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FACULDADE DE FARMÁCIA
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ADENOSINE RECEPTORS IN VASCULAR SYMPATHETIC NEUROTRANSMISSION: THE ROLE OF ENDOTHELIUM AND OF NITRIC OXIDE-MEDIATED EFFECTS AND THEIR RELATION WITH HYPERTENSION

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Trabalho realizado sob a orientação da Professora Doutora Carmen Diniz Pereira, Professora Auxiliar da Faculdade de Farmácia da Universidade do Porto e co-orientação da Professora Doutora Paula Maria Façanha da Cruz Fresco, Professora Associada da Faculdade de Farmácia da Universidade do Porto.

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DECLARAÇÃO

Ao abrigo do nº2 do artigo 8º do Decreto-Lei nº388/70, declara-se que fazem parte integrante desta tese os seguintes trabalhos publicados ou submetidos. Para esses trabalhos, o autor da tese contribuiu maioritariamente na execução das experiências laboratoriais, interpretação de resultados e preparação dos manuscritos.

PUBLICAÇÕES ACEITES OU SUBMETIDAS DURANTE O DOUTORAMENTO

Capítulos de livros

Leitão-Rocha A, Sousa JB and Diniz C. Adenosinergic System in the Mesenteric Vessels. In book: The Cardiovascular System - Physiology, Diagnostics and Clinical Implications, 2012. David Gaze (Ed.). Source: InTech

Sousa JB, Fresco P and Diniz C. Imaging receptors with Laser Scanning Confocal Microscopy: qualitative and quantitative analysis. *Microscopy: advances in scientific research and education*, 2014. A. Méndez-Vilas (Ed.)

Artigos Científicos

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É essa emoção fundamental que está na raiz de toda ciência e toda arte."

Albert Einstein

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ABSTRACT

Increased sympathetic activity has been implicated in hypertension. Adenosine provides a link between local mechanisms of blood flow autoregulation and systemic mechanisms of autonomic cardiovascular regulation. Endothelial cells interact with adenosine mechanisms in many different ways. Endothelium is widely recognized as an important regulator of blood vessel tone *via* release of various endothelium-derived endogenous substances, such as nitric oxide. Endothelial cells are known to have a very active adenosine metabolism, characterized by a large capacity for uptake and release of this nucleoside. Adenosine may modulate endothelial function via activation of cell membrane receptors. The precise nature of the interaction between adenosine receptor subtypes and endothelial cells and their role in the regulation of endothelial function is not completely understood. However, adenosine-induced production of nitric oxide by endothelial cells is recognized.

The present study intends to clarify the neuromodulatory role exerted by endogenous adenosine and nitric oxide (generated by endothelial and neuronal nitric oxide synthases) in sympathetic neurotransmission, to evaluate if endothelium dysfunction impairs vascular sympathetic neurotransmission and to explore the distribution profiles of adenosine A_1 and A_{2A} receptors and of neuronal nitric oxide synthase isoform in the adventitia layer of mesenteric and tail arteries of both normotensive and hypertensive rats.

Electrically-evoked tritium overflow (100 pulses/5 Hz) was evaluated on Wistar Kyoto and spontaneously hypertensive rats mesenteric and tail arteries, pre-incubated with [^{3}H]-noradrenaline. The role of endogenous adenosine and nitric oxide in sympathetic neurotransmission was studied in the presence of adenosine receptor agonists and antagonists; enzymes substrates and inhibitors; nitric oxide donors and a nucleoside transporter inhibitor. Purine content was determined by HPLC with fluorescence detection. The distribution profiles of adenosine A_1 and A_{2A} receptors and of neuronal nitric oxide synthase isoform in the adventitia layer of mesenteric and tail arteries of Wistar Kyoto and Spontaneously Hypertensive rats was investigated by Laser Scanning Confocal Microscopy.

Results indicate a higher electrically-evoked noradrenaline release from hypertensive mesenteric and tail arteries comparatively to those from normotensive vessels. In intact Wistar Kyoto arteries, a facilitation of tritium overflow was observed in the presence of an selective adenosine A_1 receptor antagonist. This effect can be ascribed to a tonic inhibitory effect mediated by A_1 receptors. In denuded and hypertensive arteries, in the presence of an adenosine A_{2A} receptor antagonist a reduction of tritium overflow was observed. Moreover, when endogenous adenosine bioavailability was increased by the nucleoside transporter inhibitor, tritium overflow increased in intact, denuded and

hypertensive arteries, suggesting the occurrence of a tonic activation of A_{2A} receptors. The tonic inhibitory modulation of noradrenaline release is mediated by A_1 adenosine receptors and is lacking in arteries from hypertensive animals, despite their purine content being higher comparatively to normotensive ones.

Nitric oxide donors reduced noradrenaline release in mesenteric arteries while, in tail arteries, caused the opposite effect. In mesenteric arteries, both a neuronal nitric oxide synthase inhibitor and an endothelial nitric oxide synthase inhibitor, increased noradrenaline release. However, in intact tail arteries, inhibition of endothelial nitric oxide synthase lead to a reduction of noradrenaline release whereas inhibition of neuronal nitric oxide synthase was devoid of effect showing that only endothelial nitric oxide synthase is involved in vascular sympathetic neuromodulation. Nitric oxide modulatory role on sympathetic neurotransmission differs in the mesenteric and tail arteries depending on the nitric oxide synthase isoform, endothelial nitric oxide synthase and/or neuronal nitric oxide synthase, involved in its production.

Immunohistochemistry revealed an adenosine A_1 receptors redistribution from sympathetic fibers to Schwann cells, in hypertensive mesenteric arteries adventitia which can explain, at least in part, the absence of effect of these receptors in hypertensive arteries. Moreover, immunohistochemical studies also revealed neuronal nitric oxide synthase locatization presence in Schwann cells which seem to be the main source of nitric oxide to perivascular sympathetic nerves. Moreover, lower amounts of neuronal nitric oxide synthase were found in tail arteries comparatively to those observed in mesenteric arteries.

