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FELIPE FERNANDES JACINTHO

CATECOLAMINAS ENDOTELIAIS COMO MODULADORES DA REATIVIDADE VASCULAR EM AORTA DE *Chelonoidis carbonaria*

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FELIPE FERNANDES JACINTHO

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

ORIENTADOR: PROF. DR. GILBERTO DE NUCCI

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RESUMO

O endotélio é um órgão capaz de regular o tônus vascular através da liberação de agentes relaxantes tais como óxido nítrico e prostaciclina, ou contráteis, como endotelinas e angiotensina. O conceito fisiológico atual propõe que tal regulação também pode ocorrer através da liberação de catecolaminas de origem exclusivamente neural, mas os tecidos isolados de répteis com endotélio constituem uma fonte de catecolaminas, visto que a contração da aorta induzida por estimulação por campo elétrico é abolida por antagonistas adrenérgicos e pela remoção do endotélio. Imunohistoquímica identificou a presença da enzima tirosina hidroxilase no endotélio de tecido humano, serpentes e em *Chelonoidis carbonaria*. Este estudo teve por objetivo identificar o(s) mediador(es) responsável(eis) pelas contrações induzidas por estímulo de campo elétrico (EFS) dos anéis aórticos de *Chelonoidis carbonária* e compreender a sua função fisiológica. Os anéis aórticos foram montados em banho de órgãos isolados com solução de Krebs-Henseleit oxigenada e aquecida. As contrações foram avaliadas na presença e ausência de L-NAME, tetrodotoxina, fentolamina, fenoxibenzamina, prazosina, idazoxan, atropina, D-tubocurarina ou indometacina. A contração induzida por EFS também foi realizada em anéis sem endotélio. A contração induzida por EFS foi investigada pelo ensaio sanduíche. A EFS em 16 Hz contraiu a aorta de *Chelonoidis*, que foi quase abolida pela remoção do endotélio. A adição de L-NAME aumentou a resposta do EFS. Nos anéis aórticos tratados com L-NAME, a tetrodotoxina não alterou a resposta EFS. A indometacina, prazosina, idazoxan atropina e d-tubucurarina também não afetaram a resposta do EFS. A fentolamina a 10 μM não alterou a contração induzida por EFS, porém, a 100 μM reduziu. A fenoxibenzamina a 1 μM reduziu em 76% e a 10 μM em 90% a resposta EFS. A imunohistoquímica identificou tirosina hidroxilase no endotélio e no cérebro, enquanto a 58 proteína S100 foi encontrada apenas no cérebro. As concentrações de dopamina, noradrenalina e adrenalina foram analisadas por cromatografia líquida acoplada à espectrometria de massas tandem. As contrações causadas pela dopamina e EFS foram feitas na ausência e presença do inibidor da síntese de óxido nítrico (NO) L-NAME, do inibidor da guanilil ciclase sensível ao NO ODQ, do antagonista do receptor tipo D1 SCH-23390, do receptor tipo D2 antagonistas risperidona, quetiapina, haloperidol e os inibidores da tirosina hidroxilase salsolinol e 3-iodo-L-tirosina. Concentrações basais de dopamina, noradrenalina e adrenalina foram detectadas na solução de Krebs contendo os anéis aórticos. As concentrações de catecolaminas foram significativamente reduzidas em anéis aórticos sem endotélio. L-NAME e ODQ potencializaram significativamente as contrações induzidas pela dopamina. Os antagonistas do receptor do tipo D2 inibiram as contrações induzidas por EFS dos anéis aórticos tratados com L-NAME, enquanto o SCH 23390 não teve efeito. Resultados semelhantes foram observados nas contrações induzidas pela dopamina em anéis aórticos tratados com L-NAME. Estes resultados indicam que as catecolaminas, liberadas pelo endotélio regulam as contrações induzidas por EFS. Isto pode constituir um mecanismo adequado pelo qual os répteis modulam a distribuição do fluxo sanguíneo em órgãos específicos.

Palavras-chave: 6-nitrodopamina, catecolaminas, endotélio, sistema cardiovascular.

ABSTRACT

The endothelium is an organ capable of regulating vascular tone through the release of relaxing agents such as nitric oxide and prostacyclin, or contractile agents, such as endothelins and angiotensin. The current physiological concept proposes that such regulation can also occur through the release of catecholamines of exclusively neural origin, but isolated tissues of reptiles with endothelium constitute a source of catecholamines, since aortic contraction induced by electric field stimulation is abolished by adrenergic antagonists and by removing the endothelium. Immunohistochemistry identified the presence of the enzyme tyrosine hydroxylase in the endothelium of human tissue, snakes and Chelonoidis carbonaria. This study aimed to identify the mediator(s) responsible for the contractions induced by electric field stimulation (EFS) of the aortic rings of Chelonoidis carbonaria and understand their physiological function. The aortic rings were mounted in an isolated organ bath with oxygenated and heated Krebs-Henseleit solution. Contractions were assessed in the presence and absence of L-NAME, tetrodotoxin, phentolamine, phenoxybenzamine, Prazosin, idazoxan, atropine, D-tubocurarine, or indomethacin. EFS-induced contraction was also performed in rings lacking endothelium. EFS-induced contraction was investigated by sandwich assay. EFS at 16 Hz contracted Chelonoidis's aorta, which was nearly abolished by removal of the endothelium. The addition of L-NAME increased the EFS response. In aortic rings treated with L-NAME, tetrodotoxin did not alter the EFS response. Indomethacin, Prazosin, idazoxan atropine and d-tubucurarine also did not affect the EFS response. Phentolamine at 10 μM did not alter the contraction induced by EFS, however, at 100 μM it reduced it. Phenoxybenzamine at 1 μM reduced the EFS response by 76% and at 10 μM by 90%. Immunohistochemistry identified tyrosine hydroxylase in the endothelium and brain, while the S100 protein was found only in the brain. The concentrations of dopamine, norepinephrine and adrenaline were analyzed by liquid chromatography coupled to tandem mass spectrometry. The contractions caused by dopamine and EFS were performed in the absence and presence of the nitric oxide (NO) synthesis inhibitor L-NAME, the NO-sensitive guanylyl cyclase inhibitor ODQ, the D1-type receptor antagonist SCH-23390, the D1-type receptor antagonist D2 antagonists risperidone, quetiapine, haloperidol and the tyrosine hydroxylase inhibitors salsolinol and 3-iodo-L-tyrosine. Basal concentrations of dopamine, norepinephrine and adrenaline were detected in Krebs solution containing the aortic rings. Catecholamine concentrations were significantly reduced in aortic rings without endothelium. L-NAME and ODQ significantly potentiated dopamine-induced contractions. D2-like receptor antagonists inhibited EFS-induced contractions of L-NAME-treated aortic rings, whereas SCH 23390 had no effect. Similar results were observed in dopamine-induced contractions in aortic rings treated with L-NAME. These results indicate that catecholamines released by the endothelium regulate EFS-induced contractions. This may constitute a suitable mechanism by which reptiles modulate the distribution of blood flow in specific organs.

Keywords: 6-nitrodopamine, catecholamines, endothelium, cardiovascular system.

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1. INTRODUÇÃO

1.1. CATECOLAMINAS

As catecolaminas, conhecidas pela adrenalina, noradrenalina e dopamina, são um grupo de compostos orgânicos que possuem em sua estrutura o grupo catecol (3,4-diidroxibenzeno) conectado a um grupo amina por uma ponte etil. Essas substâncias são essenciais para a neurotransmissão no Sistema Nervoso Central (SNC), assim como participam de processos endócrinos no qual integra o sistema nervoso simpático e a medula supra-renal, formada por uma unidade anatômica e fisiológica, conhecida como sistema simpático-adrenal[1,2].

A primeira etapa da síntese das catecolaminas ocorre a partir da catálise da enzima tirosina hidroxilase que converte o aminoácido tirosina em L-DOPA (l-3,4-diidroxifenilalanina) por oxidação da posição 3 do anel de benzeno[1,3]. É importante ressaltar que a tirosina pode ser encontrada no corpo por uma fonte exógena, como dieta de peixes, ovos, abacate, carnes e cereais, conhecida também por aminoácido não essencial, ou endógena, através da sua síntese no fígado. A L-DOPA é transformada em dopamina pela enzima DOPA descarboxilase (ou aminoácido aromático descarboxilasa; AADC). É possível encontrá-la no encéfalo, e mais especificamente, nos núcleos dopaminérgicos. A AADC cliva o grupo carboxila do carbono α da cadeia lateral de etilamina, liberando dióxido de carbono, processo no qual requer o cofator fosfato de piridoxal[1,3].

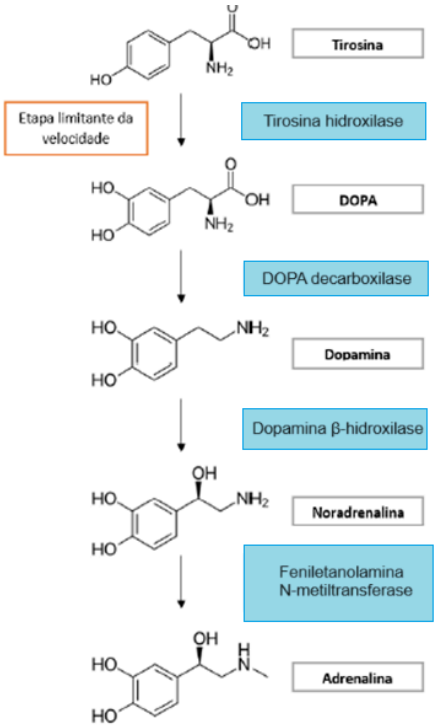

Figura 1 - Síntese das catecolaminas. Fonte: Guilherme Murari - grupo Prof. Dr. Gilberto De Nucci 2019 (adaptado).

A transformação de dopamina em noradrenalina, ou hidroxilação da dopamina, é feita pela enzima Dopamina-β-hidroxilase. Essa enzima requer oxigênio molecular, usa ácido ascórbico como cofator e está relacionado geneticamente e estruturalmente com a tirosina hidroxilase[4,5].

Compreende-se que a dopamina é o precursor metabólico da noradrenalina e é um transmissor, ou neuromodulador, no sistema nervoso central. 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 (o qual é secretado pela medula supra-renal) pela feniletanolamina N-metiltransferase[1].

Na neurotransmissão química, grande parte da noradrenalina liberada pelo neurônio é capturada e reacondicionada dentro de vesículas para nova utilização, enquanto uma pequena porção é capturada por células gliais. Como ilustrado na Figura 2, a captura neural é feita pela família das proteínas transportadoras de neurotransmissores específicas - NET (transportador de norepinefrina, do inglês *norepinephrine transporter*). Tais transportadores também atuam como cotransportadores 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 do inglês *extraneural monoamine transporter*)[3].

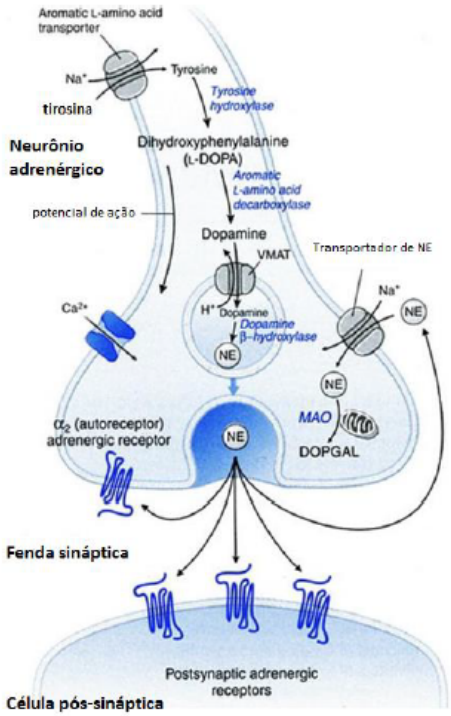

Figura 2 - Síntese das catecolaminas e seus transportadores. Fonte: Lordfred 2018.

