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LUIS FREDERICO GERBASE DE OLIVEIRA

LIBERAÇÃO E AÇÃO FARMACOLÓGICA DA 6-NITRODOPAMINA EM ARTÉRIA E VEIA POPLÍTEA HUMANA

RELEASE OF 6-NITRODOPAMINE FROM HUMAN POPLITEAL ARTERY AND VEIN

Campinas

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

Orientador: Andre Almeida Schenka

Coorientador: José Britto Junior

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Dedico esta dissertação a todos os pesquisadores que, dia após dia, trabalham incansavelmente na busca do conhecimento e sabedoria, não para eles próprios, mas para o benefício de todos. Dedico esta obra também a todos os pacientes que contribuíram direta ou indiretamente para a linha de pesquisa em que este trabalho se insere.

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EPÍGRAFE

“Quem se apoia na própria sabedoria nunca é sábio.”

Samer Agi (podcast)

RESUMO

A 6-nitrodopamina (6-ND) é uma nova catecolamina liberada pelo endotélio de vasos umbilicais humanos e, neste estudo, foi avaliado se esta substância é também liberada do endotélio de artérias e veias poplíteas humanas obtidas de membros amputados por doença arterial periférica e sua ação nestes vasos. O estudo demonstrou a liberação basal da 6-ND pelo endotélio através da cromatografia líquida acoplada à espectrofotometria de massa em tandem, mas com redução importante após remoção do endotélio ou após inibição da produção de óxido nítrico (NO) através do tratamento com N-gama-Nitro-L-arginina-metil-éster, um inibidor da síntese de NO. Em vasos pré-contraídos com U-46619 (mimético do tromboxano A2), o efeito de relaxamento da musculatura lisa vascular destes vasos pela 6-ND foi demonstrada, sendo concentração-dependente, de maneira similar ao antagonista seletivo dos receptores D2-*like* da dopamina L-741,626. Pela primeira vez, comprovamos a liberação de 6-ND em vasos poplíteos humanos; a sua ação antagonista da vasoconstrição causada pela dopamina através dos seus receptores D2-*like* poderá abrir novas possibilidades terapêuticas desta substância no tratamento das doenças arteriais obstrutivas periféricas.

Palavras-chave: dopamina; Catecolaminas; Doença arterial oclusiva periférica.

ABSTRACT

The 6-nitrodopamine (6-ND) is a novel catecholamine released by the endothelium of human umbilical vessels, and in this study, we investigated whether this substance is also released from the endothelium of human popliteal arteries and veins obtained from limbs amputated due to peripheral arterial disease and its action in these vessels. The study demonstrated the basal release of 6-ND by the endothelium through liquid chromatography coupled with tandem mass spectrometry, but with a significant reduction after endothelium removal or inhibition of nitric oxide (NO) production through treatment with L-NAME (N-gama-Nitro-L-arginina-metil-ester), an NO synthesis inhibitor. In vessels pre-contracted with U-46619 (Tromboxane A2 -*like*), the relaxation effect of vascular smooth muscle by 6-ND was demonstrated, being concentration-dependent, similar to the selective dopamine D2-*like* receptor antagonist L-741,626. For the first time, we confirmed the release of 6-ND in human popliteal vessels; its antagonistic action against dopamine-induced vasoconstriction through its D2-*like* receptors may open new therapeutic possibilities for this substance in the treatment of peripheral obstructive arterial diseases.

Key-words: dopamine; catecholamines; lower limb peripheral artery disease.

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1. INTRODUÇÃO

A doença arterial periférica obstrutiva (DAOP) é uma das principais causas de morbidade vascular no mundo, em conjunto com a doença coronariana e o infarto cerebral isquêmico (1). Ela caracteriza-se pela obstrução das artérias, principalmente dos membros inferiores, por placas de ateroma, que progressivamente vão estreitando o lúmen arterial até causar a cessação do fluxo sanguíneo e causar a trombose das artérias (2). Com a evolução da doença, ocorrem sintomas como a claudicação intermitente, progressão para dor isquêmica ao repouso e lesões tróficas espontâneas ou por pequenos traumas, que não cicatrizam devido à isquemia tecidual (3). Cerca de 10% dos pacientes com DAOP necessitarão de algum procedimento de revascularização para salvar o membro e evitar a sua amputação, situação esta que é inevitável em alguns pacientes (4).

A disfunção endotelial tem sido implicada na gênese da aterosclerose, sendo esta disfunção uma consequência da hipertensão arterial, da dislipidemia, do uso do tabaco e da própria isquemia local causada pela trombose do lúmen do vaso, entre outros fatores. O endotélio participa ativamente do equilíbrio entre relaxamento e contração dos vasos sanguíneos através da produção de substâncias vasoativas, como o óxido nítrico, prostaglandinas, prostaciclinas, angiotensina-II, tromboxano A2 (5,6). Nesta doença, ocorre relaxamento anormal endotélio-dependente das células musculares lisas, cuja mediação normal é realizada pela via do óxido nítrico (NO)-cGMP (7). A estimulação da guanilato-ciclase solúvel é o mecanismo mais aceito pelo qual o NO causaria vasodilatação (8).

As catecolaminas são um grupo de substâncias vasoativas, que também modulam o equilíbrio entre vasoconstrição e vasodilatação, principalmente através da produção neural. O conceito de que poderíamos ter estas substâncias originadas do endotélio, com ação local sem participação neuronal é um conceito relativamente novo (6). Vários estudos têm demonstrado que a dopamina, por exemplo, tem sua liberação basal oriunda do endotélio em diferentes tecidos de variadas espécies (6).

A via de síntese das catecolaminas, classicamente ocorrendo nas terminações neurais, inicia-se com a enzima tirosina hidroxilase (TH), que catalisa a conversão do aminoácido tirosina em L-DOPA (L-3,4-di-hidroxifenilalanina). Pela ação da dopa descarboxilase ocorre a quebra de diversos outros aminoácidos L-aromáticos, como a L-histidina e L-triptofano, que são precursores na síntese de histamina e, também,

da L-DOPA em dopamina. A dopamina-β-hidroxilase (DBH), localizada em vesículas sinápticas, converte a dopamina em noradrenalina (NA). A feniletanolamina N-metiltransferase (FNMT) catalisa a N-metilação da noradrenalina para adrenalina (9,10) nas células cromafins da medular adrenal.

Os efeitos fisiológicos gerados pela dopamina são mediados por receptores acoplados à proteína G (GPCR), denominados receptores dopaminérgicos, os quais são divididos em dois grupos: D1-*like* (D1 e D5) e D2-*like* (D2, D3, D4) (10). Em células musculares lisas vasculares, os receptores do tipo D1-*like* estão acoplados a proteína G estimulatória (Gs), que ativa a enzima adenilato ciclase (adenilil ciclase), resultando em aumento da produção de AMPc, ativação de proteína quinase A (PKA) e, consequentemente, relaxamento da túnica média vascular/vasodilatação (10). Os receptores do tipo D2-*like*, por sua vez, estão acoplados à proteína inibitória (Gi) e desencadeiam eventos opostos (i.e., inibição da adenilil ciclase [AC], redução dos níveis de AMPc, diminuição da ativação de PKA e, consequentemente, vasoconstrição) (10).

Sabe-se que, em pH fisiológico e através de diferentes mecanismos bioquímicos, o óxido nítrico (NO) reage com diferentes catecolaminas (e.g., a dopamina e a noradrenalina) formando nitrocompostos, em particular, os 6-nitroderivados (11). A 6-nitrodopamina (6-ND) é uma nitro-catecolamina com liberação comprovada em vasos de cordão umbilical humano (12), aorta de *Chelonoidis carbonarius,* (6) e de *Panterophis guttatus (13)*, além de aorta torácica e artéria pulmonar de *Callithrix spp (36)*. Sua síntese/liberação está ligada à síntese do NO, uma vez que o uso de antagonistas da sintetase do NO, como o L-NAME, reduzem significantemente a produção da 6-ND nessas estruturas vasculares (12-14). Sua ação nesses vasos é vasodilatadora, agindo como antagonista de receptores dopaminérgicos D2 vasculares (14), uma vez que a dopamina exerce sua ação vasoconstritora nas células musculares lisas através desses mesmos receptores (15,16)). O equilíbrio entre relaxamento e vasoconstrição pode também se dar através da 6-ND sintetizada pelo endotélio, como mais uma via pela qual o NO causa vasodilatação (17-19). No presente estudo, tanto a liberação basal de 6-ND em vasos poplíteos como suas ações farmacológicas foram avaliadas pela primeira vez.