In the experimental conditions of this work, the inhibition of enzymes involved in reactive oxygen species or prostaglandins production (with apocynin and allopurinol or indomethacin, respectively) failed to alter tritium overflow.

Data highlight the role of purines in hypertension by revealing that an increase in sympathetic activity in hypertensive arteries is occurring due to a higher noradrenaline and ATP release from sympathetic nerves and the loss of endogenous adenosine inhibitory tonus. The observed nerve-to-glial redistribution of inhibitory A_1 adenosine receptors in hypertensive arteries may explain the latter effect. Furthermore, among the endothelium-derived factors studied that could alter vascular sympathetic transmission, adenosine and adenosine receptor mediated mechanisms were clearly impaired by endothelium injury/dysfunction.

Keywords: mesenteric artery, tail artery, adenosine, nitric oxide, noradrenaline, hypertension.

RESUMO

O aumento da atividade simpática tem sido implicado na hipertensão. A adenosina fornece uma ligação entre os mecanismos locais de autoregulação do fluxo sanguíneo e mecanismos sistémicos de regulação cardiovascular do sistema nervoso autónomo. O endotélio é um componente vascular amplamente reconhecido como sendo um importante regulador do tónus vascular através da libertação de diversas substâncias endógenas, como por exemplo o monóxido de azoto. As células endoteliais são reconhecidas como tendo um metabolismo de adenosina muito ativo, caracterizado por uma grande capacidade de captação e libertação do nucleosídeo. A adenosina pode, ainda, modular a função endotelial por meio da ativação de recetores de membrana. O tipo de interação entre os subtipos de recetores de adenosina e as células endoteliais bem como o seu papel na regulação da função endotelial está longe de estar esclarecido. No entanto, a produção de monóxido de azoto pelas células endoteliais induzida pela adenosina é amplamente reconhecida.

Com o presente estudo pretendeu-se esclarecer o papel neuromodulador exercido pela adenosina endógena e pelo monóxido de azoto na neurotransmissão simpática, avaliar se a disfunção endotelial afeta a neurotransmissão simpática vascular e explorar o perfil de distribuição dos recetores da adenosina A1 e A2A e da sintase neuronal do monóxido azoto na camada adventícia de artérias mesentéricas e artérias da cauda de ratos normo e hipertensos.

No trabalho experimental deste trabalho, realizaram-se ensaios de libertação de trítio por estimulação elétrica (100 pulses/5 Hz) com a artéria mesentérica e artéria da cauda de ratos Wistar Kyoto e de ratos espontaneamente hipertensos, pré-incubadas com [^{3}H]-noradrenalina. O papel da adenosina endógena e do monóxido de azoto na neurotransmissão simpática foi estudada na presença de agonistas e antagonistas de recetores; substratos e inibidores de enzimas; dadores de monóxido de azoto e um inibidor dos transportadores de adenosina. O teor de purinas libertado foi, ainda, determinado por HPLC com deteção por fluorescência.

O perfil de distribuição dos recetores de adenosina A_1 e A_{2A} e da sintase neuronal do monóxido de azoto, na camada adventícia da artéria mesentérica e da artéria da cauda de ratos Wistar Kyoto e espontaneamente hipertensos foi investigado por microscopia confocal.

Os resultados revelaram ocorrer uma maior libertação de noradrenalina por estimulação elétrica na artéria mesentérica e na artéria da cauda de ratos hipertensos comparativamente com os normotensos. Em artérias intactas de ratos normotensos, foi observado um aumento da libertação de trítio na presença do antagonista dos recetores de adenosina A_1. Este efeito pode ser atribuído a um efeito tónico inibitório mediado pelos

recetores A1. Na presença do antagonista dos recetores de adenosina A_{2A}, em artérias onde o endotélio foi removido e nas artérias de ratos hipertensos, foi observada uma redução na libertação de trítio. Adicionalmente, quando a biodisponibilidade de adenosina endógena foi aumentada pelo inibidor dos transportadores de adenosina, verificou-se um aumento da libertação de trítio em artérias intactas, em artérias sem endotélio e em artérias de ratos hipertensos, sugerindo a ocorrência de uma ativação tónica dos recetores de adenosina A_{2A}. A modulação inibitória tónica de libertação de noradrenalina é mediado pelos recetores de adenosina A1 que parece estar ausente em artérias de animais hipertensos, apesar do seu teor em purinas ser maior comparativamente ao dos normotensos.

Por outro lado, os dadores de monóxido de azoto promoveram uma redução na libertação de noradrenalina na artéria mesentérica, enquanto na artéria da cauda, causaram o efeito oposto. Na artéria mesentérica, tanto o inibidor específico da enzima sintase neuronal do monóxido de azoto como o inibidor específico da sintase endotelial do monóxido de azoto, promoveram um aumento da libertação de noradrenalina. No entanto, na artéria da cauda intacta, a inibição da sintase endotelial do monóxido de azoto reduziu a libertação de noradrenalina enquanto a inibição da sintase neuronal do monóxido de azoto não teve qualquer efeito, mostrando que apenas a sintase endotelial do monóxido de azoto está envolvida na neuromodulação simpática da artéria da cauda. Assim sendo, o papel modulador do monóxido de azoto na neurotransmissão simpática difere nas duas artérias em estudo e depende da isoforma da sintase do monóxido de azoto, endotelial ou neuronal, envolvida na sua produção.

Estudos imunohistoquímicos revelaram uma redistribuição dos recetores A_1 de adenosina das fibras simpáticas para as células de Schwann, na camada adventícia de artérias mesentéricas de ratos hipertensos, o que pode explicar, pelo menos em parte, a falta de efeito destes recetores nestas artérias. Para além disso, os estudos imunohistoquímicos revelaram a presença da sintase neuronal do monóxido de azoto em células de Schwann parecendo ser esta enzima a fonte principal de monóxido de azoto em nervos simpáticos perivasculares. Além disso, verificou-se uma menor quantidade de sintase neuronal do monóxido de azoto na artéria da cauda, comparativamente com a observada na artéria mesentérica.