A metabolização das catecolaminas ocorre pela ação das enzimas monoamino-oxidase (MAO-A e MAO-B) e catecol-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 pela enzima aldeído desidrogenase ao ácido carboxílico correspondente. A COMT envolve a metilação de um dos grupos hidroxila do catecol, que irá produzir um derivado metoxi. Diferente da MAO, a COMT está presente na medula da suprarrenal e em outras células e tecidos. 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 é convertido em ácido vanilmandélico - VMA[6, 7, 8].

As catecolaminas atuam sobre receptores adrenérgicos α1 e 2, e β1, 2 e 3[9], tendo respostas diferentes para cada receptor e tecido[2, 10, 11]. 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[2].

As catecolaminas também são desenvolvidas e utilizadas pelas células do sistema imunológico. Os macrófagos são responsáveis pela sua produção e então as catecolaminas atuam como mensageiros químicos (autócrino) nos receptores adrenérgicos que irão regular a produção de interleucina 1 pelos macrófagos[12].

1.2. ENDOTÉLIO E O PAPEL CARDIOVASCULAR DAS CATECOLAMINAS

O endotélio, ou conjunto de células endoteliais, é parte integrante da túnica íntima – camada mais interna dos vasos sanguíneos - de maneira que percorre todo o aparelho vascular, todos os órgãos e tecidos do corpo. Assim, o endotélio funciona como um órgão e entre suas atribuições fisiológicas está a capacidade de regular o tônus vascular através da liberação de agentes vasoativos que controlam a contratilidade e proliferação das células musculares lisas de vasos sanguíneos que possuem túnica média[12, 13].

Histologicamente, o endotélio é um tecido epitelial simples pavimentoso que constitui a estrutura mais interna de todo sistema circulatório (no coração adquire o nome endocárdio), ou seja, sua túnica íntima separa o sangue da túnica média vascular e do interstício[14], como mostra a Figura 3. Células endoteliais comunicam-se com células do músculo liso vascular por meio de junções “*gap*” (do inglês, *gap junctions*) mioendoteliais que permitem a distribuição do potencial elétrico, assim como a transferência de íons ou pequenas moléculas[15].

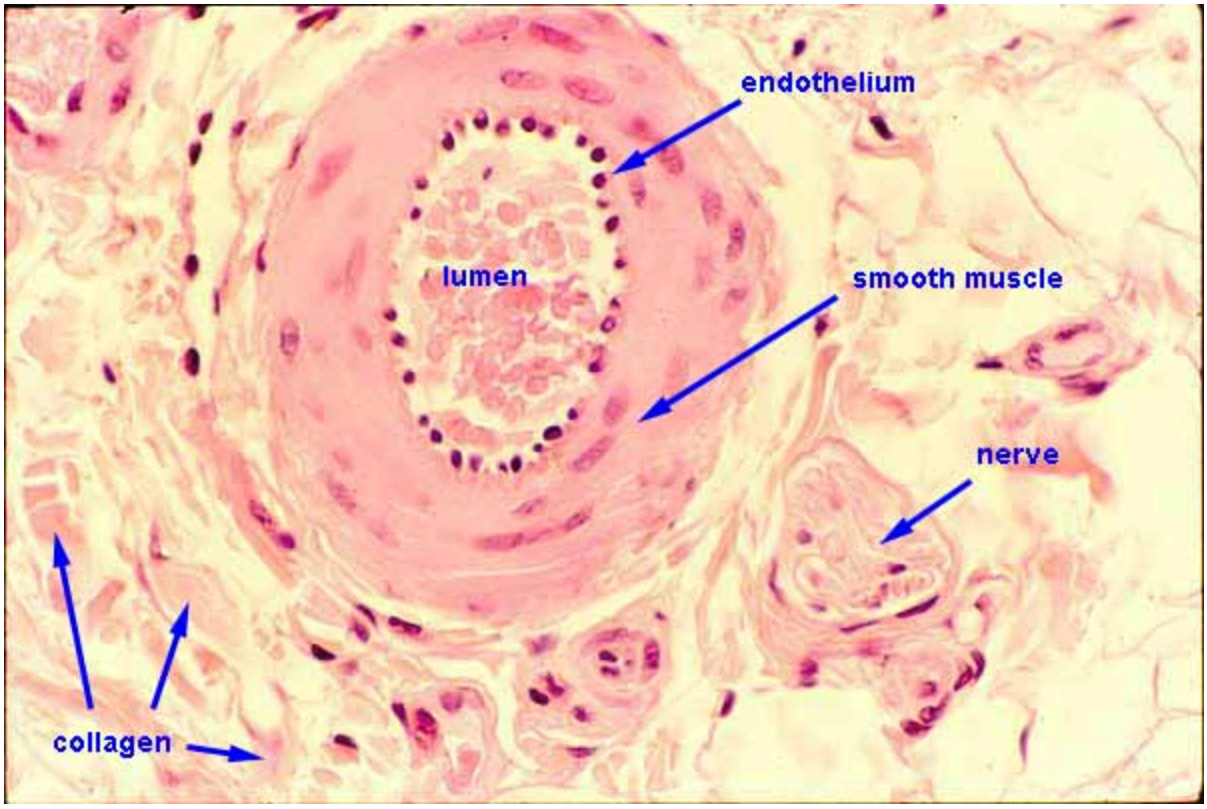

Figura 3 - corte histológico de um vaso sanguíneo. Lumen: lúmen do vaso; smooth muscle: musculatura lisa; endothelium: endotélio; collagen: colágeno; nerve: nervo. Fonte: Southern Illinois University 2022.

O endotélio possui três características morfológicas de acordo com a sua localização (Figura 4): contínuo, fenestrado e sinusoidal[16, 17]. O endotélio contínuo é encontrado no cérebro, pele, pulmão, coração e músculo. No sistema nervoso central (SNC) o endotélio repousa sobre a membrana basal e uma camada permeável de células periendoteliais, também camadas de pericitos (células mesenquimais indiferenciadas)[18], que serve como barreira, suporte e possibilita troca de íons. A morfologia do endotélio também possui importância para a distribuição dos fármacos[19]. O endotélio fenestrado é encontrado nos tecidos das glândulas endócrinas, trato gastrointestinal, rins e túbulos renais, facilitando a filtração e a transferência de hormônios e outras moléculas para a corrente sanguínea[20]. O fígado possui um endotélio sinusoidal, tem papel importante na função metabólica e de eliminação, além de estar envolvida na endocitose e metabolismo de macromoléculas como glicoproteínas, lipoproteínas e componentes de matrizes extracelulares[21].

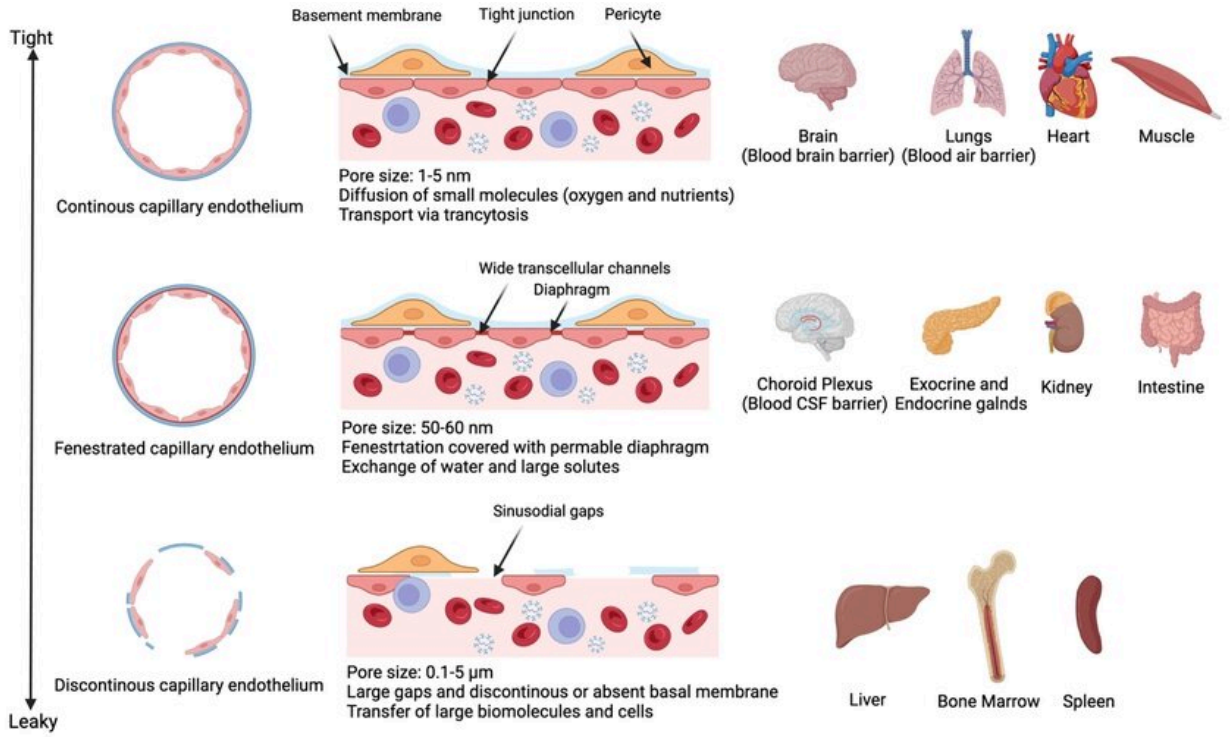

Figura 4 - Diferenças morfológicas do endotélio e as suas localizações. Continuous: contínuo; fenestrated: fenestrado; e discontinuous: sinusoidal. Fonte: Gupta, Dhanu & Wiklander, Oscar & Wood, Matthew & Andaloussi, Samir. (2023)

Além disso, o endotélio é fonte de mediadores como endotelina[22, 23], prostanóides[24], óxido nítrico[25] e fatores hiperpolarizantes derivado de endotélio (Edhfs)[26].

A Figura 5 ilustra a comparação entre o sistema circulatório répteis com o dos mamíferos. Os répteis possuem duas saídas de sangue, mas o sangue é apenas oxigenado pelos pulmões. O coração possui três câmaras e os ventrículos possuem uma separação parcial, fazendo com o que ocorra uma mistura de sangue com e sem oxigênio. Os mamíferos possuem em seu coração quatro câmaras que separa completamente o sangue oxigenado do sangue sem oxigênio. O sangue oxigenado é bombeado apenas para os órgãos e o sangue desoxigenado é bombeado para os pulmões[27].