2. OBJETIVOS

2.1 Objetivos gerais

Investigar a liberação endotelial e a ação farmacológica *in vitro* da 6-nitrodopamina em anéis arteriais e venosos poplíteos humanos.

2.2 Objetivos específicos

- Quantificar a liberação da 6-nitrodopamina nesses anéis vasculares por cromatografia líquida acoplada a espectrofotometria de massa em tandem (LC-MS/MS);
- Investigar o papel vasodilatador da 6-nitrodopamina nesses anéis vasculares;
- Confirmar a origem endotelial da 6-nitrodopamina, demonstrando, por meio de técnica imuno-histoquímica, a ausência de estruturas neurais que poderiam sintetizar/liberar alternativamente este novo mediador.

3. MATERIAIS E MÉTODOS/ RESULTADOS

Os materiais e métodos, assim como os resultados desta dissertação são apresentados em formato de artigo publicado na revista *Life Sciences,* sob o título: "*Release of 6-nitrodopamine from human popliteal artery and vein".* Os autores do artigo publicado foram: de Oliveira LFG, Britto-Júnior J, Lima AT, Moraes MO, Moraes MEA, de Souza VB, Schenka AA, Zakia Monica F, De Nucci G. A publicação foi realizada em formato *on-line*, em 25 de maio de 2023.

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Release of 6-nitrodopamine from human popliteal artery and vein

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ABSTRACT

6-Nitrodopamine (6-ND) is a novel catecholamine that is released from human umbilical cord vessels, and it causes vascular relaxation by acting as a dopamine D_2-receptor antagonist. Here it was investigated whether human peripheral vessels obtained from patients who have undergone surgery for leg amputation release 6-ND, and its action in these tissues. Popliteal artery and vein strips present basal release of 6-ND, as measured by liquid chromatography coupled to tandem mass spectrometry. The release was significantly reduced when the tissues were pre-treated with the nitric oxide synthase inhibitor L-NAME (100 μM), or when the endothelium was mechanically removed. In U-46619 (3 nM) pre-contracted rings, 6-ND induced concentration-dependent relaxations (pEC50 8.18 ± 0.05 and 8.40 ± 0.08, in artery and vein rings, respectively). The concentration-dependent relaxations induced by 6-ND were unaffected in tissues pre-treated with L-NAME, but significantly reduced in tissues where the endothelium has been mechanically removed. In U-46619 (3 nM) pre-contracted rings, the selective dopamine D_2 receptor antagonist L-741,626 also caused concentration-dependent relaxations (pEC_{50} 8.92 ± 0.22 and 8.79 ± 0.19, in artery and vein rings, respectively). The concentration-dependent relaxations induced by L-741,626 were unaffected in tissues pre-treated with L-NAME, but significantly reduced in tissues where the endothelium has been mechanically removed. This is the first demonstration that 6-nitrodopamine is released from human peripheral artery and vein rings. The results also indicate that endothelium-derived dopamine is a major contractile agent in the popliteal artery and vein, and that selective dopamine D_2-receptor antagonists such as 6-ND, may have therapeutic potential in the treatment of human peripheral vascular diseases.

1. Introduction

Lower limb peripheral artery disease is the third leading cause of atherosclerotic vascular morbidity after coronary heart disease and stroke [1]. Atherosclerosis has been associated to endothelial cell dysfunction [2], which is characterized by abnormal endothelial-dependent smooth muscle relaxation, due to impairment of the NO-cGMP pathway [3]. The stimulation of soluble guanylate cyclase is presently considered the main mechanism by which NO causes vasodilation [4]. 6-nitrodopamine (6-ND) is a nitro-catecholamine that is released by the endothelium of human cord vessels [5], *Chelonoidis carbonarius* aortic rings [6], *Panterophis guttatus* aortic rings [7], and *Callithrix spp* thoracic aorta and pulmonary artery rings [8]. The synthesis/release of 6-ND is coupled to nitric oxide (NO) synthesis, since it is significantly reduced when the vascular tissues are pre-treated with the NO synthase inhibitor L-NAME [6–8]. In these vascular tissues, 6-ND is a potent vasorelaxant, acting as a highly and truly selective dopamine $D_{2\text{-like}}$ receptor antagonist [8]. Indeed, vascular endothelial cells can synthetize dopamine and other catecholamines [9], and human umbilical cord vessels do present release of dopamine [5,11]. Dopamine contracts vascular smooth muscle via $D_{2\text{-like}}$ receptors [10,11], thus biosynthesis of 6-ND should be considered as another mechanism by

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which NO causes vasodilation [6–8].

In this manuscript, the release of 6-ND was evaluated by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) from human popliteal artery and vein, obtained from patients who have undergone surgery for leg amputation. Since 6-ND has been characterized as a potent vasorelaxant, its action on pre-contracted vessels was evaluated in the presence of the nitric oxide (NO) synthase inhibitor L-NAME and in endothelium-denuded vessels.

2. Material and methods

2.1. Study participants

Twenty-six patients (17 male and 9 female), aged from 60 to 95 years, with diagnostic of diabetic foot (15 patients) or arterial insufficiency (11) have undergone surgery for leg amputation in the Hospital Santa Casa de Misericórdia (Sorocaba-SP, Brazil). The investigation followed the principles outlined in the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Institute of Biomedical Sciences of the University of São Paulo – ICB/USP (protocol number 3.583.667), and the patients were asked to sign an informed consent.

2.2. Vessels preparation

The human popliteal artery and vein were removed, with special care not to damage the endothelial layer or to over distend the vessels during the procedure. The vessels were placed in a container with Krebs-Henseleit's solution (KHS; pH 7.4, 95%O_2:5%CO_2) at 8 °C.

2.3. Basal release of catecholamines from human popliteal vessels

The vessels were dissected free of connective tissue and bisected into 15-mm-length rings and suspended in 5-mL organ baths containing KHS with ascorbic acid (1 mM) continuously gassed with a mixture of 95% O_2:5%CO_2 at 37 °C for 30 min. The release of 6-ND, dopamine, noradrenaline, and adrenaline was evaluated in intact vessels and in vessels whose endothelia was mechanically removed. The release of the catecholamines was also assessed in intact vessels that were pre-incubated (30 min) in the absence and presence of the NO synthesis inhibitor N_ω-nitro-L-arginine methyl ester (L-NAME, 100 μM). A 2 mL KHS aliquot was transferred to a tube and stored at −20 °C until further analysis by LC-MS/MS.

2.4. Determination of catecholamines in KHS by tandem mass spectrometry (LC-MS/MS)

The extraction of 6-ND, dopamine, noradrenaline, and adrenaline, from 1 mL of KHS was performed by solid phase extraction. To 1 mL of KHS was added 50 μL of the internal standard (100 ng/mL of 6-nitrodopamine-d_4). The samples were homogenized for 10 s. The Strata™-X 33 mm Polymeric Reversed Solid Phase Extraction (SPE) cartridges were pre-conditioned with 1 mL of methanol and then balanced by 2 mL of deionized water. The samples were injected into the cartridge, and the cartridge was subsequently washed 3 times with deionized water. The samples were then eluted with 0.9 mL methanol/water (90/10, *v*/v) with 0.1 % formic acid. The eluate was evaporated under N_2 flow at 50 °C. The residue was dissolved with 100 μL of acetonitrile/water (50/50, v/v) with 0.1 % formic acid and transferred to vials ready for injection. The LC–MS/MS system consisted of LC ADVp Liquid Chromatograph Shimadzu System (Shimadzu Corporation, Kyoto, Japan) coupled to an 8060 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan) operating in electrospray positive-ionization mode. Samples were injected into the system by means of a SIL-30 AC autoinjector, at a temperature of 8 °C. The chromatography separation was performed at room temperature using a GIST-HP C_{18} column (150 mm × 3.0 mm, 3 mm) column (Shimadzu, Duisburg, Germany). A 75 % mobile phase A consisting of deionized water with 0.1 % formic acid (*v*/*v*) and 25 % mobile phase B consisting of acetonitrile/water (90/10, *v*/*v*) with 0.1 % formic acid at a flow rate of 0.35 mL/min were used. The mobile phase perfused a LC ADVp Liquid Chromatograph Shimadzu System coupled to a Shimadzu 8060 triple quadrupole mass spectrometer operating in ESP^+ mode at 350 μL/min. The dissolved residues were injected by a SIL-30 AC autoinjector, at a temperature of 8 °C. The transitions monitored by electrospray multiple reaction monitoring (MRM), injection volume, run-time and limit of quantitation were described elsewhere [12,13].