Nas condições experimentais deste estudo, a inibição das enzimas envolvidas na produção de espécies reativas de oxigénio ou de prostaglandinas (com apocinina e alopurinol ou indometacina, respetivamente) não conseguiu alterar a libertação de noradrenalina.

Os resultados deste trabalho destacam o papel das purinas na hipertensão, revelando que um aumento na atividade simpática em artérias de ratos hipertensos ocorre devido a uma maior libertação de noradrenalina e ATP de nervos simpáticos e a perda do

tónus inibitório da adenosina endógena. A redistribuição nervo-glial observada dos recetores de adenosina A_1 inibitórios em artérias de ratos hipertensos pode explicar este aumento da libertação de noradrenalina e ATP observado. Para além disso, entre as substâncias/fatores derivados do endotélio estudados, que poderiam alterar a transmissão simpática vascular, a adenosina e os mecanismos mediados por este nucleósido estão claramente comprometidos pela disfunção ou lesão endotelial.

Palavras-chave: artéria mesentérica, artéria da cauda, adenosina, monóxido de azoto, noradrenalina, hipertensão.

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LISTA DE ABREVIATURAS

5′-N- 5′-nucleotidase
AC- Adenylyl cyclase
ADA- Adenosine deaminase
ADP- Adenosine diphosphate
AK- Adenosine kinase
AMP- Adenosine monophosphate
AR- Adenosine receptors
ATP- Adenosine triphosphate
BH_4 - Tetrahydrobiopterin
Ca^{2+}- calcium
cAMP- cyclic AMP
CGS 21680- 2-p–(2-carboxyethyl)phenethylamino-5´-N-ethylcarboxamidoadenosine hydrochloride
CNT- Concentrative transporters
COX- Ciclooxigenase
CPA- N6-cyclopentyladenosine
DAF-2- 4,5-Diaminofluorescein
DAF-FM- 4-Amino-5-Methylamino-2',7'-Difluorofluorescein
DHE- dihydroetidium
DMSO- Dimethylsulphoxide
DPCPX- 8-cyclopentyl-1,3-dipropylxanthine
eNOS- Endothelial nitric oxide synthase
ENT- Equilibrative nucleoside transporters
GFP- green fluorescent protein
HPLC- High-performance liquid chromatography
ITU- 5-iodotubericidin
K^+- potassium
L-NAME- Nω-Nitro-L-arginine methyl ester hydrochloride
LSCM- Laser scanning confocal microscopy
NA- Noradrenaline
Na^+- sodium
NADPH oxidase- Nicotinamide adenine dinucleotide phosphate oxidase
NBTI- S-(4-Nitrobenzyl)-6-thioinosine
nNOS- Neuronal nitric oxide synthase

NO- Nitric oxide

NOS- Nitric oxide synthase

$ONOO^-$- Peroxynitrite

PGI2- Prostaglandin 12

PKA- Protein kinase A

PLA2- Phospholipase A2

RCFP- reef coral fluorescent protein

RNS- Nitrogen oxygen species

ROS- Reactive oxygen species

SAH- S-adenosyl-homocysteine

SAHH- S-adenosyl-homocysteine hydrolase

SCH 58261- 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine

SHR- Spontaneously hypertensive rats

TH- tyrosine hydroxilase

WKY- Wistar Kyoto

CHAPTER I

General Introduction

1. INTRODUCTION

1.1 Vessels

Blood vessels are an intricate part of the circulatory system, and are the structures through which blood flows throughout the body. Blood vessels are involved in every pathological condition, including cancer, inflammation and cardiovascular disease. There are several types of vessels, each designed for a special purpose in various parts of the body. Arteries and veins vary largely in the thickness of their walls, the diameters of their lumen or their elasticity.

Arteries and veins have a similar structure (Figure 1). Surrounding the lumen is the innermost layer, the tunica intima, which is the thinnest layer, made up of simple endothelial cells and lines the entire circulatory system. Also included in this thin layer are circular, elastic bands, the internal elastic lamina. The second layer, the tunica media, is made up of more circular elastic bands, the external elastic lamina. The tunica media contains vascular smooth muscle which helps regulate the size of the lumen and those of arteries contains more smooth muscle than the tunica media of their counterpart, the veins, and this allows arteries to constrict and dilate to adjust the volume of blood needed by the tissues that they support.

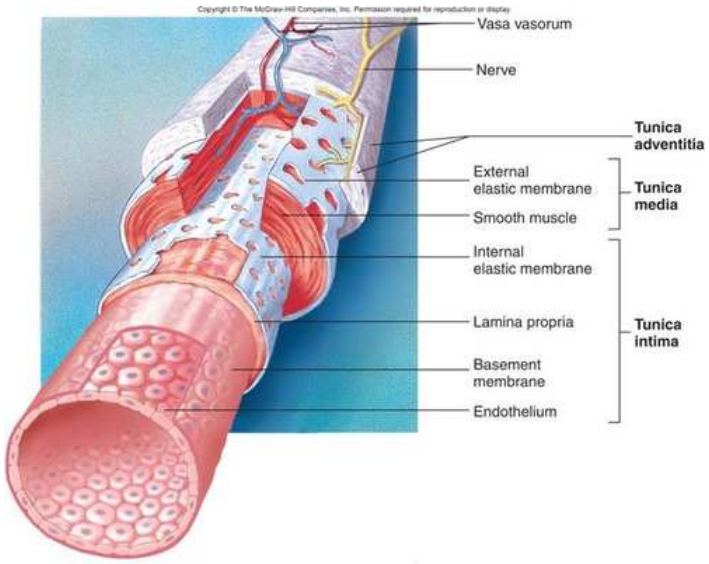

Figure 1 – Schematic representation of vessels wall. (adapted from Copyright McGraw-Hill Companies, Inc)