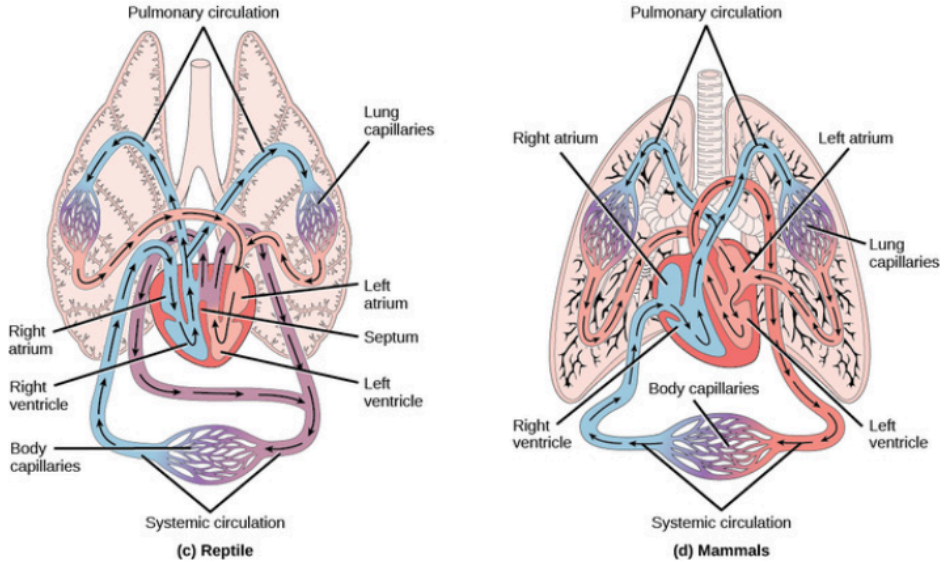

Figura 5 - Anatomia comparativa do sistema circulatório de (c) répteis e (d) mamíferos. Fonte: Boudless. Boundless biology. In: Overview of the Circulatory System - Types of Circulatory Systems in Animals. 2013.

A primeira demonstração da importância do endotélio na regulação do tônus vascular foi pela análise da relação entre acetilcolina (ACh) e a vasodilatação na aorta isolada de um coelho. Observaram que em alguns protocolos, a ACh produziu vasodilatação e em outros, houve vasoconstrição. Após a condução do protocolo, concluiu-se que a vasodilatação ocorreu em vasos com endotélio intacto e vasoconstrição em vasos com endotélio comprometido[28].

Assim, esse fenômeno foi atribuído à presença do “fator relaxante derivado do endotélio” (do inglês, EDRF ou *endothelium-derived-relaxing-factor*), mas que depois foi demonstrado que tal fator se tratava do óxido nítrico (NO). Isso se deu através de ensaios farmacológicos que empregavam bloqueadores competitivos da enzima eNOS, isto é, substâncias que competiam pelo sítio de ligação da L-arginina na eNOS, para abolir a vasodilatação induzida por ACh. Entre as substâncias empregadas estão: N-monometil L-arginina (L-NMMA), NG-nitro-L-arginina (L-NNA), N-nitro-L-arginina metil-éster (L-NAME). Após a ligação desses compostos à eNOS o NO deixava de ser produzido e não mais proporcionava vasodilatação[29, 30].

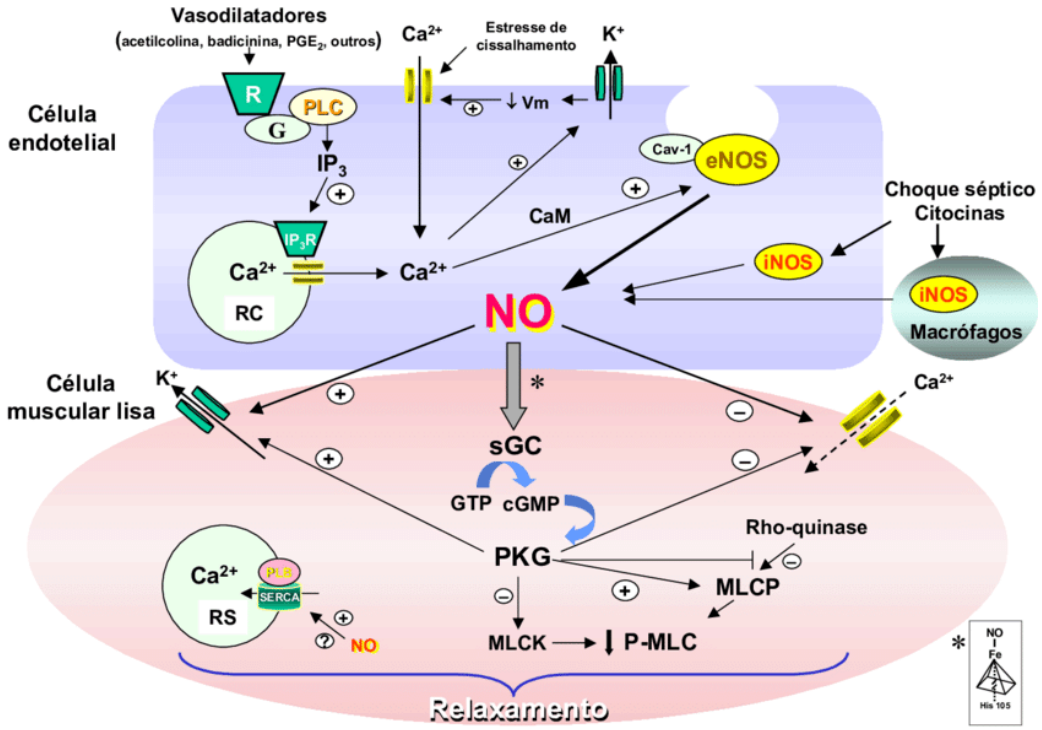

Figura 6 - Esquema do endotélio na contração e relaxamento. Fonte: Dias, 2007.

As prostaglandinas (PG), da classe dos prostanóides, são responsáveis pela regulação do sistema imune, febre e dor associados à inflamção, homeostase e pressão arterial. São produzidas nas células por estímulo externo e se ligam os seus respectivos receptores, divididos em receptores PG-E, PG-D, PG-F, PG-I, e TXA (tramboxano A). O TXA2, considerado um dos mais importantes prostanoides, é um mediador lipídico e tem como mecanismo de ação a vasoconstrição, ativação de agregação plaquetária e modula a resposta das células endoteliais[31].

No grupo das endotelinas, a endotelina 1 (ET-1) é o vasopressor mais potente e está associada às alterações vasculares, como a hipertensão, disfunção renal aguda e angina pectoris. Em termos de potência a ET-1 *in vitro* induz a contração do vaso na mesma proporção que eleva a pressão arterial *in vivo* e possui ação direta no endotélio, uma vez que a sua produção ocorre neste mesmo tecido, exceto para o tipo 2 e tipo 3. A partir da sua produção e liberação, a endotelina atua em receptores específicos das células musculares lisas vasculares e ativa canais de cálcio dependentes de voltagem através das proteínas Gi, aumentando o influxo de cálcio extracelular e consequentemente ocorrendo a vasoconstrição[32].

Os fatores hiperpolarizantes derivados do endotélio (EDHF) diferem por espécie e leito vascular, mas agem aumentando a condutância do potássio (K+), resultando na subsequente propagação da despolarização das células musculares lisas vasculares e

no relaxamento, uma vez que o vaso continua relaxando após a exclusão do NO e endotelina 1. EDHFs tendem a deixar os canais de K+ abertos, localizados na musculatura lisa, e dessa forma permite o efluxo de K+, resultando em hiperpolarização da membrana. Entende-se então que EDHF atua como relaxamento dependente do endotélio induzido por agonista que não é bloqueado por inibidores da NO sintase ou da ciclooxigenase, mas pode ser inibido, pelo menos em parte, por bloqueadores dos canais de K+[33].

Além do sistema vascular periférico, as câmaras cardíacas do coração também possuem células endoteliais chamadas de endocárdio (histologicamente analisada por Nagayo em 1909[34])[35] que modulam a contratilidade do coração como o aumento de sensibilidade de canais de cálcio voltagem-dependente através da liberação de mediadores endoteliais e na presença de catecolaminas[36].

No ponto de vista clínico de encontro com o sistema cardiovascular e o endotélio, condições de hipóxia e hipertensão arterial estão relacionadas à maior vasoconstrição, pois são caracterizadas por modificações estruturais em vasos. Estudos *in vitro* mostraram que as células endoteliais nessas condições expressaram, em maior quantidade, todas as enzimas para síntese de catecolaminas e contribuíram para a vasoconstrição induzida pelo endotélio[37, 12]. A isquemia aumenta a concentração plasmática de noradrenalina, contribuindo para a arteriogênese e angiogênese em quadros como a isquemia dos membros posteriores e para a angiogênese no processo de cicatrização de feridas. Sorriento et al. 2015[38] demonstrou a capacidade das células endoleiais em sintetizar e liberar catecolaminas em resposta à hipóxia (*in vitro*) e à isquemia (*in vivo*) e o envolvimento deste fenômeno na regulação da angiogênese.

1.3. A DESCOBERTA DAS CATECOLAMINAS ENDOTELIAIS

O interesse pelas catecolaminas de origem endotelial iniciou-se através da informação de que a cópula de serpentes pode durar até 24 horas e esse mecanismo via sistema nervoso autônomo pode não ter uma relação exata, principalmente porque o sistema nervoso autônomo é conhecido como “luta e fuga”. Desse modo, a ereção peniana precisa de algum mecanismo que faz com que o pênis fique em estado ereto por um período de tempo prolongado e constante e a relação de que o endotélio da musculatura lisa cavernosa faz sentido. A ereção peniana é um evento neurovascular dependente do relaxamento da musculatura lisa cavernosa e da elevação do fluxo sanguíneo local[39,40]. A contração dos músculos cavernosos em mamíferos ocorre pelo estado de detumescência, o qual é o principal mecanismo fisiológico, em resultado da liberação das catecolaminas através das terminações nervosas adrenérgicas. O óxido

nítrico (NO) é o principal componente responsável por iniciar e manter o estado tumescente, promovendo o relaxamento da musculatura lisa cavernosa[41, 42, 43, 44].

Canais de sódio dependentes de voltagem (CDVS) são importantes canais iônicos envolvidos na despolarização nervosa[45]. O tratamento com tetrodotoxina (TTX; bloqueador dos canais de sódio dependentes da voltagem) ou outros inibidores de CDVS abole o relaxamento nitrérgico induzido pela estimulação de campo elétrico (EFS; técnica que aplica o estímulo uniformemente a um tecido isolado em um pequeno pulso de ondas curtas, frequentemente utilizado em ensaios farmacológicos para a estimulação de nervos intramurais[46, 47, 48] em preparações de corpos cavernosos de coelhos, macacos e humanos[49, 50, 51]. No corpo cavernoso de *Crotalus durissus terrificus* (cascavel), o relaxamento induzido por EFS não é afetado pelo TTX[52], indicando a possível presença de um canal de sódio insensível ao TTX[44].

Foi encontrado a enzima tirosina hidroxilase no corpo cavernoso de *Crotalus durissus terrificus* e *Bothrops jararaca* (jararaca) e as contrações induzidas por EFS nesse tecido era insensível à tetrodotoxina. A contração da aorta da cascavel induzida pelo EFS era inibida por antagonistas α-adrenérgicos (fentolamina e guanitidina) e através da remoção do endotélio, indicando pela primeira vez um potencial fisiológico das catecolaminas endoteliais[52].

A Figura 7 abaixo ilustra a presença de tirosina hidroxilase em tecido endotelial em aorta de *Crotalus durissus terrificus* (A) e *Bothrops jararaca* (B). Não se observa proteína S100 (um marcador de tecido neural), indicando ausência de fibras nervosas na parede da aorta de *Crotalus durissus terrificus* (C) e *Bothrops jararaca* (D)[52].