2.5. Effect of 6-ND in pre-contracted vessels rings

Briefly, rings (5-mm) of human popliteal artery and popliteal vein were suspended vertically between two metal hooks in 10-mL organ baths containing KHS, continuously gassed with a mixture of 95%O_2:5% CO_2 (pH 7.4) at 37 °C. Tissues were allowed to equilibrate under a resting tension of 10 mN, and the isometric tension was registered using a PowerLab system (ADInstruments, Sydney, Australia).

Vessels rings with intact and denuded endothelium and endothelium-intact rings pre-treated with NO synthase inhibitor L-NAME (100 μM), were pre-contracted with the thromboxane A_2 (TXA_2) mimetic U-46619 (3 nM). After a sustained contraction was obtained, cumulative concentration-response curves to 6-ND (10 pM – 300 nM) were performed. The relaxation responses were expressed as percentage of the contractile response. The removal of the endothelium was done mechanically by gently rubbing the vessels with forceps.

2.6. Histological analysis

After 48 h of fixation in 10 % neutral buffered formalin, the samples of human popliteal artery and vein were processed, embedded in paraffin blocks and cut into 5-μm sections using a microtome. All tissue sections were submitted to H&E staining. Images from these slides were obtained of a trinocular Eclipse 50i microscope (Nikon, Tokyo, Japan) coupled to a 10MP CMOS digital camera (Mu1003, Amscope, USA).

2.7. Immunohistochemistry

The immunohistochemistry slides were prepared, processed and stained manually. Briefly, 5-μm sections of formalin-fixed, paraffin-embedded samples of human popliteal artery and vein ($N = 3$, one sample per artery or vein) were deparaffinized in xylene and rehydrated in a series of ethanol baths of increasing concentration. Then, they were treated for 10 min with 3 % H_2O_2 to neutralize endogenous peroxidase, washed and incubated in citrate buffer at pH 6.0 (regardless of primary antibody) in a steamer set for 20 min (at 95° C). Later, the sections were incubated for 2 h at room temperature (25° C) with the following primary antibodies: (1) a rabbit monoclonal anti-human calretinin antibody (catalog code: ab92341; clone: EP1798; immunogen: synthetic peptide,[2] dilution: 1:100 in PBS; Abcam, Cambridge, UK) and (2) a mouse monoclonal anti-S100 (catalog code: ab4066; clone: 4C4.9; immunogen: full length protein; dilution: 1:100 in PBS; Abcam, Cambridge, UK). 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). Then, the sections were counter-stained with Harris' hematoxylin and cover-slipped in Entellan. Formalin-fixed, paraffin-embedded sections of human basal ganglia (brain tissue) were used as positive controls for the presence of calretinin and S100. All slides were examined using a

[2] The information regarding its sequence is proprietary to Abcam.

trinocular Eclipse 50i microscope (Nikon, Tokyo, Japan) coupled to a 10MP CMOS digital camera (Mu1003, Amscope, USA).

2.8. Data analysis

Nonlinear regression analysis to determine the pEC_{50} was carried out using GraphPad Prism (GraphPad Software, version 9.5, San Diego, CA, USA). The values of pEC_{50} data represent the mean ± standard error of the mean (S.E.M.) of *n* experiments. Values of E_{max} were expressed in % of relaxation (100 % relaxation represents the tension of the tissue not pre-incubated with the contractile agent). Student's one-tail unpaired *t*-test was employed and the differences between groups and $p < 0.05$ was considered significant. For E_{max} and pEC_{50} analysis, unpaired Student's t-test was used. In the pharmacological experiments, the number of experiments in expressed as x/y, where x represents the number of patients and y the number of rings employed in the experiment.

3. Results

3.1. Effect of L-NAME and mechanical removal of the endothelium on the basal release of catecholamines

Basal release of 6-ND was detected in KHS obtained from popliteal artery (Fig. 1A and C) and popliteal vein (Fig. 1B and D). This was significantly reduced when the rings were pre-incubated (30 min) with L-NAME (100 μM; Fig. 1A and B), or when the endothelium was mechanically removed from the popliteal artery (Fig. 1C) and from the popliteal vein (Fig. 1D) rings. The levels of dopamine, noradrenaline and adrenaline were below the limit of quantitation (0.1 ng/mL) in control rings and in rings pre-incubated with L-NAME (data not shown).

3.2. Relaxing effects of 6-ND on pre-contracted human popliteal vessels rings

In pre-contracted (U-46619, 3 nM) vessels, 6-ND induced concentration-dependent relaxations of popliteal artery (pEC_{50} 8.17 ± 0.02 and E_{max} 66.78 ± 3.23 %; Fig. 2A) and popliteal vein (pEC_{50} 8.26 ± 0.09 and E_{max} 83.24 ± 9.84 %; Fig. 2B). The mechanical removal of the endothelium caused a significant inhibition of 6-ND induced relaxation of the popliteal artery (Fig. 2A) and popliteal vein (Fig. 2B).

The relaxations induced by 6-ND were not significantly affected when the vascular tissues were pre-treated (30 min) with L-NAME (100 μM; Fig. 2C-D). The values of E_{max}, pEC_{50} and *p*-values are displayed in Table 1.

3.3. Relaxing effects of L-741,626 on pre-contracted human popliteal vessels rings

In pre-contracted (U-46619, 3 nM) popliteal artery and vein rings, the dopaminergic selective D_2 antagonist L-741,626 induced concentration-dependent relaxations of popliteal artery (pEC_{50} 8.73 ± 0.28 and E_{max} 58.08 ± 8.85 %; Fig. 3A) and popliteal vein (pEC_{50} 9.12 ± 0.17 and E_{max} 68.66 ± 6.73 %; Fig. 3B) rings. The mechanical removal of the endothelium caused significant inhibitions of L-741,626 induced relaxations on the popliteal artery (Fig. 3A) and popliteal vein (Fig. 3B) rings.

The relaxations induced by L-741,626 were not significantly affected when the vascular tissues were pre-treated (30 min) with L-NAME (100 μM; Fig. 3C-D). The values of pEC_{50}, E_{max}, and *p*-values, are displayed in Table 2.