When the smooth muscle found within the tunica media is stimulated, it contracts, squeezing the walls of the artery and narrowing the lumen vessel. The term vasoconstriction is used to describe the narrowing of the blood vessel due to contraction of the muscular wall. When an artery constricts, the blood flow decreases and the pressure within the vessel rises. Vasoconstriction is a mechanism that body uses to regulate important functions, such as body temperature and blood pressure. Vasodilation is the opposite of vasoconstriction and consists in the widening of the blood vessel due to relaxation of the muscular wall. When a blood vessel dilates, blood is able to flow through the vessel with less resistance. Therefore, vasodilation has the opposite effect of vasoconstriction on such functions as body temperature and blood pressure. The tunica adventitia is the outermost layer of the arteries and veins. This outer layer is composed of connective tissue that allows the blood vessel to withstand forces acting on the vessel wall. It also contains strong collagen fibers that help anchor the blood vessel to surrounding tissues, and this gives the vessel some stability.

Over the last years, endothelium has emerged as a key regulator of vascular homeostasis, and is no longer looked as having only a barrier function but also as an active signal transducer for circulating influences that modify the vessel wall phenotype. Alteration in endothelial function precedes the development of morphological atherosclerotic changes and can also contribute to lesion development and later clinical complications.[1]

Endothelium is able to respond to physical and chemical signals by production of a wide range of factors that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation. Endothelium effect on vascular tone is achieved by production and release of several vasoactive molecules that relax or constrict the vessel, as well as by response to and modification of circulating vasoactive mediators. This vasomotion plays a direct role in the balance of tissue oxygen supply and metabolic demand by regulation of vessel tone and diameter, and is also involved in the remodeling of vascular structure and long-term organ perfusion.[2]

Veins and arteries both have a similar three-layer structure which contain smooth muscle that is regulated by the sympathetic nervous system. In veins, nerves are distributed throughout the medial smooth muscle coat, often in close apposition (50 nm) to smooth muscle cells. In arteries, nerves are located at the adventitial-medial border, few closer than 2 nm to smooth muscle cells, often with interposing connective tissue and Schwann cell processes.[3] When necessary, the sympathetic nervous system signals arteries to relax or contract, which changes the lumen size of the lumen and thereby regulates blood flow and also affects blood pressure.

The autonomic nervous system is often defined as a motor neuronal system, generally concerned with involuntary body functions, in contrast to the somatic nervous

system, which has both motor and sensory neurons responsible for voluntary muscle function and general sensation. The autonomic nervous system innervates smooth muscle, cardiac muscle, and glands. Physiologically, smooth and cardiac muscles are not completely dependent on autonomic motor neurons for contraction. The first-order neuronal cell body is located in a central nervous system nucleus, and its fiber (axon) travels peripherally to synapse with a second-order neuron, which is located in a peripheral nervous system ganglion. In the peripheral nervous system, the larger diameter axons are surrounded by a lipid-rich myelin sheath formed by the Schwann cells. Many nerve fibers in the central and peripheral nervous system are unmyelinated. In the peripheral nervous system, however, even the unmyelinated fibers are enveloped in Schwann cells. The Schwann cell's plasma membrane does not spiral repeatedly around the fiber as it does in a myelin sheath, but folds once around each fiber and somewhat overlaps itself along the edges. Most nerve fibers travel through individual channels in the Schwann cell, but small fibers are sometimes bundled together within a single channel.[4] The Schwann cells (in the peripheral nerves) and the satellite cells (in the ganglia) are glial cells (supporting cells) of the peripheral nervous system. Glial cells provide support and protection for neurons. They are thus known as the "supporting cells" of the nervous system. The four main functions of glial cells are: to surround neurons and hold them in place, to supply nutrients and oxygen to neurons, to insulate one neuron from another, and to destroy and remove dead neurons.[5, 6]

The small arteries and arterioles are responsible for the major part of the total resistance to blood flow in the circulatory system. Normally these vessels are maintained in a state of functional constriction (vascular tone) determined by the interaction between intrinsic (myogenic tone) and extrinsic (neurotransmitters, hormones, autacoids, and products of metabolism) factors. Myogenic tone is a property of arteriolar smooth muscle that is independent of any other influence and is the reference tone for the control of vascular resistance by extrinsic factors. Neurogenic mechanisms occupy a central position in the normal regulation of vascular tone and coordinating the degree of transient changes in vascular tone in many regions and organs, enable the rapid redistribution of blood flow to areas functionally important to specific activities.

The renin-angiotensin-aldosterone system plays an important role in regulating blood volume and systemic vascular resistance, which together influence cardiac output and arterial pressure.[7] Renin, which is primarily released by the kidneys and also by other cell types,[8] stimulates the formation of angiotensin in blood and tissues, which in turn stimulates the release of aldosterone from the adrenal cortex.[9, 10] Angiotensin II facilitates noradrenaline release from sympathetic nerve endings and inhibits noradrenaline re-uptake by nerve endings, thereby enhancing sympathetic adrenergic function.[11]

1.2 Autonomic Nervous System

The autonomic nervous system plays an important role in the regulation of cardiac and vascular function. The sympathetic division is the most important nervous division of the autonomic nervous system influencing both the whole-body hemodynamics and the local vascular tone in many areas. The activity of sympathetic neurons usually elicits vasoconstriction roughly proportional to the level of neural activity. This vasoconstriction is determined by various combinations of noradrenaline, neuropeptides, and purines.[12] The sympathetic nerves that originate in the spinal cord run through the spinal cord, where they synapse with preganglionic cell bodies. From these cell bodies run preganglionic fibers that synapse with the cell bodies of postganglionic sympathetic fibers (Figure 2).