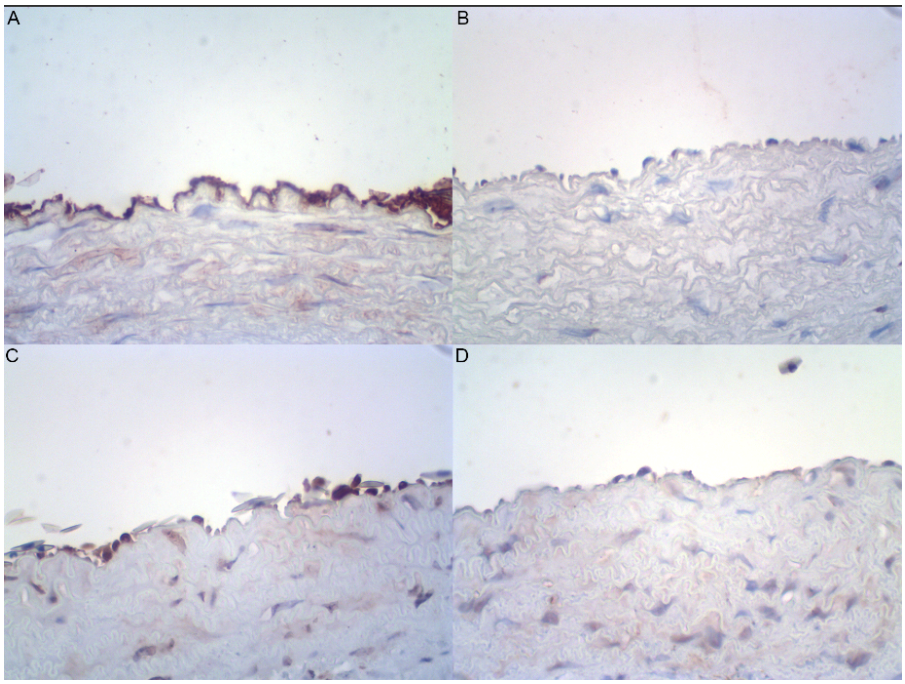

Figura 7 - Fotomicrografia representativa ilustrando a presença de tirosina hidroxilase em células endoteliais de *Crotalus durissus terrificus* (n=4) (A) e *Bothrops jararaca* (n=6) (B) em amostras de aorta. Ausência de

proteína S100 indicando ausência de fibras nervosas e parede de aorta de Crotalus spp (n=4) (C) e Bothrops spp (n=6). Immunoperoxidase, 400x (aumento original). Fonte: Campos et al., 2018a.

O óxido nítrico[25, 28] é responsável pelo mecanismo de vasodilatação e fator importante para o mecanismo vascular seja regularizada em mamíferos e répteis[53]. Fica claro a importância da compreensão dos mecanismos fisiológicos e suas adaptações do sistema cardiovascular em diferentes espécies de animais.

A infusão de doador de óxido nítrico (SNP) em cascavel, tartarugas e lagartos induziu uma vasodilatação sistêmica[54]. Na tartaruga tolerante à anóxia *Trachemys scripta elegans,* o óxido nítrico também participa da anóxia e reoxigenação[55, 56]. Os experimentos *in vitro* demonstraram a acetilcolina (ACh) provocou um relaxamento dependente de NO em anéis aórticos isolados do crocodilo *Crocodylus porosus*[57]. A via de sinalização do NO foi caracterizada por análises farmacológicas, imunohistoquímicas e transcriptômicas em anéis aórticos isolados da cascavel *Crotalus durissus terrificus*[58]. Os receptores colinérgicos muscarínicos ao serem ativados promovem um aumento do tônus parassimpático da tartaruga *Pseudemys scripta* resultando em vasoconstrição na circulação pulmonar[59]*.* A administração de atropina *in vivo* aumenta o fluxo pulmonar na tartaruga *Chelonoidis carbonaria*[60] e a via de relaxamento NO – sGC (guanilato ciclase solúvel) – PDE5 (fosfodiesterase tipo 5) está presente na aorta e possui semelhança funcional e genômica com vasos de mamíferos[61], permitindo utilizar o modelo animal e comparar com o modelo mamífero.

1.4. A 6-NITRODOPAMINA

1.5.

A 6-nitrodopamina (6-ND) é um integrante do grupo 6-nitrocatecolaminas e um resultado putativo da nitração dependente de óxido nítrico da dopamina[62, 63]. *In vitro*, a 6-ND impossibilita a ação neuronal do óxido nítrico sintase (nNOS)[63].

A aorta de *Chelonoidis carbonária in vitro* libera 6-nitrodopamina derivada do endotélio em níveis basais, assim como dopamina, noradrenalina e adrenalina, quantificados através de amostras de solução de Krebs-Henseleit por cromatografia líquida acoplada à espectrometria de massa em tandem (LC-MS/MS). O protocolo utilizou amostras de aorta com e sem endotélio e foram deixadas em solução de Krebs por 30 minutos[64].

A incubação de L-NAME inibe a liberação basal de 6-ND em *Chelonoidis carbonaria* e inibe a contração da aorta, diferente do que ocorre com a dopamina, noradrenalina e adrenalina. A 6-ND antagoniza seletivamente as contrações induzidas pela dopamina sem afetar as contrações induzidas pela noradrenalina ou pela adrenalina em aorta de *Chelonoidis carbonária*[65, 66].

Ainda no que se refere ao *Chelonoidis carbonaria*, o EFS causou contrações dependentes da frequência dos anéis aórticos[65] e essa contração é dependente da liberação de dopamina derivada do endotélio[67]. A 6-ND inibiu as contrações induzidas por EFS dos anéis aórticos e em anéis aórticos pré-contraídos pelo mimético de tromboxano U-46619[68], a 6-ND causou relaxamentos dependentes do endotélio, mas independentes de L-NAME. Estes resultados indicam que esta nova catecolamina derivada do endotélio modula a reatividade do músculo liso de aorta de *Chelonoidis carbonaria*, agindo como um antagonista altamente seletivo da dopamina, atuando em receptor D2-like. A risperidona[69], haloperidol[70] e a quetiapina[71] – antagonistas do receptor do tipo D2 da dopamina – geraram desvios relevantes para a direita das contrações induzidas pela dopamina, demonstrando a diminuição da potência da dopamina[67].

O haloperidol[72] e a risperidona[73] são parcialmente mais potentes que a 6-ND, a seletividade para os receptores dopaminérgicos do tipo D2 é menor, dado que também se ligam aos receptores α1 adrenérgicos. Em contraste, a 6-ND comporta-se como um antagonista extremamente seletivo para receptores do tipo D2, pois não afetou a noradrenalina e as contrações induzidas pela adrenalina nos experimentos com anéis de aorta de *Chelonoidis carbonaria*[66].

A estimulação do campo elétrico contrai os anéis aórticos de *Chelonoidis carbonaria*, que eram sensíveis ao antagonista duplo dos receptores adrenérgicos α1 e α2 fentolamina, mas não ao antagonista seletivo do receptor α1 prazosina ou ao antagonista seletivo do receptor α2 idazoxan, sugerindo que a fentolamina era possivelmente atuando nos receptores dopaminérgicos[65]. Os resultados farmacológicos demonstrando que a 6-ND inibiu as contrações induzidas por EFS afirma que a dopamina de origem endotelial, agindo em receptores semelhantes a D2, é o principal modulador da contração do anel aórtico de *Chelonoidis carbonaria* após estimulação elétrica, atuando claramente como um mediador fisiológico para a manutenção do sistema cardiovascular.

2. OBJETIVOS

2.1. GERAL

I. Investigar o papel fisiológico das catecolaminas produzidas pelo endotélio em *Chelonoidis carbonaria*.

2.2. ESPECÍFICOS

I. Avaliar o papel do endotélio como fonte de catecolaminas na contração induzida por EFS em aorta de *Chelonoidis carbonaria* na presença e ausência do endotélio íntegro;

II. Caracterizar o mecanismo farmacológico das contrações induzidas por EFS em músculo liso vascular de *Chelonoidis carbonaria*;

III. Identificar e localizar as enzimas envolvidas na síntese de catecolaminas em vasos isolados de *Chelonoidis carbonaria*.

3. METODOLOGIA E RESULTADOS
3.1. ARTIGOS CIENTÍFICOS

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

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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.

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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.

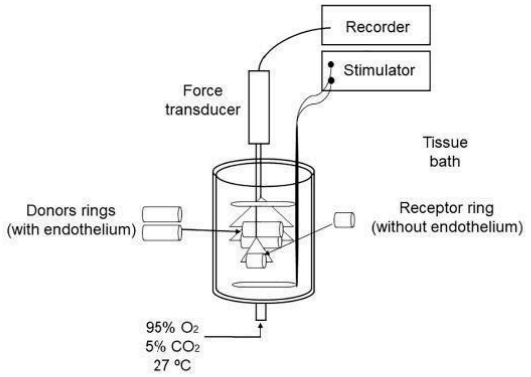

Fig. 1. Schematic illustration of the sandwich assay performed in *Chelonoidis aortic rings.*

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

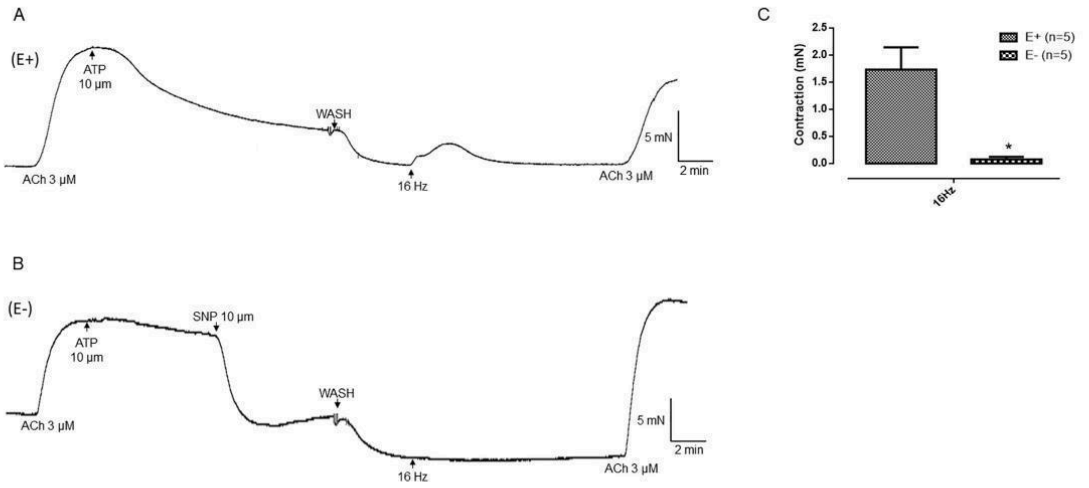

Fig. 2. Representative tracing of a n of 5 experiments for EFS-induced contraction in intact (A) and endothelium-denuded (B) *Chelonoids* aortic rings. Panel (C) shows the expressed as mean ± S.E.M. ($n = 5$, for each group; unpaired *t*-test (E^+ Vs E^-; * = $p < 0.05$).