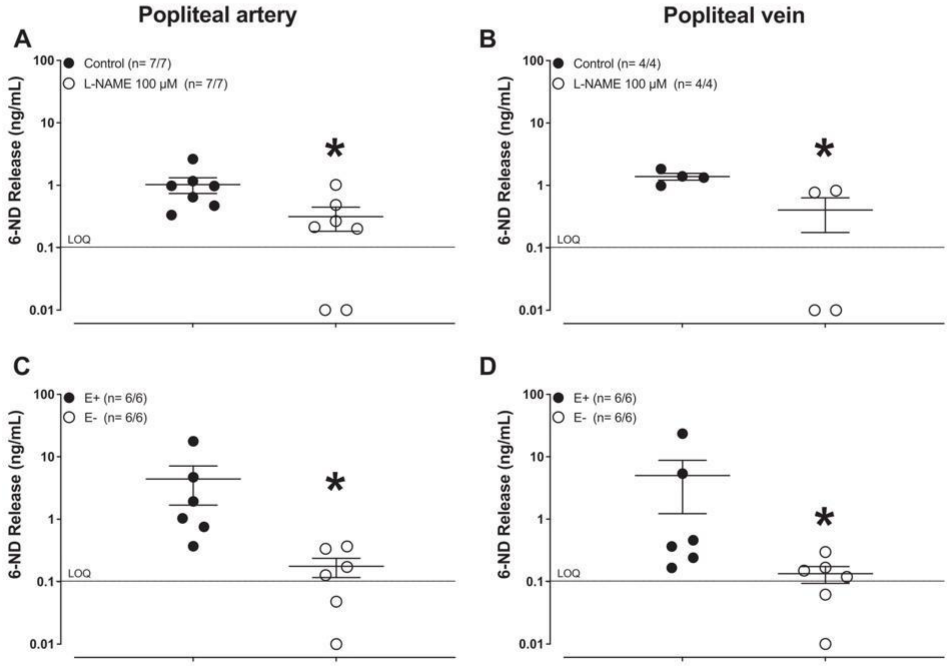

Fig. 1. Basal release of 6-nitrodopamine (6-ND) from human popliteal artery and vein.
Panels A and B show the effect of pre-incubation (30 min) of L-NAME (100 μM) on basal release of 6-ND from popliteal artery ($n = 7$) and popliteal vein ($n = 4$), respectively. Panels C and D show the effect of endothelium removal (*E-*) on the basal release of 6-ND from popliteal artery ($n = 6$) and popliteal vein (n = 6), respectively. Data are reported as means±SEM. *$P < 0.05$ (Student's unpaired *t*-test).

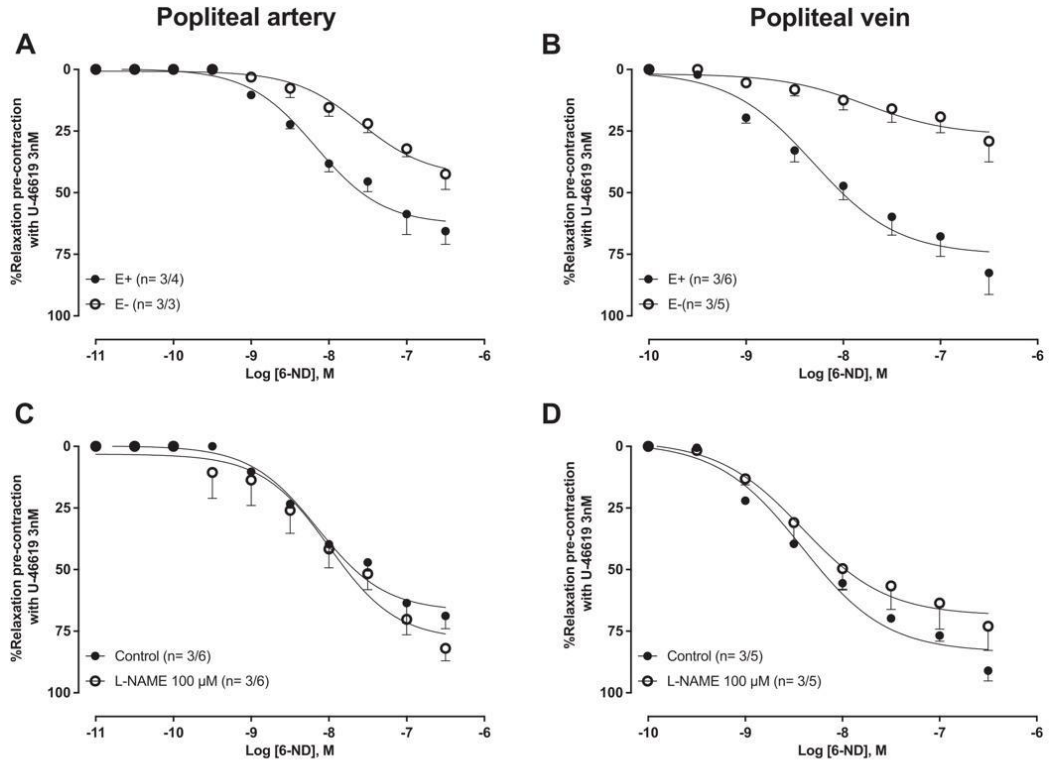

Fig. 2. Relaxations induced by 6-nitrodopamine (6-ND) in U-46619-pre-contracted human popliteal artery and vein rings. Panels A and B show the effect of endothelium removal (E-) on the relaxations induced by 6-ND on human popliteal artery and vein. Panels C and D show the effect of pre-incubation (30 min) of L-NAME (100 µM) on the relaxations induced by 6-ND on popliteal artery and popliteal vein rings. The number of experiments (n) is reported as x/y, where x represents the number of patients and y the number of rings employed.

Table 1
The potency (pEC_{50}) and the maximum response (E_{max}) of the relaxing effects induced by 6-ND on pre-contracted (U-46619 3 nM) human popliteal artery and vein rings. *P* values were obtained by Student unpaired, two tail *t*-test, comparing treated versus control (not treated) vessels.

6-Nitrodopamine	pEC_{50} (log[M])	*P-value*	E_{max} (%)	*P-value*	*n*
Artery E+	8.17 ± 0.04		62.26 ± 5.40		3/4
Artery E-	7.70 ± 0.22	0.1203	40.31 ± 6.25	0.0370	3/3
Vein E+	8.24 ± 0.09		87.64 ± 8.82		3/6
Vein E-	7.72 ± 0.92	0.1316	33.39 ± 8.44	0.0019	3/5
Artery E+ control	8.18 ± 0.05		75.66 ± 6.59		3/6
Artery E+ L-NAME	8.09 ± 0.32	0.3831	81.55 ± 5.69	0.1576	3/6
Vein E+ control	8.40 ± 0.08		94.05 ± 3.36		3/5
Vein E+ L-NAME	8.34 ± 0.14	0.3578	80.29 ± 7.62	0.0648	3/5

3.4. Immunomorphology

All of the examined popliteal arteries and veins harbored pathological findings consistent with arteriosclerosis or phlebosclerosis, respectively (see Table 3 and Fig. 4). Arteriosclerosis was characterized by intimal fibrosis of various intensity and confluent dystrophic calcifications within tunicae media and intima. Phlebosclerosis, on the other hand, comprised two main components: smooth muscle hyperplasia of the medial layer (usually moderate to severe) and prominent fibrosis of the intima. No distinctive alterations were observed in endothelial cells of both vessel types at light microscopy, in the examined samples. Delicate nerve fibers (with an average diameter of 30 µm) were rarely seen in the outer rim of tunica adventitia and in this particular layer only (usually 1 discrete structure per tissue section, regardless of vessel type). In one sample of each vessel type, no nervus vasorum could be morphologically identified. No neural structures were observed in tunicae media or intima, and none of the detected nervi vasorum were found in close proximity to or making direct contact with either medial or intimal layers. Furthermore, no apparent differences between arteries and veins regarding number, size or topography of nerves were seen in the current set of samples.

Immunohistochemistry results are summarized and illustrated in Table 3 and Fig. 5, respectively. Using this technique, S-100 protein expression could be detected in all nervi vasorum present in both artery and vein sections. Calretinin, by contrast, was consistently negative in all morphologically identifiable nerves (but positive in all positive control sections [human brain] performed in each batch of immunohistochemistry slides). Of notice, no unspecific/background staining was observed in negative control slides.