Acetylcholine is the neurotransmitter within these ganglia, and the acetylcholine binds to nicotinic receptors on postganglionic neurons. From these neurons arise relatively long postganglionic fibers that travel to their target organ and release noradrenaline as the primary neurotransmitter. Some of the preganglionic sympathetic fibers, instead of synapsing within paravertebral ganglia, synapse in prevertebral ganglia located within the abdomen (celiac, superior mesenteric, and inferior mesenteric ganglia). Postganglionic sympathetic fibers then travel from the prevertebral ganglia to innervate tissues, such as blood vessels, where they release noradrenaline as the primary neurotransmitter. Therefore, sympathetic postganglionic fibers can originate in either paravertebral or prevertebral ganglia (Figure 2).

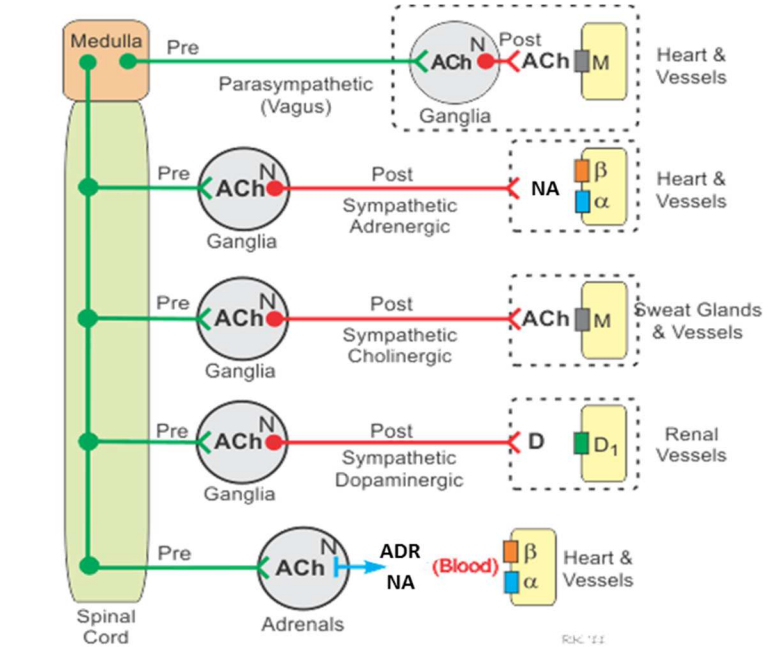

Figure 2 – Schematic representation of autonomic nervous system regulation of cardiac and vascular function. (adapted from Cardiovascular Physiology Concepts, Richard E. Klabunde, Lippincott Williams & Wilkins)

Activation of sympathetic adrenergic nerves in the heart releases noradrenaline that binds to adrenergic receptors (primarily beta-adrenoceptors), increasing heart rate, contractility and velocity of electrical impulse conduction. Together, these changes increase cardiac output and arterial blood pressure. Sympathetic adrenergic activation also constricts blood vessels, through the actions of noradrenaline binding to alpha-adrenoceptors leading to increase of arterial blood pressure. Therefore, the cardiovascular effects of vagal activation are primarily mediated through the heart, whereas sympathetic activation affects both the heart and vasculature. Preganglionic sympathetic nerves that synapse in the adrenal glands stimulate catecholamine release that circulates in the blood and affect heart, blood vessels and other organs by activating adrenergic receptors.[13, 14] Moreover, under basal resting conditions, vagal and sympathetic adrenergic nerves are tonically active. In the heart, because vagal influences override sympathetic effects, there is a resting vagal tone that is responsible for maintaining a low heart rate at rest. In contrast, most blood vessels, which have little or no vagal innervation, are dominated by sympathetic adrenergic influences the sympathetic vascular tone, at rest.

1.3 Sympathetic Neurotransmission

Noradrenaline is the classical neurotransmitter, released from sympathetic nerve terminals and is responsible for tonic and reflexive changes in cardiovascular tones. Noradrenaline is one of the catecholamine neurotransmitters that are synthesized from tyrosine by three consecutive enzymatic steps. While tyrosine hydroxylase is the enzyme responsible for the first step of catecholamine biosynthesis, converting tyrosine to l-dopa (the enzymatic rate-limiting step in catecholamine synthesis), and is expressed in all catecholamine neurons, dopamine-β-hydroxylase is the one responsible for conversion of dopamine to noradrenaline and is specifically expressed in noradrenergic neurons (Figure 3).

A presynaptically localized noradrenaline transporter retrieves released noradrenaline to limit the spread and duration of synaptic excitability and allows repackaging of noradrenaline into synaptic vesicles. During sympathetic discharge, noradrenaline produces vasoconstriction by activating α-adrenoceptors located on vascular smooth muscle.

Most of the noradrenaline contained in adrenergic nerves is stored in granular vesicles. Two types of granular vesicles, small and large dense-cored vesicles, both storing noradrenaline, have been identified. Vesicles generated near the Golgi apparatus, in cell bodies, travel by axonal transport to the nerve terminals. Noradrenergic vesicles may also be formed, by endocytosis, within the axons. Noradrenaline is believed to be stored in the vesicles in a complex with adenosine triphosphate (ATP), but the vesicles also contain enzymes involved in noradrenaline synthesis, and other proteins. It is currently accepted that the ATP released together with noradrenaline from sympathetic nerves may act as a sympathetic cotransmitter.[15, 16] Noradrenaline taken up into the axoplasm is subject to two fates, translocation into storage vesicles and deamination by monoamine oxidase. The combination of enzymatic breakdown and vesicular uptake constitute an intraneuronal way to keep axoplasmic noradrenaline concentrations very low. Sympathetic stimulation releases noradrenaline, and binding of noradrenaline to adrenoceptors on cardiovascular smooth muscle cells causes the cells to contract.