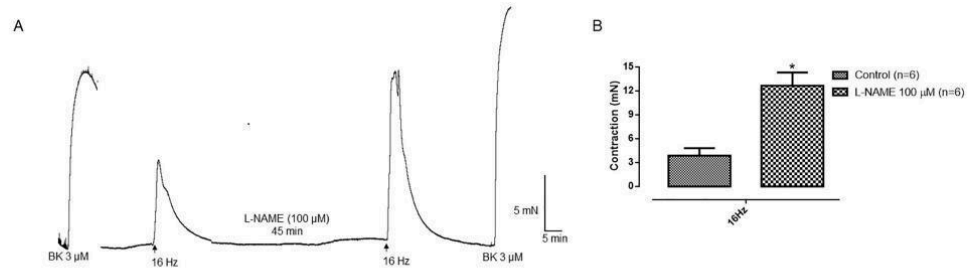

Fig. 3. Representative tracing of a n of 6 experiments of the effect of L-NAME in the EFS-induced contraction in endothelium-intact *Chelonoids* aortic rings (A). The histograms in Panel (B) represent the mean ± S.E.M ($n = 6$) for EFS-induced contractions in endothelium-intact aortic rings before and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

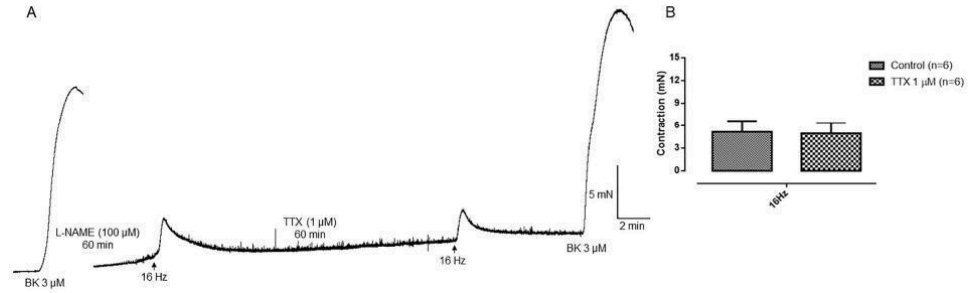

Fig. 4. Panel A shows a representative tracing of a n of 6 experiments of the effect of tetrodotoxin (TTX, 1 mM) in endothelium-intact *Chelonoids* aortic rings treated with L-NAME (100 mM). The histograms in Panel (B) represent the mean ± S.E.M (n = 6) for EFS-induced contractions in endothelium-intact 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

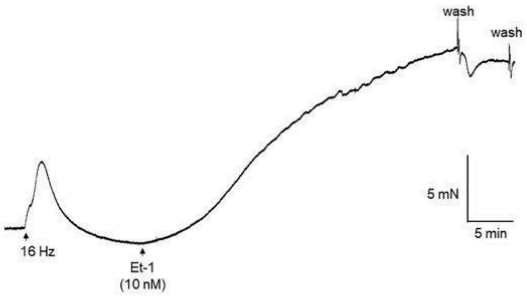

Fig. 5. Representative tracing of a n of 5 experiments comparing the contraction of the *Chelonoids* aortic rings induced by EFS and by ET-1 (10 nM).

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

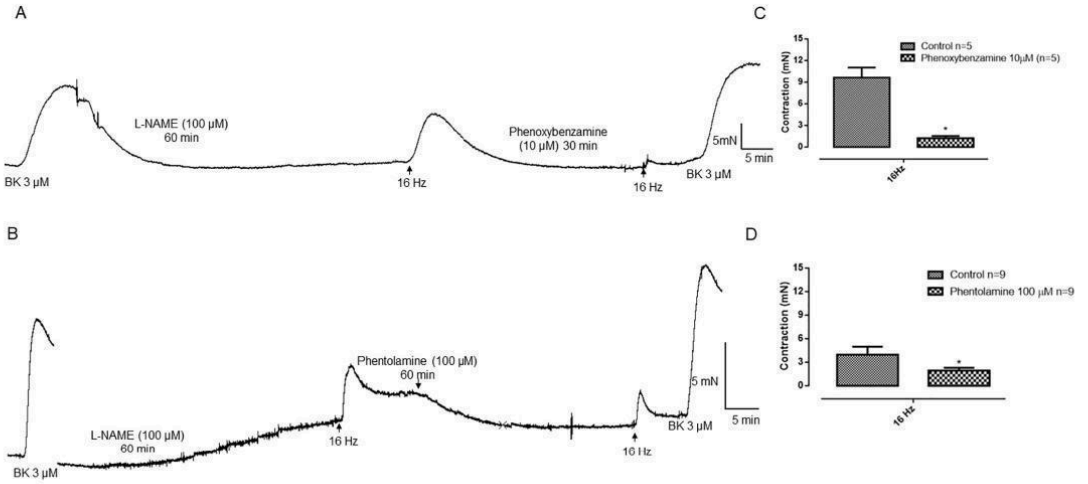

Fig. 6. Panel A shows a representative tracing of a n of 5 experiments of the effect of phenoxybenzamine (10 mM) in endothelium-intact *Chelonoids* aortic rings treated with L-NAME (100 mM). The histograms in Panel (C) represent the mean ± S.E.M (n = 5) for EFS-induced contractions in endothelium-intact 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.

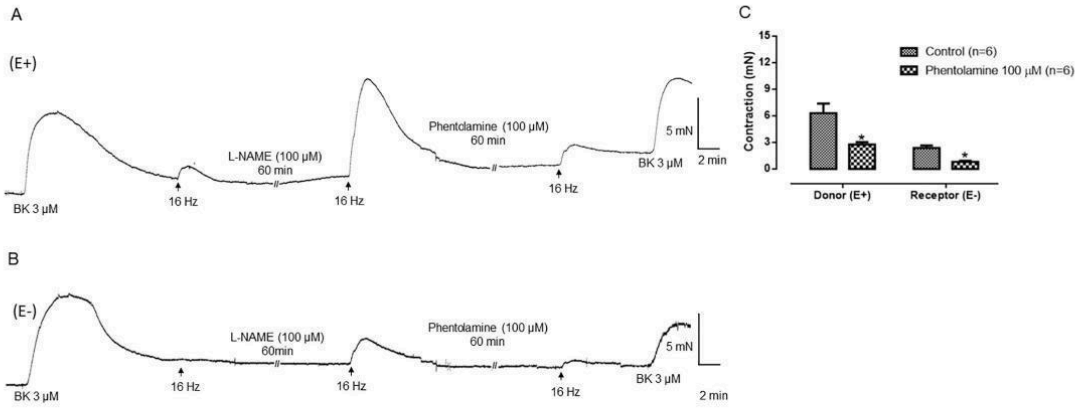

Fig. 7. The sandwich experiment. Two rings with endothelium-intact (E^+, donor tissues) and one ring with endothelium-removed (E^-, receptor tissue) were mounted in the same organ bath. Representative tracings of a n of 6 experiments of EFS-induced contractions in endothelium-intact (A) and endothelium 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).

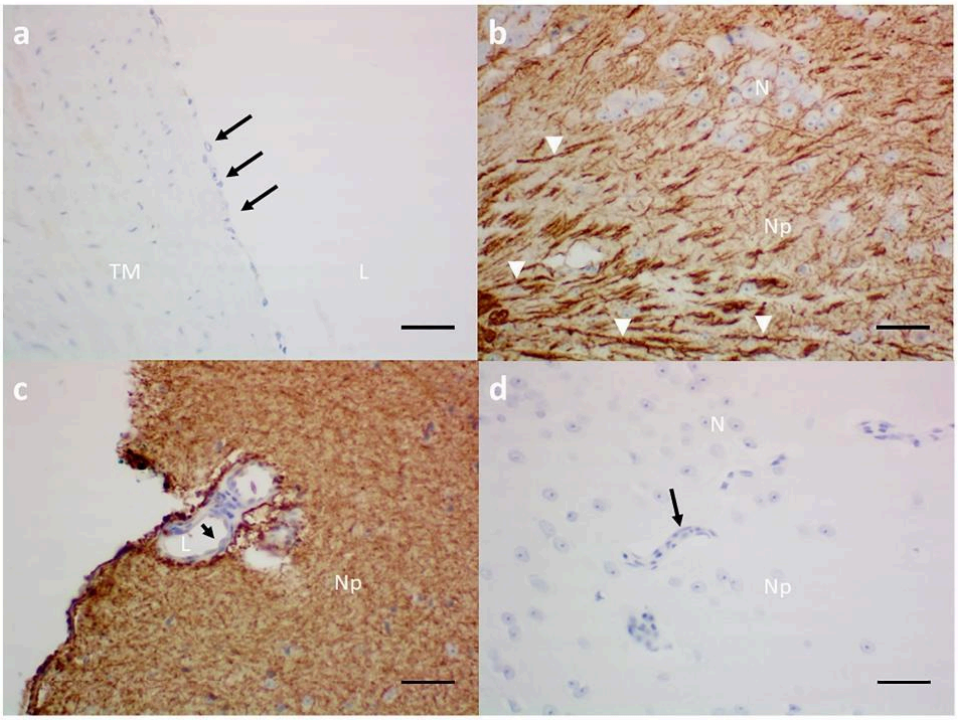

Fig. 8. Detection of S100 protein by immunohistochemistry in *Chelonoidis* aorta (a) and central nervous system (b-d): (a) negative endothelium (arrows) and absence of neural structures within aortic walls; (b) positive neurons (mostly dendrites and axons), and (c) negative vascular endothelium (arrow), 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

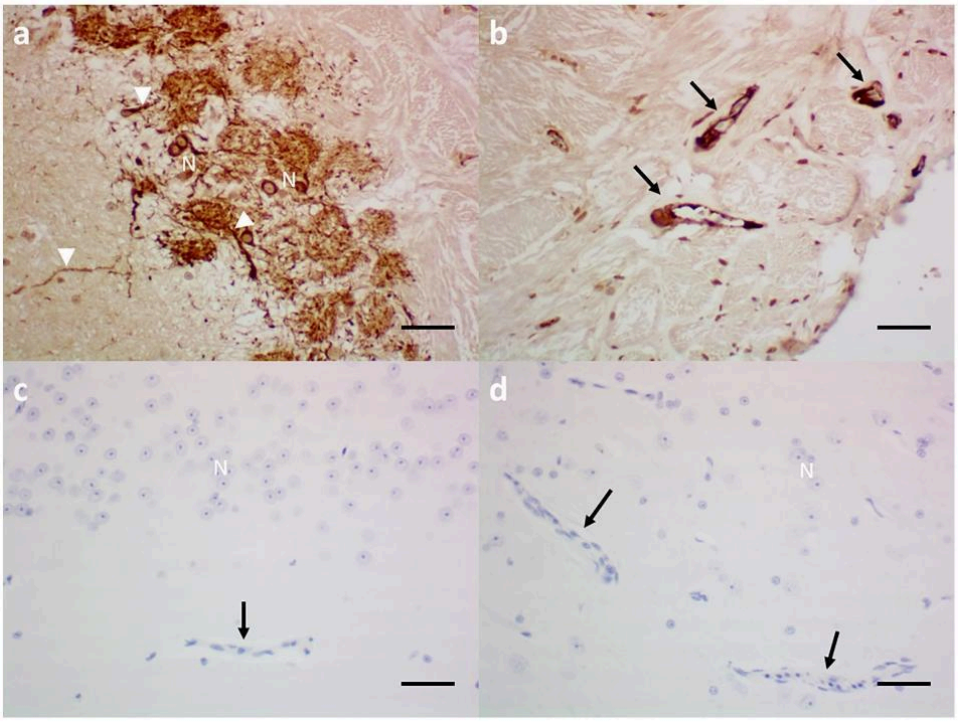

Fig. 9. Detection of tirosine hydroxylase by immunohistochemistry in *Chelonoidis carbonaria* (central nervous system): (a) neurons (cell body, dendrites and axons) and (b) vascular endothelia (arrows); (c) and (d): negative control sections (omission of primary antibody) showing absence of positivity 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-