4. Discussion

This is the first demonstration that 6-nitrodopamine is released from human peripheral artery and vein rings. 6-Nitrodopamine was the major catecholamine released from human popliteal artery and vein rings, and the release was significantly reduced when the endothelium was mechanically removed. Indeed, the basal release of 6-ND was significantly decreased in human umbilical cord vessels following mechanical

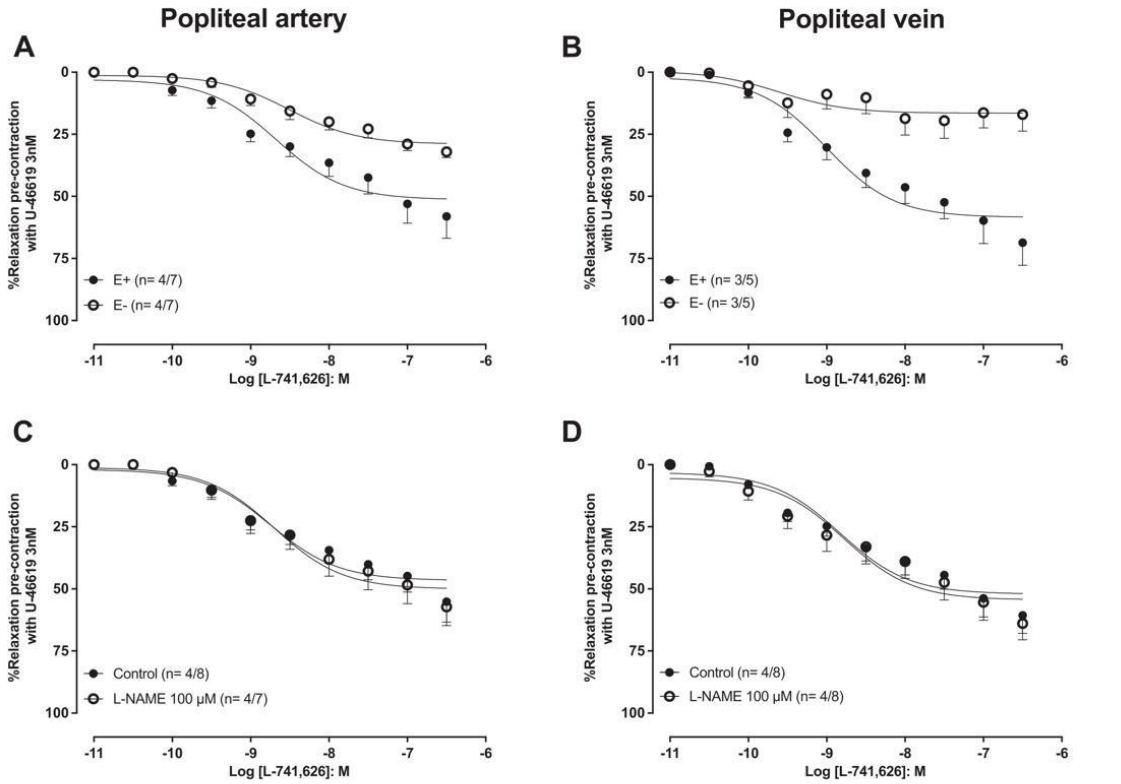

Fig. 3. Relaxations induced by L-741,626 in U-46619-pre-contracted human popliteal artery and vein rings. Panels A and B show the effect of endothelium removal (E-) on the relaxations induced by L-741,626on human popliteal artery and vein. Panels C and D show the effect of pre-incubation (30 min) of L-NAME (100 µM) on the relaxations induced by L-741,626 on popliteal artery and popliteal vein rings. The number of experiments (n) is reported as x/y, where x represents the number of patients and y the number of rings employed.

Table 2
The potency (pEC_{50}) and the maximum response (E_{max}) of the relaxing effects induced by L-741,626 on pre-contracted (U-46619 3 nM) human popliteal artery and vein rings. P values were obtained by Student unpaired, two tail t-test, comparing treated versus control (not treated) vessels.

L-741,626	pEC_{50} (log[M])	*P-value*	E_{max} (%)	*P-value*	*n*
Artery E+	8.27 ± 0.21		63.30 ± 8.86		4/7
Artery E-	8.61 ± 0.28	0.4854	34.98 ± 2.37	0.0111	3/5
Vein E+	9.31 ± 0.17		69.35 ± 9.15		3/5
Vein E-	8.74 ± 0.57	0.1095	10.01 ± 6.77	0.0018	4/8
Artery E+ control	8.92 ± 0.22		58.60 ± 8.60		4/8
Artery E+ L-NAME	8.68 ± 0.22	0.2321	57.24 ± 7.59	0.4538	4/7
Vein E+ control	8.79 ± 0.19		60.66 ± 7.27		4/8
Vein E+ L-NAME	8.74 ± 0.39	0.4496	63.90 ± 6.65	0.3734	4/8

Table 3
Human popliteal artery and vein innervation: immunomorphological features using rabbit polyclonal antibodies against neural markers S-100 and Calretinin protein.

	Popliteal vein $n = 3$	Popliteal artery $n = 3$
Morphology		
– Morphologically identifiable nerves (location)	2/3 (adventitia)	2/3 (adventitia)
– Pathological findings (arteriosclerosis or phlebosclerosis):	3/3	3/3
– Main components:		
■ Intimal fibrosis:	3/3	3/3
■ Medial smooth muscle hyperplasia	2/3	0/3
Immunohistochemistry[a]		
– S-100-positive nerve fibers:	2/3 (adventitia)	2/3 (adventitia)
– Calretinin-positive nerve fibers:	0/3	0/3

[a] No significant background reaction was observed on the slides stained for S-100 or Calretinin proteins.

removal of the endothelium [5]. Human umbilical cord vessels apparently do not present innervation, since no cholinergic or adrenergic nerve fibers have been identified by fluorescence [13]. Immunohistochemistry assays performed with monoclonal and polyclonal antibodies against neuron-associated antigens have also proven negative [14]. Aortae obtained from *Chelonoidis carbonarius* [15], *Panterophis guttatus* [7] and *Callithrix spp* [8] were all negative for S-100, as evaluated by immunohistochemistry. Thus, the finding that the basal release of 6-ND from popliteal artery and vein rings was significantly reduced when the endothelium was mechanically removed, coupled to the evidence that the immunohistochemistry examination revealed immunoreactivity for the neuromarkers S-100 [16,17] only in the nervi vasorum, supports the concept that the basal release of 6-ND is endothelium-derived. In contrast to the S-100 neuromarker, immunoreactivity for calretinin was not detected, though nerve terminals were morphologically identified in both artery and vein slices. Calretinin is a member of the EF-hand family

