Contributor shall obtain written permission from the copyright owners for all uses as set forth in the standard permissions form and the Journal's Author Guidelines, and show credit to the sources in the Contribution; (vii) the Contribution and any submitted Supporting Information contain no libelous or unlawful statements, do not infringe upon the rights (including without limitation the copyright, patent or trademark rights) or the privacy of others, do not breach any confidentiality obligation, do not violate a contract or any law, do not contain material or instructions that might cause harm or injury, and only utilize data that has been obtained in accordance with applicable legal requirements and Journal policies; and (viii) there are no conflicts of interest relating to the Contribution, except as disclosed. Accordingly, the Contributor represents that the following information shall be clearly identified on the title page of the Contribution: (1) all financial and material support for the research and work; (2) any financial interests the Contributor or any co-Contributors may have in companies or other entities that have an interest in the information in the Contribution or any submitted Supporting Information (e.g., grants, advisory boards, employment, consultancies, contracts, honoraria, royalties, expert testimony, partnerships, or stock ownership); and (3) indication of no such financial interests if appropriate.

Notwithstanding acceptance, the Owner or Wiley may (but is not obliged to) require changes to the Contribution, including changes to the length of the Contribution, and/or elect not to publish the Contribution if for any reason, in the Owner's or Wiley's reasonable judgment, such publication would be inconsistent with the Core Practices and associated guidelines set forth by the Committee on Publication Ethics (a not-for-profit organization based in the UK: https://publicationethics.org/core-practices) or would result in legal liability, violation of Wiley's ethical guidelines, or violation of journal ethical practices.

H. USE OF INFORMATION

The Contributor acknowledges that, during the term of this Agreement and thereafter, the Owner (and Wiley where Wiley is not the Owner) may process the Contributor's personal data, including storing or transferring data outside of the country of the Contributor's residence, in order to process transactions related to this Agreement and to communicate with the Contributor, and that the Publisher has a legitimate interest in processing the Contributor's personal data. By entering into this Agreement, the Contributor agrees to the processing of the Contributor's personal data (and, where applicable, confirms that the Contributor has obtained the permission from all other contributors to process their personal data). Wiley shall comply with all applicable laws, statutes and regulations relating to data protection and privacy and shall process such personal data in accordance with Wiley's Privacy Policy located at: https://www.wiley.com/en-us/privacy.

[X] I agree to the COPYRIGHT TRANSFER AGREEMENT as shown above, consent to execution and delivery of the Copyright Transfer Agreement electronically and agree that an electronic signature shall be given the same legal force as a handwritten signature, and have obtained written permission from all other contributors to execute this Agreement on their behalf.

Contributor's signature (type name here): José Britto-Júnior

Date: August 07, 2022

SELECT FROM OPTIONS BELOW:

[X] Contributor-owned work

[ ] U.S. Government work

Note to U.S. Government Employees

*A contribution prepared by a U.S. federal government employee as part of the employee's official duties, or which is an official U.S. government publication, is called a "U.S. government work", and is in the public domain in the United States. If the Contribution was not prepared as part of the employee's duties, is not an official U.S. government publication, or if at least one author is **not** a U.S. government employee, it is not a U.S. government work. If at least one author is **not** a U.S. government employee, then the non-government author should also sign the form, selecting the appropriate ownership option. If more than one author is not a U.S. government employee, one may sign on behalf of the others.*

[ ] U.K. Government work (Crown Copyright)

Note to U.K. Government Employees

The rights in a contribution prepared by an employee of a UK government department, agency or other Crown body as part of his/her official duties, or which is an official government publication, belong to the Crown and must be made available under the terms of the Open Government Licence. Contributors must ensure they comply with departmental regulations and submit the appropriate authorisation to publish. If your status as a government employee legally prevents you from signing this Agreement, please contact the Journal production editor. If this selection does not apply to at least one author in the group, this author should also sign the form, indicating transfer of those rights which that author has and selecting the appropriate additional ownership selection option. If this applies to more than one author, one may sign on behalf of the others.

[ ] Other

Including Other Government work or Non-Governmental Organisation work

Note to Non-U.S., Non-U.K. Government Employees or Non-Governmental Organisation Employees

If you are employed by the Australian Government, the World Bank, the World Health Organization, the International Monetary Fund, the European Atomic Energy Community, the Jet Propulsion Laboratory at California Institute of Technology, the Asian Development Bank, the Bank of International Settlements, USDA Agricultural Research Services, or are a Canadian Government civil servant, please download a copy of the license agreement from http://www.wileyauthors.com/licensingFAQ and upload the form to the Wiley Author Services Dashboard. If your status as a government or non-governmental organisation employee legally prevents you from signing this Agreement, please contact the Journal production editor.

Name of Government/Non-Governmental Organisation:

_____

[ ] Company/institution owned work (made for hire in the course of employment)

If you are an employee of Amgen, please download a copy of the company addendum from http://www.wileyauthors.com/licensingFAQ and return your signed license agreement along with the addendum. If this selection does not apply to at least one author in the group, this author should also sign the form, indicating transfer of those rights which that author has and selecting the appropriate additional ownership selection option. If this applies to more than one author, one may sign on behalf of the others.

Name of Company/Institution:

_____

Authorized Signature of Employer: _____

Date: _____

Signature of Employee: _____

Date: _____