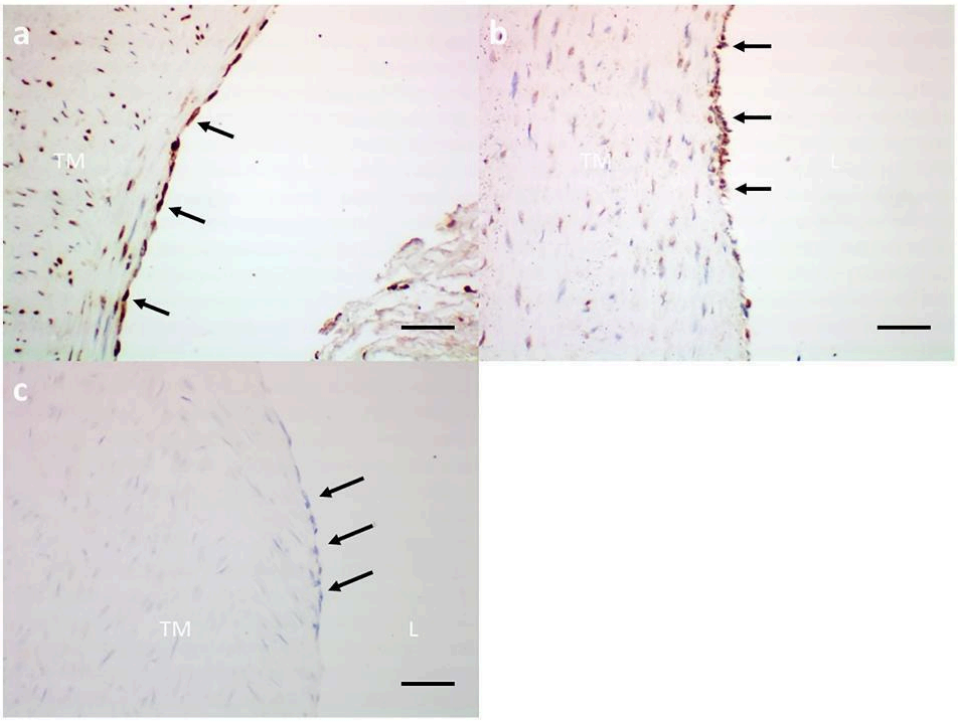

Fig. 10. Detection of tirosine hydroxylase by immunohistochemistry in *Chelonoidis carbonaria* (Aorta): positive aortic endothelium (arrows within a and b); (c): negative control sections (omission of primary antibody) showing absence of positivity in aortic endothelium (arrows). TM = tunica media, L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

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RESEARCH ARTICLE

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

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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.

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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

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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

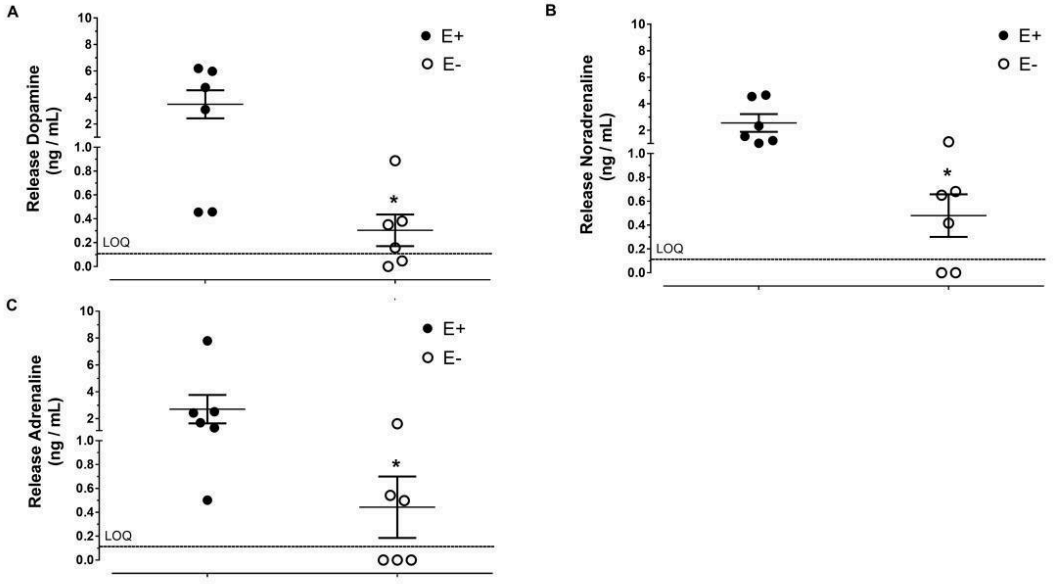

Fig. 1. Basal release of catecholamines in endothelium-intact aortic rings of *Chelonoidis carbonaria*. The basal release of dopamine (A), noradrenaline (B) and adrenaline (C) in Krebs-Henseleit solution after 30 min incubation with endothelium-intact (E+; n=6/6) and endothelium-denuded aortic rings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

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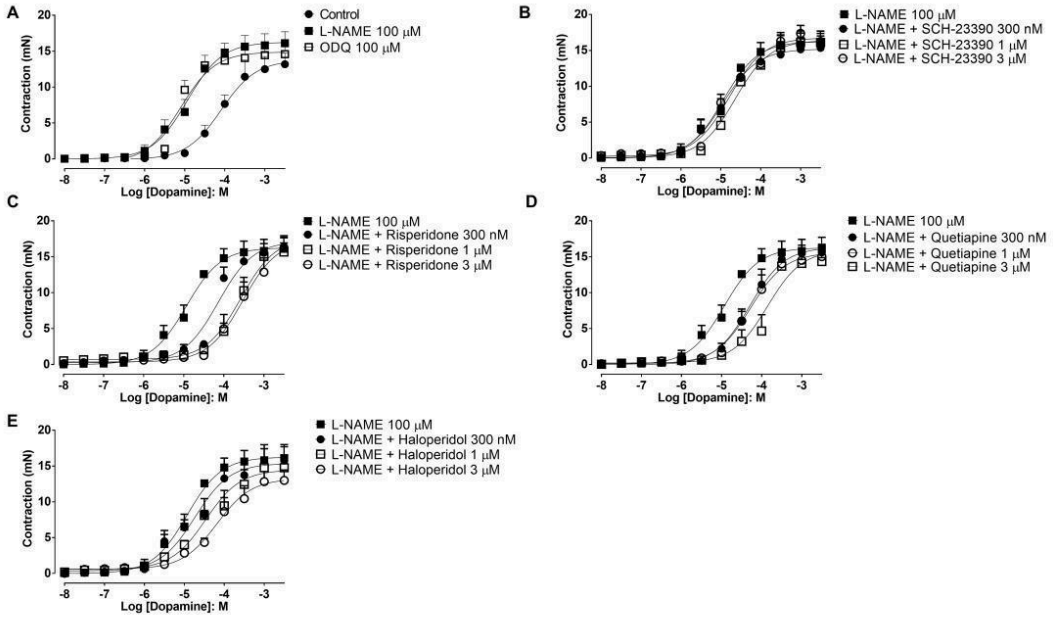

Fig. 2. Concentration-response curves to dopamine in *Chelonoidis carbonaria* aortic rings. Cumulative concentration-response curves to dopamine in *Chelonoidis carbonaria* aortic rings was performed in presence and absence of L-NAME (100 μM; *n*=5/6) and ODQ (100 μM; *n*=5/5; panel (A). Concentration-response 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).

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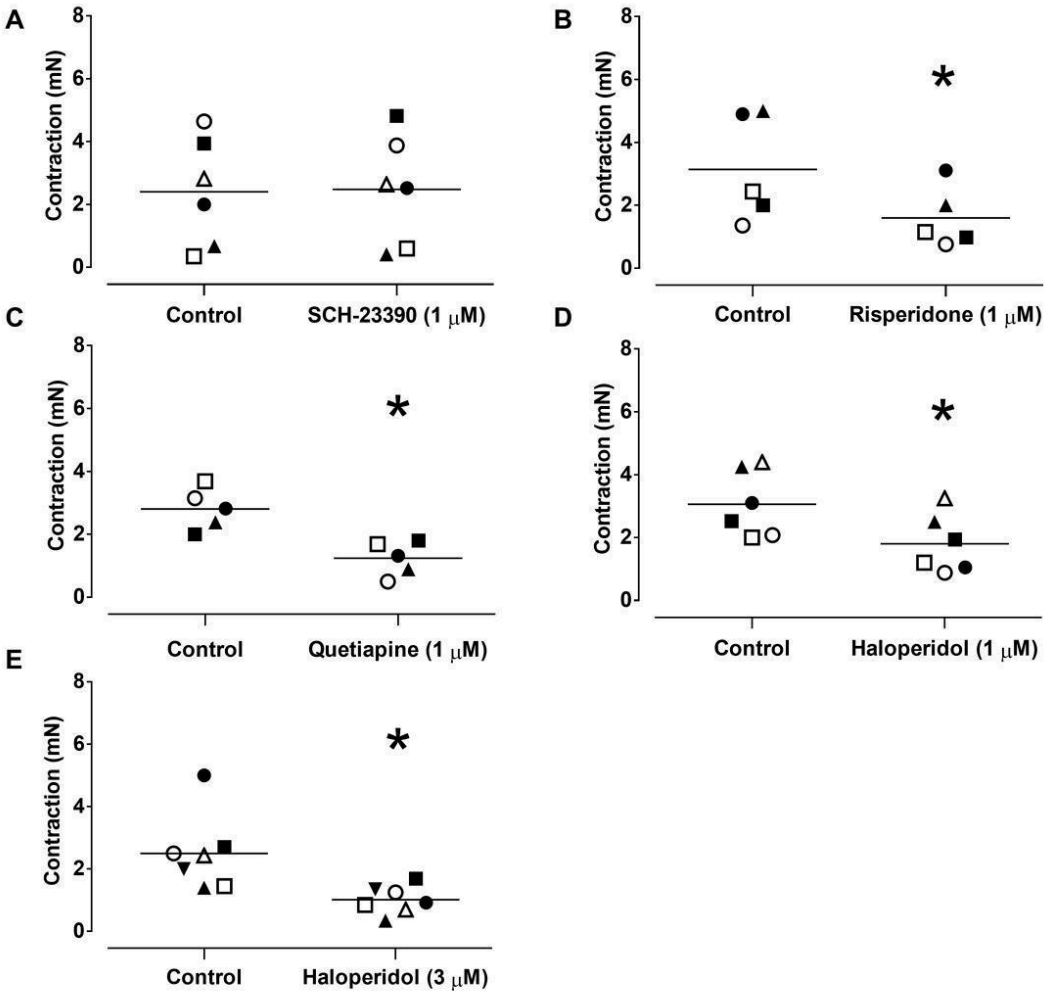

Fig. 3. Effects of D1-like and D2-like receptor antagonists on EFS-induced contractions of aortic rings of *Chelonoidis carbonaria*. Scatter plots show the individual values of the effects of the D1-like receptor antagonist SCH-23390 (*n*=4/6; 1 µM; A) and the D2-like receptor antagonists risperidone (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

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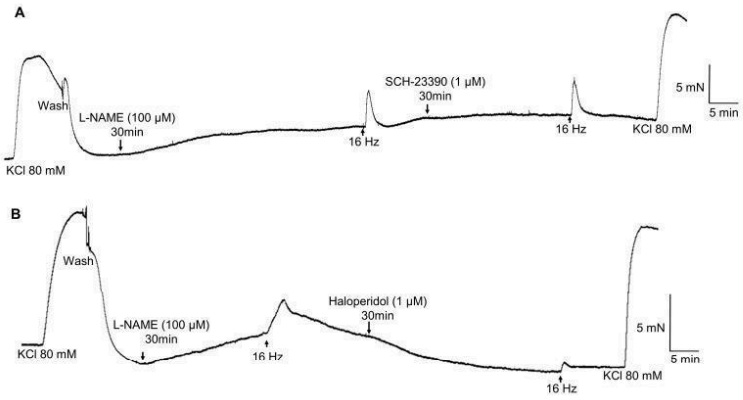

Fig. 4. Representative tracing of the effect of the D1-like receptor antagonist SCH 23390 (1 µM; *n*= 4/6) and the D2-like receptor antagonist haloperidol (1 µM; *n*=4/6) on EFS (16 Hz)-induced contractions of aortic rings of *Chelonoidis carbonaria* pretreated with L-NAME (100 µM).