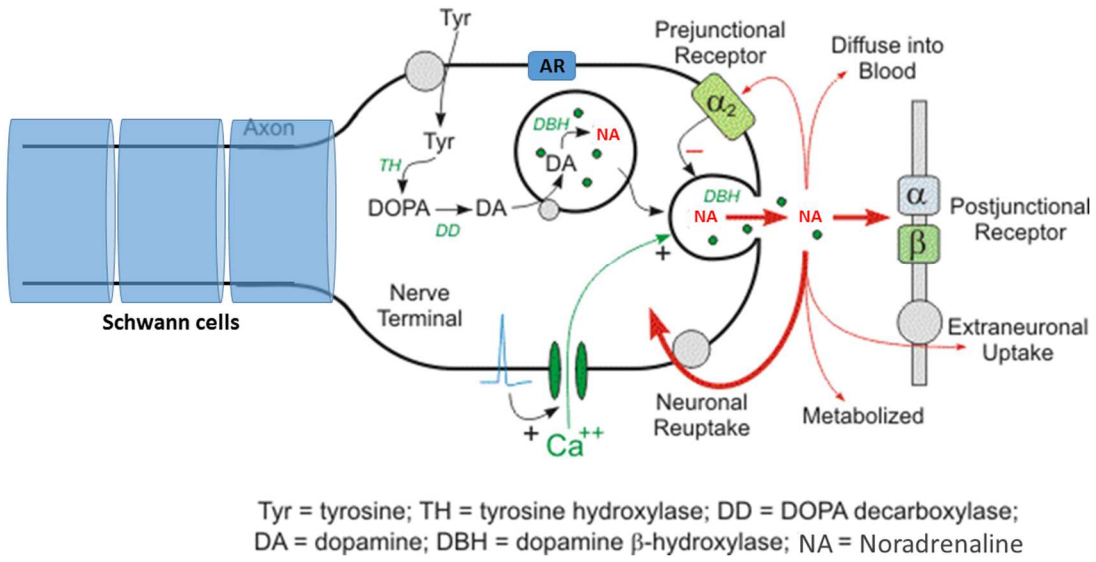

Figure 3 – Noradrenaline synthesis, storage, release and action. (adapted from Cardiovascular Pharmacology Concepts. Richard E. Klabunde. Lippincott Williams & Wilkins, Second Edition)

Sympathetic vascular innervation varies widely among vascular beds, with dense innervation present on resistance vessels. The density of sympathetic nerves increases as the arterial caliber decreases, so that small arteries and arterioles, the smallest nutrient vessels possessing smooth muscle cells, have the densest innervation. Sympathetic stimulation in these beds produces profound vasoconstriction. Different stressors can elicit different patterns of sympathoneural outflows and therefore differential noradrenaline release in the various vascular beds. Local sympathoneural release of noradrenaline also markedly affects cardiac function and glandular activity.

A search for new transmitters, other than acetylcholine and noradrenaline, was prompted by the discovery that nerves that relax some smooth muscles did not involve the release of noradrenaline or acetylcholine. A variety of nonadrenergic, noncholinergic transmitters were identified, including ATP, vasoactive intestinal polypeptide, nitric oxide (NO), and neuropeptide Y. The notion of the presence of multiple transmitters is now firmly established becoming clear that within a single neuron multiple transmitter systems may exist, and that within a given ganglion the variety and pattern of neurotransmitters may be quite extensive.

1.3.1 Sympathetic Neurotransmission: NA/ATP co-transmission

Purinergic neurotransmission expands the cotransmission subject. ATP is a primitive extracellular signaling molecule and is now established as a cotransmitter in most nerve types both in the peripheral and central nervous systems. There is purinergic cotransmission together with noradrenaline and neuropeptide Y, from sympathetic nerves; with acetylcholine, from parasympathetic nerves; with calcitonin gene–related peptide and substance P, from sensory-motor nerves, with nitric oxide and vasoactive intestinal polypeptide, from enteric nerves and with glutamate, dopamine, noradrenaline, g-aminobutyric acid, and 5-hydroxytryptamine, from subpopulations of central neurons.[17] Sympathetic purinergic cotransmission has been demonstrated in a variety of blood vessels and the proportion of noradrenaline to ATP is extremely variable in the sympathetic nerves supplying different blood vessels.[18-20] ATP contributes to vasoconstriction produced after activation of the perivascular nerves in SHR. This involves a synergistic interaction with noradrenaline to causes enhanced arterial vasoconstriction, which may contribute to the hypertension.[21]
Distinct presynaptic and postsynaptic mechanisms are observed: ATP is in high concentration in sympathetic synaptic vesicles and, after release, is rapidly catabolized to other adenosine derivatives.[22]

1.4 Adenosine

Adenosine is a naturally endogenous occurring purine nucleoside formed by the degradation of ATP (Figure 4) during energy-consuming processes. ATP is the primary energy source in cells for transport systems and many enzymes. Most ATP is hydrolyzed to ADP, which can be further dephosphorylated to AMP. Most ADP and AMP formed in the cell are rephosphorylated in the mitochondria by enzymatic reactions requiring oxygen. If there are large amounts of ATP hydrolyzed, and especially if there is insufficient oxygen available (i.e., hypoxia), then some of the AMP can be further dephosphorylated to adenosine by the cell membrane associated enzyme, 5'-ectonucleotidase.

Adenosine is rapidly transported into red blood cells (and other cell types) where it is rapidly deaminated, by adenosine deaminase, to inosine, which is further broken down to hypoxanthine, xanthine and uric acid, which is then excreted by the kidneys. Adenosine deamination also occurs in the plasma, but at a lower rate than that occurring within cells. Adenosine can be acted on by adenosine kinase and rephosphorylated to AMP. This salvage pathway helps maintain adenine nucleotide pools in the cell. Extracellular concentrations

of adenosine are increased when energy demands exceed oxygen supply that is, during ischemia.[23]