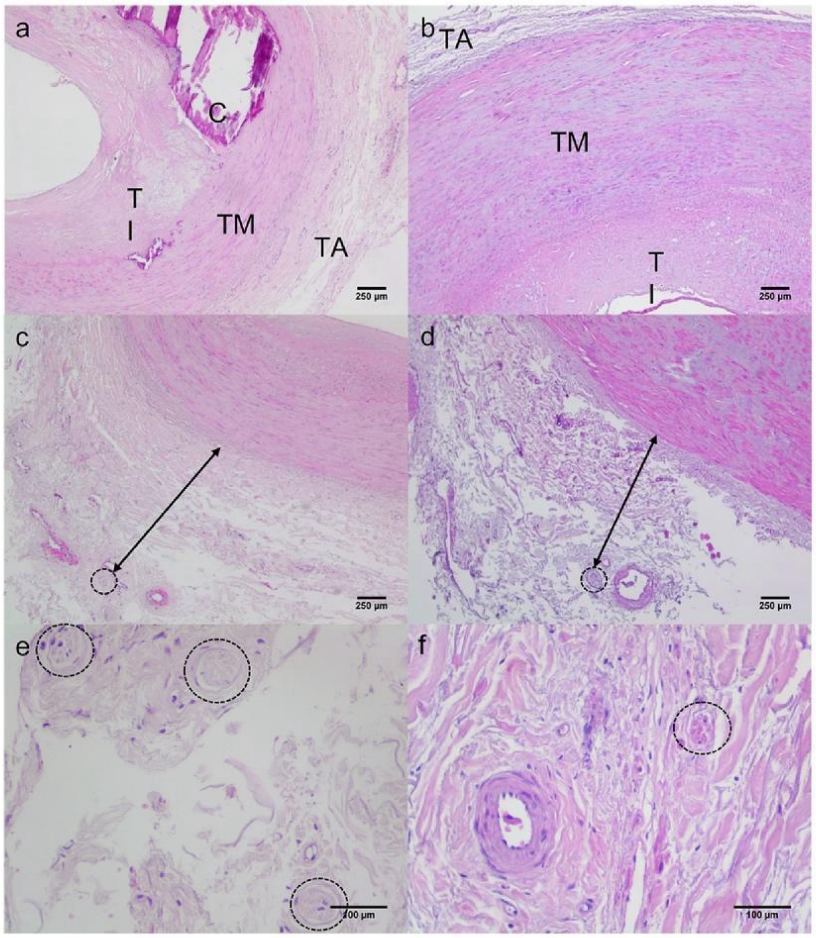

Fig. 4. Morphological aspects of human popliteal artery (a, c, e) and vein (b, d, f) in this series. (a) Popliteal artery (Pa), low-power field, showing severe fibrosis of tunica intima (TI), dystrophic calcifications within TI and tunica media (TM), with no alterations in tunica adventitia (TA). (b) Popliteal vein (Pv), low-power field, exhibiting intimal fibrosis and moderate medial smooth muscle hyplerplasia. (c) and (d) illustrate the presence of rare, delicate nerve fibers (dashed circles) within TA only (c = Pa; d = Pv), and the distance (double-arrowed lines) between these neural structures and TM (of notice, no neural structures were observed penetrating TM or reaching TI). (e) and (f) represent the main distinctive histomorphological aspects of TA nerve fibers (nervi vasorum, see dashed circles) which allow for their prompt identification even without the aid of neural immunohistochemical markers. Hematoxylin-eosin (H&E), original magnifications: 40× (a-d), 200× (e,f). TI = tunica intima, TM = tunica media, TA = tunica adventitia, C = calcifications.

of calcium-binding proteins [18] and is found in specific neuronal subpopulations throughout the rodent [19], primate brain [20] and pituitary nerve terminals [21]. Immunoreactivity for calretinin was also present in nerve terminals of the rat sympathetic nervous system [22]. Thus, the conclusion is that calretinin cannot be considered a reliable marker for nerve structures in human vessels.

Nitroglycerin causes smooth muscle relaxation in popliteal ring preparations pre-contracted with 5-hydroxytryptamine [23]. It is interesting that the pEC_{50} of GTN in this preparation was approximately 6.5, whereas that of 6-ND is almost one hundred times more potent, and the pEC_{50} of the D_2-dopamine antagonist L-741,626 was over one hundred times more potent. These results indicate that endothelium-derived dopamine is a major contractile agent in the popliteal artery and vein. It is interesting that noradrenaline elicits very poor contractile response in these preparations [23], indicating that α_1-adrenoceptors are probably not relevant in these peripheral vessels. As a vasodilator in human vascular tissues, 6-ND is 10–30 times more potent than acetylcholine [24,25], 10 times more potent than histamine [26], similar potency to bradykinin [26] and 10 times less potent that vascular endothelial growth factor (VEGF) [27]. However, it is important to emphasize that in contrast to 6-ND, the relaxation induced by all these other endogenous mediators are affected by inhibition of NO synthase. This characteristic increases the potential therapeutic use of 6-ND in diseased vessels.

The action of dopamine in the vasculature is quite heterogenous, depending on species, vessels, and doses. For instance, in human and monkey epicardial coronary arteries, dopamine induced contractions, whereas in the dog coronary artery, it induces vasorelaxation [28]. Even within the same species, the mechanism has been considered complex, since dopamine in an intact dog can produce either an increase or decrease in coronary circulation [29]. This heterogeneity has been associated with its actions on α-adrenergic and dopaminergic receptors responsible for vasoconstriction and vasodilation, respectively [30]. Indeed, in the dog cerebral arteries, dopamine causes contraction, but when pretreated with nonselective α-blocker phenoxybenzamine [31], it caused relaxation [32]. However, the expression of dopamine receptors in the vasculature is complex; five genes encoding dopamine receptors have been characterized and these receptors have been classified into two subfamilies: the D_1-like receptor subtypes (D1R and D5R), whose activation stimulates adenylate cyclase, and the D_2-like receptors subfamily (D2R, D3R and D4R), linked to Gi, which inhibits adenyl cyclase [33]. All five dopamine receptors have been identified in vascular beds in vitro by radioligand-receptor binding, autoradiographic techniques and immunohistochemistry [34–36]. Our results indicate that dopamine

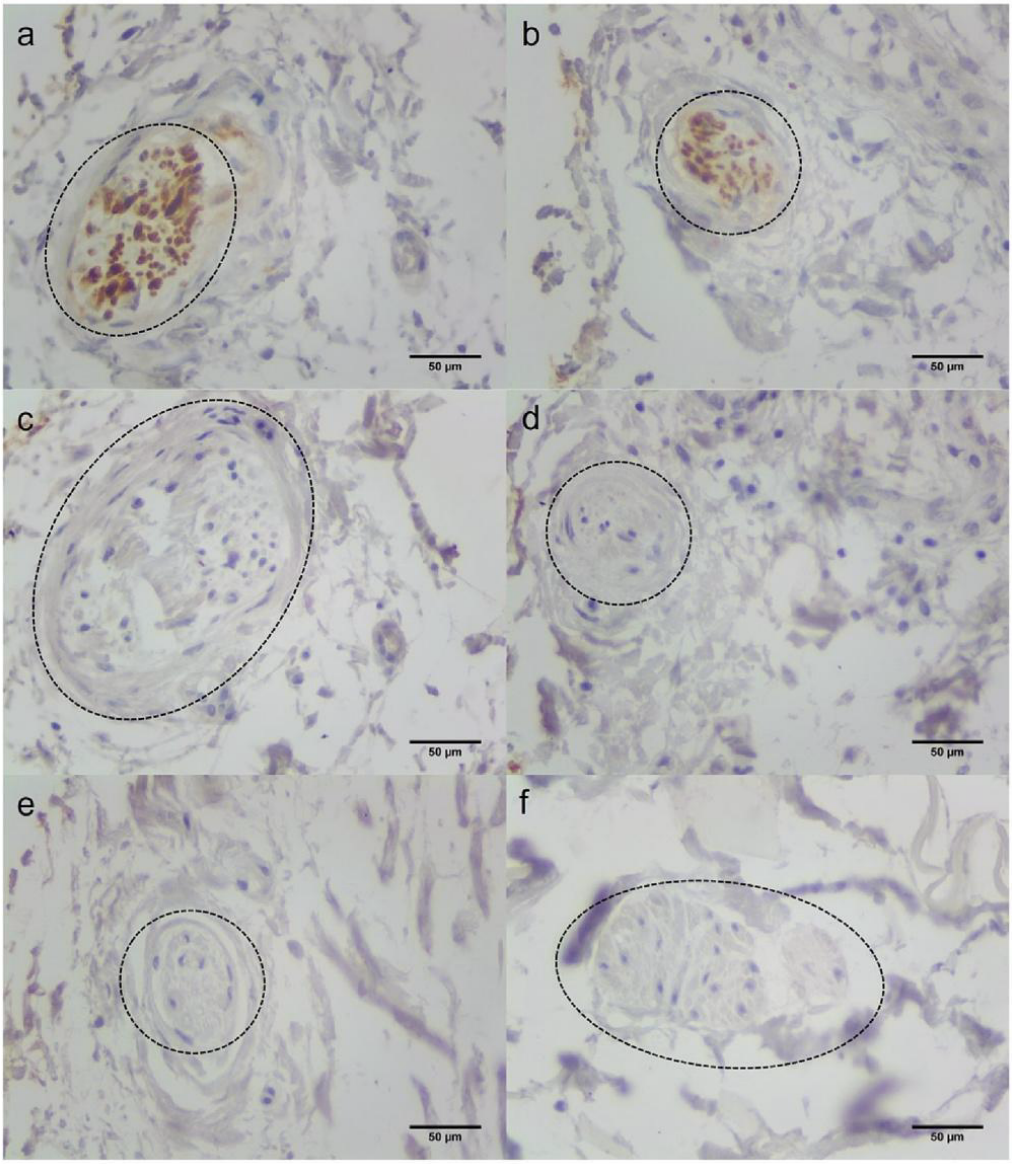

Fig. 5. Immunohistochemical detection of nervi vasorum in human Popliteal artery (a, c, e) and vein (b, d, f): representative images. S-100 protein positive nerve fibers (dashed circles) in Popliteal artery (a) and vein (b) tunica adventitia (TA). Popliteal artery (c) and vein (d) nerve fibers of the TA showing no detectable expression of calretinin protein. Similarly, negative controls (absence of primary antibody) show no brown chromogen (DAB) deposition in nervi vasorum (dashed lines) in both Popliteal artery (e) or vein (f). No positivity for either neural marker was observed in tunicae intima and media. Immunoperoxidase/DAB, 400× (original magnification). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

causes vasoconstriction by acting on dopamine D2-like receptors, since both 6-ND and L-741,626 cause relaxation. Identification and localization of these receptors in human popliteal artery and vein by immunohistochemistry and fluoresce in situ hybridization should clarify the potential modulatory role of dopamine in human peripheral vessel tonus.