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

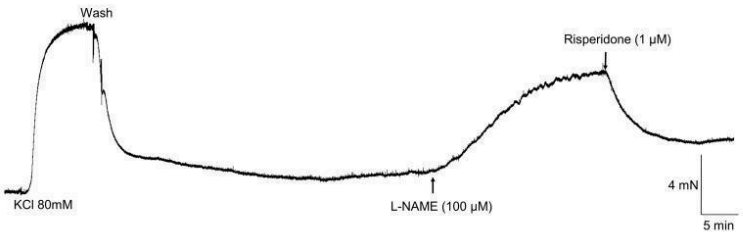

Fig. 5. Representative tracing showing the reversal by risperidone (1 µM; *n*=5/5 of the elevated tonus induced by L-NAME (100 µM) in aortic rings of *Chelonoidis carbonaria*.

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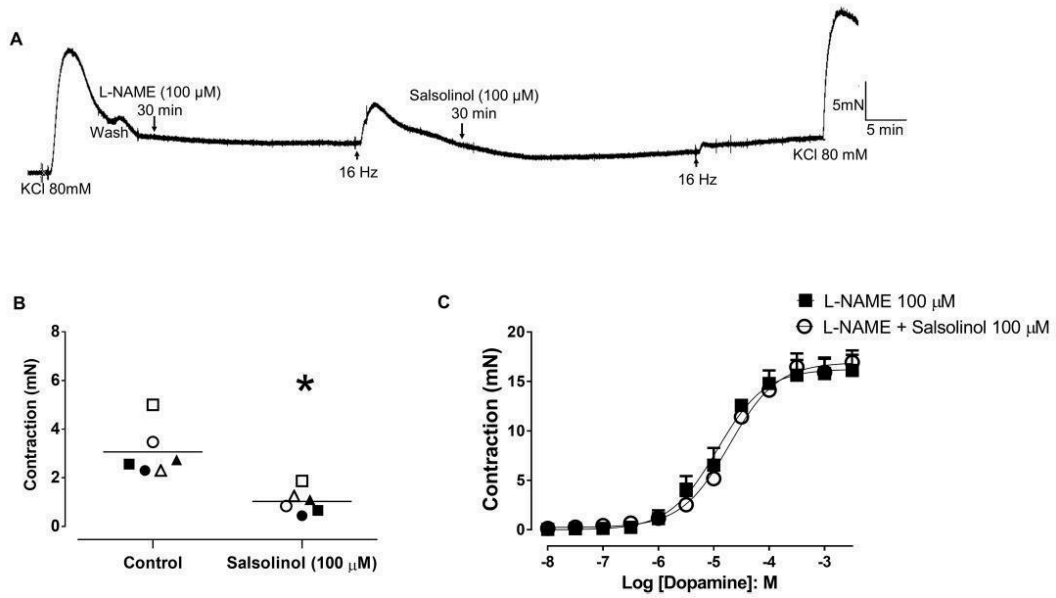

Fig. 6. Effect of the tyrosine hydroxylase inhibitor salsolinol on EFS-induced contraction of aortic rings of *Chelonoidis carbonaria*.
(A) Representative tracing displaying the inhibitory effect of salsolinol (100 µM) on EFS (16 Hz)-induced contraction of aortic rings pretreated with L-NAME (100 µM; *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

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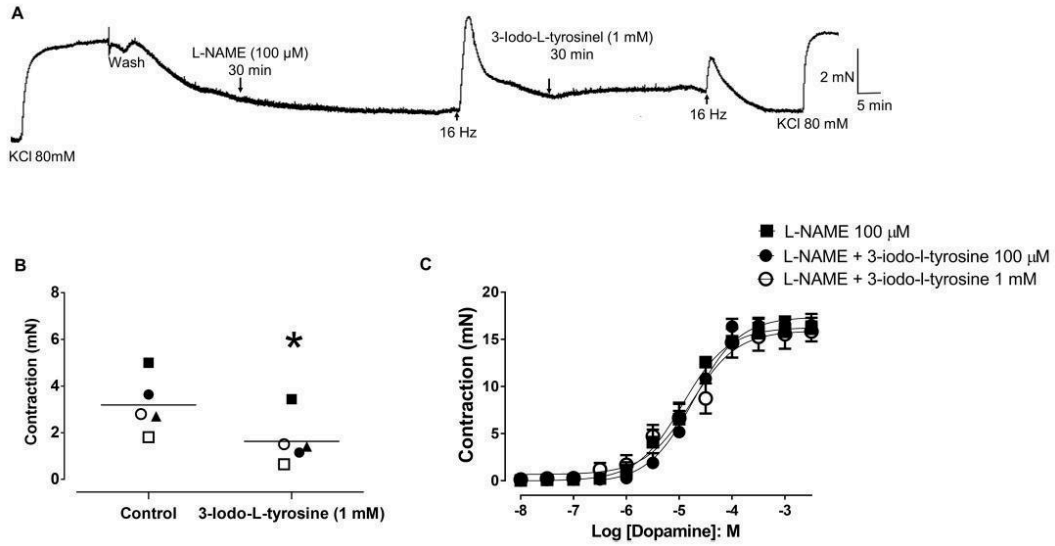

Fig. 7. Effect of the tyrosine hydroxylase inhibitor 3-iodo-tyrosine on EFS-induced contraction of aortic rings of *Chelonoidis carbonaria*.
(A) Representative tracing of the inhibitory effect of 3-iodo-L-tyrosine (1 mM) on EFS (16 Hz)-induced contraction of aortic rings pretreated with L-NAME (100 µM; *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), Nᵒ-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

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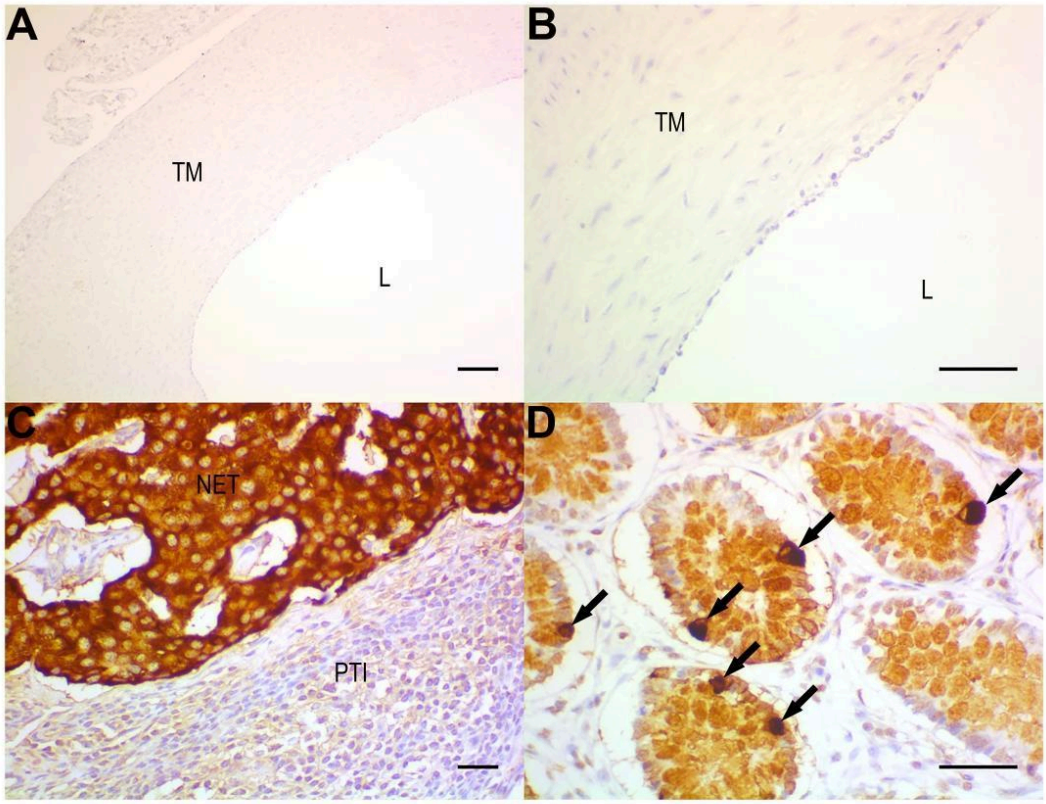

Fig. 8. Chromogranin A detection by immunohistochemistry. (A) lack of positivity for chromogranin A (CgA) in Chelonoidis aortic smooth muscle cells of the tunica media (TM) and in endothelial cells lining the lumen (L), low-power field (100X, original magnification); (B) same as in previous photomicrograph, 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).

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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.