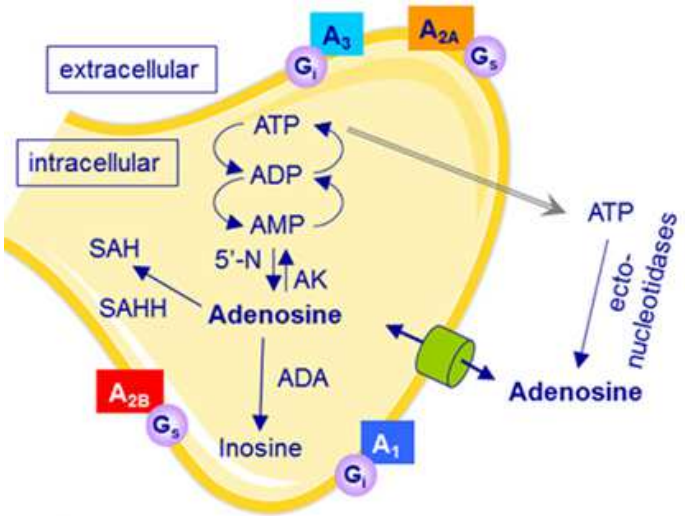

Figure 4 - Schematic representation of adenosine formation, metabolism and transport, and G-protein coupled adenosine receptors (rectangles representing A_1, A_{2A}, A_{2B}, and A_3 receptors). Neurons can release adenosine and adenosine-tri-phosphate (ATP). All cell types express adenosine receptors, adenosine transporters (cylinder), and ecto-nucleotidases that convert ATP into adenosine. ADP, adenosine-di-phosphate; AMP, adenosine-mono-phosphate; SAH, S-adenosyl-homocysteine; 5′-N, 5′-nucleotidase; AK, adenosine kinase; ADA, adenosine deaminase; SAHH, S-adenosyl-homocysteine hydrolase. Activation of adenosine receptors either inhibits ($A_{1/3}$ receptors) or stimulates ($A_{2A/2B}$ receptors) adenylate cyclase and the cyclic AMP (cAMP) pathway. (adapted from Landolt HP et al, 2012)

Adenosine modulates many physiological processes through activation of four subtypes of G protein–coupled membrane receptors: A_1, A_{2A}, A_{2B}, and A_3 (Figure 4).[24] It is well established that adenosine effects occur *via* activation of A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors classically considered to be coupled to Gi/o, Gs, Gs/Gq and Gi/o/Gq, respectively.[25] Adenosine receptors are broadly grouped into two main categories: A_1 and A_3 receptors, which couple to inhibitory G proteins, and A_{2A} and A_{2B} receptors, which couple to stimulatory G proteins. However, adenosine receptors are pleiotropic; they can couple with various G proteins and transduction systems according to their degree of activation and their particular cellular or subcellular location.[26] Its physiologic importance depends on the affinity of these receptors and the extracellular concentrations reached. Adenosine

may have tonic actions even during physiologic conditions, mostly through activation of high-affinity A_1 and A_{2A} receptors.[27, 28]

Membrane transporters are responsible for the uptake of essential nutrients, modulation of concentrations of physiologically relevant chemicals, and active release of substances such as signaling molecules.[29] Transmembrane transport is a critically important physiological process in all cells and is likely to have evolved early to allow for controlled uptake and release of nonlipophilic compounds. Nucleoside transporters constitute a family of membrane proteins with different pharmacological and kinetic properties.[30] To date it is accepted that there are two types of transporters: Equilibrative Nucleoside Transporters (ENT) and Concentrative Transporters (CNT). The ENT promote equilibrative bidirectional transport processes driven by chemical gradients by facilitated diffusion. ENT are present in most, possibly all, cell types. They might mediate adenosine transporter in both directions, depending on the concentration gradient of adenosine across the plasma membrane. Until the present day, there are four subtypes described: ENT1, ENT2, ENT3 and ENT4. The CNT carry out active inwardly directed concentrative processes, driven by the Na^+ electrochemical gradient: Na^+-dependent. CNT are expressed in a tissue-specific fashion. Three subtypes were described: CNT1, CNT2 and CNT3.[31]

After stimulation, released adenosine is quickly transported back into cells by an energy-dependent uptake mechanism, which is part of a purine salvage pathway designed to maintain intracellular levels of ATP. The intra and extracellular concentration of adenosine is determined, nearby their receptors, by the existence and function of the transporters. Adenosine transporters contribute to the intra and extracellular concentration of adenosine, modulating its concentration in the vicinity of its receptors.[32-34] The effectiveness of this adenosine transport system is species dependent. It is particularly active in humans, and it is mainly responsible for the extremely short half-life of adenosine in human blood. Adenosine mechanisms are the target of commonly used drugs acting by blockade of adenosine reuptake, thus potentiating its actions or antagonizing adenosine receptors. Adenosine receptors are ubiquitous and, depending on their localization, may mediate opposite effects. This phenomenon is particularly evident in the interaction of adenosine and the autonomic nervous system; adenosine can produce either inhibition or excitation of autonomic neurons.[35] Adenosine inhibits the release of neurotransmitters putatively through presynaptic A_1 receptors both in the brain and periphery. This is true for practically all neurotransmitters studied, including noradrenaline and acetylcholine.

Interstitial levels of adenosine are elevated under conditions of increased metabolic demand (exercise) and decreased energy supply (ischemia), reaching physiologically relevant concentrations. Sympathetic activation leads to systemic vasoconstriction, increase in blood pressure, and improved perfusion pressure. This systemic vasoconstriction would

be deleterious to the ischemic organ if not for the simultaneous local inhibitory actions of adenosine, which produce vasodilation and inhibit noradrenaline release. These actions are, for the most part, circumscribed to the local ischemic tissue so that it is protected from sympathetically mediated vasoconstriction while it benefits from the improved perfusion pressure. Adenosine provides a link between local mechanisms of blood flow autoregulation and systemic mechanisms of autonomic cardiovascular regulation.[36]

Adenosine is released into the extracellular space and signals to restore the balance between local energy requirements and energy supply. Endothelial cells interact with adenosine mechanisms in many different ways. Endothelial cells are known to have a very active adenosine metabolism, characterized by a large capacity for uptake and release of the nucleoside, [37, 38] and can be an important source of adenosine released during ischemia.[39] Conversely, adenosine may modulate endothelial function via activation of cell membrane receptors. The precise nature of the interaction between adenosine receptor subtypes and endothelial cells and their role in the regulation of endothelial function is not completely understood. However, adenosine-induced endothelial cells production of nitric oxide is recognized.