The vessels used in our experiments were obtained from diseased patients, and the vessels present substantial amounts of calcification, although no major morphological alteration has been seen in the endothelium at light microscopy. Oxidative stress has been considered a reliable marker of vessel plaque instability [37]. Oxidative stress is characterized as an imbalance between reactive oxygen and nitrogen species, and antioxidants [38]. The finding that the release of 6-ND was significantly reduced when the popliteal artery and vein rings were pretreated with the NO synthase inhibitor L-NAME, as reported for other vascular tissues [6–8], indicates that there is another source of NO, independent of the metabolism of arginine by NO synthase. Indeed, incubation of dopamine with $NaNO_2$ and H_2O_2 in a cell free system

generates 6-nitrodopamine as detected by LC-MS/MS [39], and the release of 6-ND from HUA rings are significantly reduced in the presence of catalase and the peroxidase mimetic ebselen [40,41], and increased when the rings are pre-incubated with the $NOX_{1/4}$ inhibitor GKT-137831, and the NOX_2 inhibitor GSK-2795039 [42], indicating a potential role for endogenous nitrite and peroxidases as alternative biosynthetic pathways for 6-ND synthesis [43]. Unfortunately, it was not possible to evaluate the basal release of 6-ND from healthy popliteal artery and vein rings. Whether the oxidative stress generates an imbalance in the production of endothelium-derived nitrocatecholamines remains to be further investigated.

5. Conclusion

6-Nitrodopamine is the major catecholamine released from human peripheral vessels. Endothelium-derived dopamine is a major contractile agent in the popliteal artery and vein, and selective dopamine D2-receptor antagonists such as 6-ND, may present therapeutic potential in the treatment of human peripheral vascular diseases.

CRediT authorship contribution statement

Conceptualization: JBJ, GDN.
Data curation: JBJ, GDN.
Formal analysis: GDN.
Funding acquisition: FZM, GDN.
Investigation: LFGO, JBJ, ATL, VS, AAS.
Methodology: LFGO, JBJ, ATL.
Project administration: GDN.
Supervision: FZM and GDN.
Visualization: AAS, FZN, GDN.
Writing – original draft: JBJ, GDN.
The authors declare that all data were generated in-house and that no paper mill was used.

Ethical approval

The investigation followed the principles outlined in the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Institute of Biomedical Sciences of the University of São Paulo – ICB/USP (protocol number 3.583.667), and the patients were asked to sign an informed consent.

Consent to participate

Not applicable.

Consent to publish

The authors authorize the submission and publication of this article in *Life Sciences*.

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Uncited reference

[11]

Declaration of competing interest

The investigation conformed to the principles outlined in the Declaration of Helsinki and written consent was obtained from those who agreed to participate.

Data availability

The authors authorize the availability of any data used in this study.

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4. DISCUSSÃO

Este estudo foi pioneiro em demonstrar a liberação basal de 6-ND pelos vasos poplíteos humanos. 6-ND foi a catecolamina principal identificada, mas com liberação muito diminuída pela retirada do endotélio, assim como acontece nos vasos do cordão umbilical (12). Estes últimos, aparentemente, não possuem inervação (18,19).

Uma vez que não foram identificadas estruturas neurais/neuronais na túnica média e íntima, apenas na camada adventícia (20,21), e sendo a falta de endotélio um fator inibidor da liberação da 6-ND, nos parece que esta liberação da 6-ND é derivada do endotélio. Apresenta-se sendo 100 vezes mais potente do que a nitroglicerina (22); 10-30 vezes mais que a acetilcolina (23,24); dez vezes mais que a histamina (25); similar à bradicinina (25) e dez vezes menos potente que o fator de crescimento endotelial vascular (26). Vale aqui ressaltar que a vasodilatação causada por estas últimas substâncias é afetada pela inibição da sintase de NO, aumentando o potencial terapêutico da 6-ND em vasos patológicos.

A ação da dopamina no sistema cardiovascular é bem heterogênea, dependendo da espécie, vasos e doses. Pode causar vasoconstrição ou vasodilatação, dependendo da sua ação em receptores dopaminérgicos, beta-2 adrenérgicos ou alfa-1 adrenérgicos (27-31). Os receptores dopaminérgicos são agregados em duas subfamílias, D1-*like* (que inclui os receptores D1 e D5 – estimuladores da adenilato ciclase [AC]), e D2-*like* (que inclui D2, D3 e D4 – inibidores da AC) (32). Todos os cinco subtipos foram encontrados na vasculatura humana (33-35), sendo que os D2-*like* parecem causar vasoconstrição. A correta identificação e localização desses receptores em vasos poplíteos e outros vasos periféricos e centrais ajudarão a entender melhor o papel modulador vascular da dopamina e, consequentemente, o da 6-nitrodopamina.

5. CONCLUSÕES

O presente trabalho descreve de forma inédita a 6-Nitrodopamina como um novo mediador vasoativo catecolaminérgico, produzido e secretado no endotélio de vasos poplíteos humanos (e, possivelmente, na maioria dos vasos periféricos humanos) com ação vasodilatadora significante.

A 6-Nitrodopamina é a catecolamina liberada em maior quantidade nos vasos poplíteos (e, possivelmente, na maioria dos vasos periféricos humanos) e indiretamente podemos concluir que a dopamina é um importante vasoconstritor na artéria e veia poplítea.

Antagonistas seletivos dos receptores D2-*like* da dopamina, como a 6-ND, apresentam grande potencial terapêutico no tratamento de doenças vasculares periféricas (que cursam com insuficiência vascular).

Os dados farmacológicos e histoquímicos indicam que a principal fonte de dopamina e 6-nitrodopamina é o endotélio.

A calretinina não é um bom marcador neural em vasos poplíteos (e, possivelmente, em vasos periféricos humanos). Está constatação tem implicações práticas para a pesquisa básica nesta área.

6. REFERÊNCIAS

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7. ANEXOS

ANEXO 1. Parecer Consubstanciado CEP

USP - INSTITUTO DE CIÊNCIAS BIOMÉDICAS DA UNIVERSIDADE DE SÃO PAULO - ICB/USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: 009/19 - Avaliação do papel fisiopatológico das catecolaminas endoteliais de vasos de pernas de amputados devido aterosclerose grave. **Pesquisador:** GILBERTO DE NUCCI **Área Temática:**
Versão: 1
CAAE: 20139919.9.0000.5467
Instituição Proponente: Instituto de Ciências Biomédicas da Universidade de São Paulo - ICB/USP
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.583.667

Apresentação do Projeto:
O projeto acima visa avaliar o papel fisiopatológico das catecolaminas endoteliais de vasos de pernas de 200 amputados devido aterosclerose grave. Está bem redigido com toda a documentação necessária.

Objetivo da Pesquisa:
O projeto acima visa avaliar o papel fisiopatológico das catecolaminas endoteliais de vasos de pernas de amputados devido aterosclerose grave. O projeto visa utilizar amostras de segmentos de veia safena magna, artéria fibular, artéria tibial posterior, artéria
tibial coletados imediatamente após a cirurgia de amputação e que seriam jogados fora se não utilizados(descarte) e detectar em laboratório a presença e característica da produção de catecolaminas. Neste sentido este estudo não vai implicar em um contato ou intervenção direta com pacientes a não ser no momento de se obter as os consentimentos livres e esclarecidos. O scritérios de inclusão e exclusão são claros e razoáveis.