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3.2. NOVOS RESULTADOS

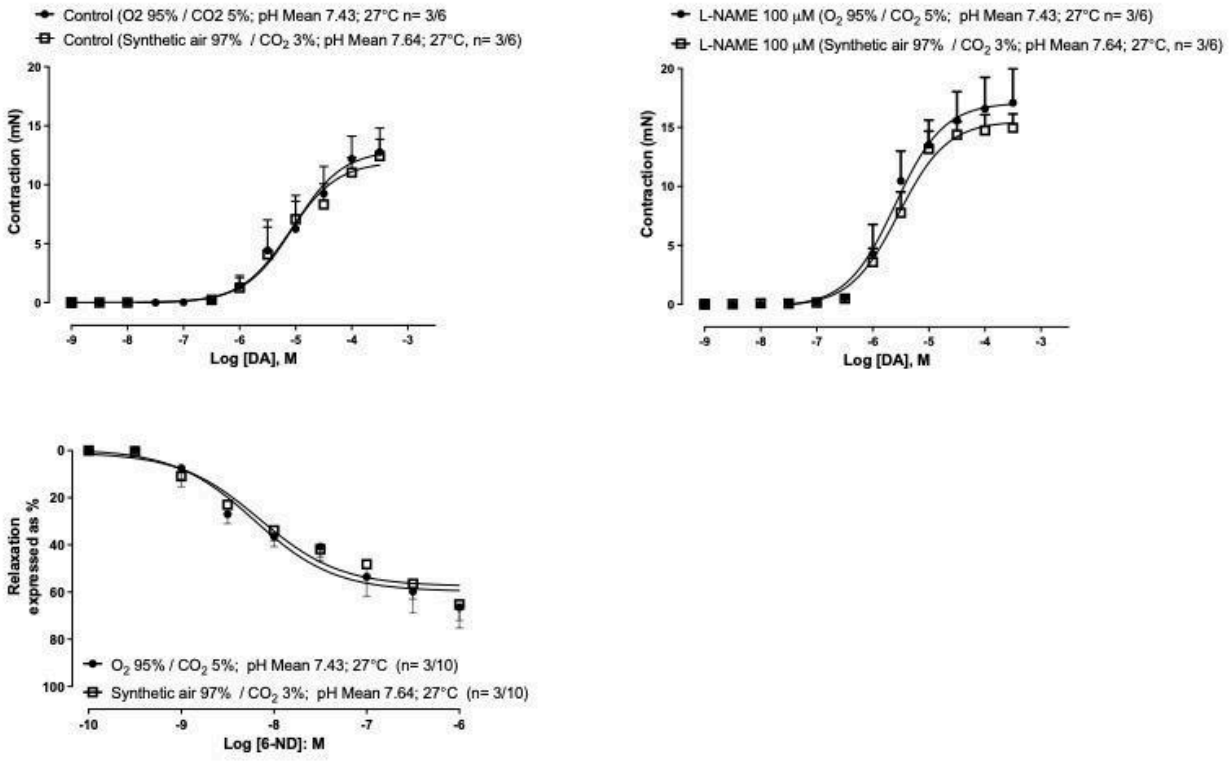

Figura 18 - Curva concentração-resposta com dopamina e 6-nitrodopamina com e sem L-Name, na presença de gás carbogênio padrão (O2 95% e CO2 5%) e modificado.

4. DISCUSSÃO

Os resultados deixam claro que a aorta de *Chelonoidis carbonaria* tem liberação basal de dopamina, noradrenalina e adrenalina, identificadas da espectrometria de massa, e essa liberação é reduzida quando se remove o endotélio e evento similar acontece em vasos de cordão umbilical humano[74]. Os novos dados ilustram que a mistura de carbogênio utilizada como padrão não demonstra diferença em *in vitro* quando comparado com uma concentração de pH menos ácida, como proposto pelo revisor. Sabe-se que uma maior concentração de dióxido de carbono resulta em uma acidificação do sangue[75] que pequenas mudanças podem alterar o metabolismo de algumas moléculas e enzimas[76].

Somente a fentolamina, bloqueador não seletivo de receptor α-adrenérgico, em grande concentração foi capaz de inibir as contrações por EFS na aorta de *Chelonoidis carbonaria*, enquanto prazosina, antagonista α1, e idazoxano, antagonista α2, não tiveram efeitos na contração da aorta. Desse modo, pode-se concluir que a inibição da fentolamina não ocorre em receptores α-adrenérgicos[65], pois também é antagonista de receptores dopaminérgicos[77]. Esses elementos atestam as contrações por EFS inibidas pelo antagonista de receptor D2-like risperidona, quetiapina e haloperidol e a não inibição pelo antagonista de receptor D1-like SCH-23390[78]. As contrações com dopamina e EFS foram abolidas na presença de antagonistas para D2-like e o mesmo evento ocorre em artérias e veias de cordão umbilical humano. Pode-se afirmar que a dopamina derivada do endotélio tem um papel modulador importante de vasoconstrição no sistema cardiovascular através do bloqueio do receptor D2-like. As células endoteliais modulam a contração por EFS, e mesmo que essas células não sejam consideradas células excitáveis, há expressão de canais de voltagem de potássio. Tal relação ainda precisa ser aprofundada.

Um estudo comparando pacientes transplantados com coração e pulmão e pacientes não transplantados, demonstra a ação das catecolaminas de origem endotelial. O grupo de transplantados teve uma frequência cardíaca e pressão diastólica maior do que o grupo de não transplantados. No momento da inclinação da cabeça, foi observado que os aumentos dos parâmetros foram semelhantes em ambos os grupos, assim como os níveis basais de adrenalina e noradrenalina. Porém, na etapa da inclinação da cabeça para cima, ocorreu um aumento significativo dos níveis plasmáticos de noradrenalina no grupo transplantado em comparação ao grupo controle. É evidente que as catecolaminas estão agindo e que não estão vindo dos ramos adrenérgicos desnervados no coração[79].

Os fatos que a dopamina, noradrenalina e adrenalina liberadas em níveis basais pelo endotélio em aorta de *Chelonoidis carbonaria* modulam as contrações provocadas por EFS e que as catecolaminas originadas do endotélio por terem a sua ação nos receptores do tipo D2, podem estabelecer um mecanismo adequado para que os

répteis consigam controlar localmente o fluxo sanguíneo. Em parâmetros fisiológicos normais, as grandes artérias não desempenham função de regular o fluxo sanguíneo e a pressão, mesmo sendo capazes de contrair e dilatar[80]. É proposto que as catecolaminas derivadas do endotélio são responsáveis por estes mecanismos nos vasos e na microcirculação. É importante destacar que a vasoatividade α-adrenérgica contribui para a regulação do fluxo sanguíneo para o fígado e casco durante a submersão anóxica a 5°C na tartaruga *Trachemys scripta*[81] e é interessante que os receptores D2 tenham sido identificados na microcirculação capilar pulmonar de coelhos[82].

Nos anéis aórticos de *Chelonoidis carbonaria*, a liberação de 6-ND também foi reduzida pela remoção mecânica da camada de endotélio, indicando que as células endoteliais são responsáveis pela produção de 6-ND. O mesmo ocorre em vasos de cordão umbilical humano.

As descobertas de que tanto a dopamina quanto a 6-ND[83] são liberados pelo endotélio, e a liberação de 6-ND está acoplada à síntese de NO, indicam que o tônus vascular pode ser influenciado por um equilíbrio dinâmico entre a atividade contrátil vascular da dopamina e a ação vasodilatadora do 6-ND. Como não há imunorreatividade à proteína S-100 nos anéis de aorta de *Chelonoidis carbonaria*, tais tecidos não possuem inervação autonômica[65]. Mesmo que a aorta seja classificada como um vaso condutor[84] e não tem papel na resistência vascular[85], a pouca evidência morfológica de inervação autonômica, como terminais nervosos simpáticos na microcirculação, sustenta-se que a liberação de catecolaminas de origem e dependentes do endotélio poderia ser o principal mecanismo para controlar o fluxo sanguíneo local.

5. CONCLUSÃO

As catecolaminas liberadas pelo endotélio regulam as contrações induzidas por EFS. Isto pode constituir um mecanismo adequado pelo qual os répteis modulam a distribuição do fluxo sanguíneo em órgãos específicos. O óxido nítrico desempenha papel importante no fluxo cardiovascular e a 6-ND atua como um antagonista altamente seletivo do receptor do tipo D2 da dopamina, de modo que a 6-ND atua na manutenção do sistema cardiovascular de *Chelonoidis carbonaria.* Ainda como ponto importante, foram encontradas no endotélio de *Chelonoidis carbonaria* a enzima tirosina hidroxilase, precursora do início da síntese das catecolaminas e as contrações dos vasos não ocorrem quando o endotélio é removido.

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ANEXOS
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CERTIFICADO

Certificamos que a proposta intitulada **Avaliação do papel do endotélio como fonte de catecolamina em Chelonoidis sp**, registrada com o nº **5265-1/2019**, sob a responsabilidade de **Prof. Dr. Gilberto De Nucci e Felipe Fernandes Jacintho, Rafael Campos**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP**, em reunião de **29/05/2019**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	01/05/2019 a 25/03/2029
Vigência da autorização para manipulação animal:	29/05/2019 a 25/03/2029
Espécie / linhagem/ raça:	Réptil** / Chelonoids sp
No. de animais:	10
Idade/Peso:	30.00 Anos / 3.00 Kilos
Sexo:	10 Machos
Espécie / linhagem/ raça:	Réptil** / Chelonoids sp
No. de animais:	10
Idade/Peso:	30.00 Anos / 3.00 Kilos
Sexo:	10 Machos
Espécie / linhagem/ raça:	Réptil** / Chelonoidis sp
No. de animais:	10
Idade/Peso:	30.00 Anos / 3.00 Kilos
Sexo:	10 Fêmeas
Espécie / linhagem/ raça:	Réptil** / Chelonoidis sp
No. de animais:	10
Idade/Peso:	30.00 Anos / 3.00 Kilos
Sexo:	10 Fêmeas
Origem:	Os animais serão adquiridos no Parque Ecológico do Tietê e serão utilizados para fins experimentais no mesmo dia da sua chegada, permanecendo menos de 24 horas no laboratório de Farmacologia.
Biotério onde serão mantidos os animais:	-Não se aplica-

A aprovação pela CEUA/UNICAMP não dispensa autorização a junto ao **IBAMA, SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, **29 de maio de 2019**.

Prof. Dr. Wagner José Favaro
Presidente

Rosangela dos Santos
Secretária Executiva

IMPORTANTE: Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos sejam submetidos.

SISBIO

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 20910-13	Data da Emissão: 25/05/2021 14:12:44	Data da Revalidação*: 01/03/2022

De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Gilberto De Nucci	CPF: 868.134.478-15
Título do Projeto: Avaliação, ex vivo, do trato urogenital de Chelonoidis carbonaria.	
Nome da Instituição: UNIVERSIDADE ESTADUAL DE CAMPINAS	CNPJ: 46.068.425/0001-33

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Seleção dos animais e início do tratamento	08/2009	08/2009
2	início do tratamento	09/2009	09/2009
3	Retirada de tecidos e estudos na Unicamp	10/2012	12/2012
4	Retirada de tecidos e estudos na Unicamp	06/2011	07/2011
5	Retirada de tecidos e estudos na Unicamp	08/2011	09/2011
6	Retirada de tecidos e estudos na Unicamp	07/2012	09/2012
7	Retirada de tecidos, análise de resultados e finalização dos estudos	01/2013	07/2013
8	Retirada de tecidos e estudos na Unicamp	10/2011	12/2011
9	Retirada de tecidos e estudos na Unicamp	04/2012	06/2012
10	Retirada de tecidos e estudos na Unicamp	01/2012	03/2012
11	Retirada de tecidos e retomada dos estudos na Unicamp	04/2015	03/2016
12	Retirada de tecidos e retomada dos estudos na Unicamp	08/2013	08/2014
13	Retirada de tecidos e retomada de estudos na unicamp	03/2016	03/2017
14	Retirada de tecidos e retomada de estudos na unicamp	05/2017	05/2018
15	Retirada de tecidos e retomada de estudos na unicamp	03/2019	03/2020
16	Retirada de tecidos e retomada de estudos na Unicamp	04/2020	04/2021
17	Retirada de tecidos e retomada de estudos na Unicamp	05/2021	05/2022

Equipe

#	Nome	Função	CPF	Nacionalidade
1	Renata Lopes Rodrigues	Executor	368.601.738-58	Brasileira
2	Rafael de Morais Campos	Executor / Médico Veterinário	020.678.733-26	Brasileira

Observações e ressalvas

1	O pesquisador somente poderá realizar atividade de campo após o término do estado de emergência devido à COVID-19, assim declarado por ato da autoridade competente.
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