Avaliação dos Riscos e Benefícios:
Não há benefício declarado para o participante e não há risco significativo evidente enquanto sigilo sobre o participante for mantido. Ademais, não há qualquer tipo de benefício ou compensação previstos. No entanto há uma apólice de seguros bem abrangente que confere proteção ampla para os participantes. Cabe considerar qual a finalidade desta apólice se não a de incentivar o

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Bairro: Cidade Universitária **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-7733 **E-mail:** cep@icb.usp.br

consnetimento de sujeitos de pesquisa, justamente numa fase pós-operatória crítica. Isto é no melhor interesse dos proponentes?

Comentários e Considerações sobre a Pesquisa:

A pesquisa tem com desfecho primário avaliar o papel do endotélio como fonte de catecolaminas na contração induzida por estímulo de campo elétrico. O desfecho Secundário é caracterizar o papel adrenérgico na contração induzida por estímulo de campo elétrico em músculo liso vascular de artéria e veia. A metodologia é bem estabelecida e não apresenta peoblemas evidentes.

Considerações sobre os Termos de apresentação obrigatória:

O TCLE é direto e breve. Claramente, dada a natureza da pesquisa e devido ao não envolvimento direto do participante, o nível de informação me parece adequado e suficiente.

Recomendações:

recomento a aprovação.

Favor apenas justificar a inclusão do seguro em grupo

Conclusões ou Pendências e Lista de Inadequações:

Não há.

Considerações Finais a critério do CEP:

O Colegiado do CEP - ICB concorda com o parecer do relator em aprovar o projeto. Cabe aos pesquisadores executantes elaborar e apresentar a este comitê relatórios anuais (parciais ou final) de acordo com o ítem II, II.19 e II.20 da resolução 466/12 do Conselho Nacional da Saúde. Com relação `as amostras biológicas, em não havendo ainda um biorepositório e se houver retenção de material deverá ser solicitado o devido cadastro conforme modelo constante no "site" do ICB. Ao pesquisador cabe também finalizar o processo junto `a plataforma Brasil quando do encerramento deste **Este parecer foi elaborado baseado nos documentos abaixo relacionados:**

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1423235.pdf	02/09/2019 16:03:23		Aceito
Outros	Carta_CEP.pdf	02/09/2019 16:01:52	GILBERTO DE NUCCI	Aceito
Outros	Apolice.pdf	02/09/2019 16:01:34	GILBERTO DE NUCCI	Aceito

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TCLE / Termos de Assentimento / Justificativa de Ausência	00919_TCLE.pdf	02/09/2019 16:01:00	GILBERTO DE NUCCI	Aceito
Projeto Detalhado / Brochura Investigador	00919_Protocolo_Catecolaminas_aterosclerose.pdf	02/09/2019 16:00:47	GILBERTO DE NUCCI	Aceito
Folha de Rosto	Folha_Rosto.pdf	02/09/2019 15:40:11	GILBERTO DE NUCCI	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SAO PAULO, 18 de Setembro de 2019

Assinado por:
Camila Squarzoni Dale
(Coordenador(a))

Endereço: Av. Profº Lineu Prestes, 2415
Bairro: Cidade Universitária **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
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ANEXO 2- TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Avaliação do papel fisiopatológico das catecolaminas endoteliais de vasos de pernas de amputados devido aterosclerose grave.

Protocolo número GDN 009/19

Você está sendo convidada a participar de um projeto de pesquisa. Leia atentamente as informações abaixo e faça qualquer pergunta que desejar, para que todos os procedimentos desta pesquisa sejam esclarecidos.

Pesquisadores Responsáveis:

Guilherme Machado de Figueiredo Murari

Wesley Willian Gomes da silva

Prof. Dr. Gilberto De Nucci

DADOS DO PARTICIPANTE

Nome:
_____

Data de Nascimento: _____/_____/_____

Telefones para contato: ( ) _____ / _____

E-mail
(opcional):_____

NATUREZA E PROPÓSITO DO ESTUDO

Este estudo visa investigar a importância das catecolaminas, uma substância produzida pelos nervos e também por células endoteliais (um tipo de célula que recobre o interior dos vasos sanguíneos), presentes em vasos de pernas amputadas. O material será obtido no Hospital Estadual de Sumaré.

PROCEDIMENTOS A SEREM REALIZADOS E RESPONSABILIDADES

Durante os procedimentos, imediatamente após a cirurgia de amputação, segmentos de veia safena magna, artéria fibular, artéria tibial posterior, artéria tibial serão jogados fora. Iremos utilizar pedaços desse material (descarte) para testar a presença e característica da produção de catecolaminas.

Você não precisará receber qualquer tipo de tratamento para a participação no estudo. Não haverá contato com você. Esse pequeno pedaço de tecido será levado para um laboratório para executarmos a pesquisa.

Você não será identificado durante os experimentos e esse teste não vai lhe trazer qualquer benefício. Não se trata de exame para diagnosticar algum tipo de doença.

RESPONSABILIDADES

Nossa responsabilidade com você será manter sua identificação e sigilo.

POSSÍVEIS RISCOS E DESCONFORTOS

Esse estudo não trará nenhum risco pra você, tendo em vista que iremos coletar material destinado ao descarte, sem termos contato com você.

BENEFÍCIOS OU COMPENSAÇÕES

Não há qualquer tipo de benefício ou compensação previstos.

INTERCORRÊNCIAS (efeitos indesejáveis)

Uma vez que se trata de tecido descartado não há qualquer risco de intercorrência ou efeitos indesejados.

RESSARCIMENTO

Não haverá ressarcimento pelo uso dos tecidos vasculares descartados.

DIVULGAÇÃO DE INFORMAÇÕES QUANTO A PARTICIPAÇÃO NO ESTUDO

Os resultados obtidos deverão fazer parte de publicações científicas e de teses de alunos de pós-graduação. Sua identificação será mantida em sigilo.

CONTATOS E PERGUNTAS

Caso surja alguma dúvida você poderá contatar o **Dr. Gilberto De Nucci,** para receber informações adicionais, relacionadas à pesquisa.

Poderá também contatar a Secretaria do Comitê de Ética em Pesquisas da Universidade de São Paulo (USP) fone (11) 3091-7733, email: cep@icb.usp.br para questionamentos de aspectos éticos do estudo.

Você receberá uma via deste Termo de Consentimento Livre e Esclarecido.

Médico do estudo: Gilberto De Nucci
Endereço: Av. João Erbolato, 266 – Jardim Chapadão – Campinas/SP
Números de telefone: (19) 3243-2767 / (19) 99178-8879
Número de telefone para emergências e contato 24 horas: (19) 99178-8879

Você deverá rubricar todas as folhas deste Termo de Consentimento Livre e Esclarecido – TCLE.

O pesquisador responsável deverá, da mesma forma, rubricar todas as folhas do Termo de Consentimento Livre e Esclarecido – TCLE apondo sua assinatura na última página do referido Termo. O TCLE será emitido em duas vias, sendo que uma via ficará com o participante da pesquisa e a outra com o pesquisador responsável.

Se você concorda com o exposto acima leia e assine o documento abaixo:

Acredito ter sido suficientemente informado(a) a respeito das informações que li ou que foram lidas para mim sobre o estudo “Avaliação do papel fisiopatológico

das catecolaminas endoteliais de vasos de pernas de amputados devido aterosclerose grave".

Eu discuti com os pesquisadores responsáveis e está claro para mim quais são os propósitos do estudo, os procedimentos a serem realizados, quais são seus desconfortos, riscos e a garantia de confidencialidade dos meus dados. Entendo que sempre que eu tiver dúvidas elas serão esclarecidas e que minha participação é isenta de despesas. Concordo voluntariamente em fazer parte deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízo ou perda de qualquer benefício que eu possa ter adquirido.

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