1.5 Nitric Oxide

The greatest surprise in the study of autonomic neuroeffector transmission, after the discovery that transmitters other than noradrenaline and acetylcholine exist, was the role of endothelial cells in the relaxation of arterial smooth muscle. This pointed to the existence of a substance that, when released from endothelial cells, acts on vascular smooth muscle cells to relax them. The subsequent identification of this substance as nitric oxide led to the discovery that nitric oxide is an important transmitter at some autonomic neuromuscular junctions.[40, 41]

Nitric oxide is one substance proposed to act as a neurotransmitter in the autonomic nervous system and is very different from the classical neurotransmitters, noradrenaline and acetylcholine. NO is a free radical and potentially very toxic. It passes freely through membranes and, thus, cannot be stored in vesicles for release during an action potential. In addition, it cannot act in a stereospecific way on postjunctional receptors on the target membrane to produce a response. It is now known that NO does act as a neurotransmitter within the autonomic nervous system, particularly within the parasympathetic and enteric nervous systems. NO relaxes smooth muscle by activating soluble guanylate cyclase, which increases cGMP levels. Moreover, the complexity of autonomic control and the range of mechanisms available to peripheral sympathetic and parasympathetic neurons and their

targets are expanded by gaseous molecules (NO) and endothelium released peptides. Also, central nervous system centers involved in autonomic control contain NO synthase, and evidence suggests that NO can mediate either sympathoinhibition or sympathoexcitation. Peripherally, NO exerts tonic vasodilation and mediates acetilcholine-induced vasodilation, as well as catecholamine release and action. Nitrergic neurotransmission has been demonstrated in the gastrointestinal, urogenital, and cardiovascular systems and its loss has also been implicated in a variety of pathological conditions.

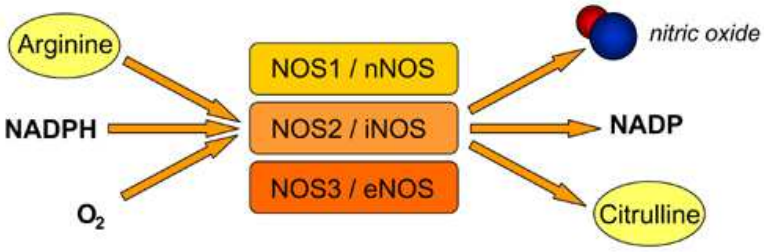

Figure 5 - Synthesis of nitric oxide by Nitric Oxide Synthases (NOS) isoforms.

NO is synthesized by the enzyme nitric oxide synthase (NOS), which can exist in three isoforms (Figure 5). The isoform involved in nitrergic neurotransmission in the autonomic nervous system is nNOS. The other sources of NO are endothelial NOS (eNOS) in the endothelium of blood vessels and inducible NOS (iNOS), which is expressed as part of an immune response.

The neuronal isoform of nitric oxide synthase (nNOS) is a highly complex enzyme (Figure 6) that exists as a dimer, in which each monomer consists of two enzymes in one. One end, the reductase domain, produces electrons during the conversion of reduced NADPH to NADP. Electrons are passed along the enzyme by flavin cofactors until they reach the oxygenase domain. An important region, the calmodulin binding region, links the two domains. In the oxygenase domain there is a heme-binding site. In the presence of electrons, heme and O_2, L-arginine is converted to citrulline, resulting in NO synthesis in a highly regulated, Ca^{2+}-dependent, manner. At basal Ca^{2+} concentrations within the nerve, nNOS is inactive.[42] During an action potential, intracellular concentrations of Ca^{2+} increase resulting in the binding of calmodulin to nNOS and activation of the enzyme. Once activated, nNOS converts L-arginine to citrulline and NO.

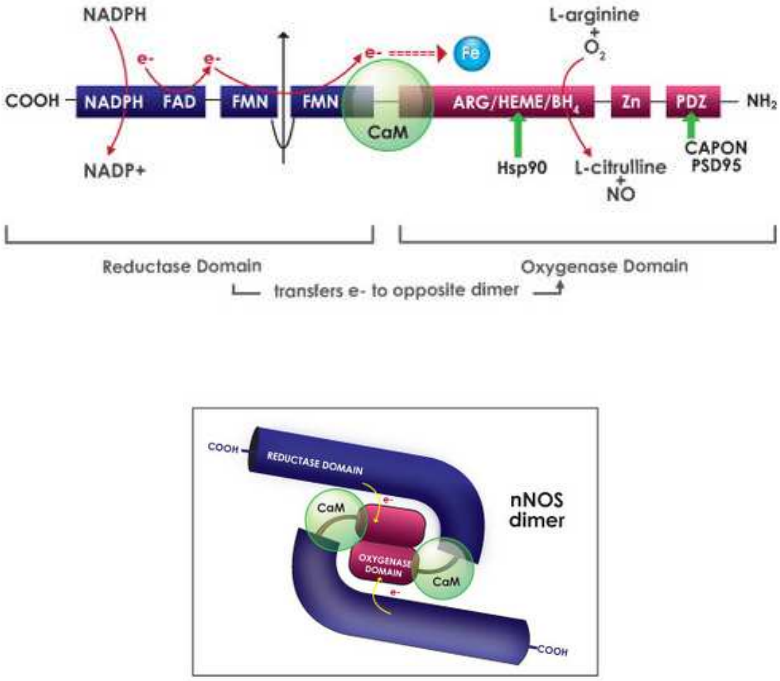

Figure 6 - Neuronal isoform of nitric oxide synthase. nNOS contains a reductase domain and a oxygenase domain which are separated by a calmodulin (CaM) binding motif. nNOS is active in dimeric form. (from Nitric Oxide Contribution in the CNS: a NO brainer, May O.)

The most important step in demonstrating nitrergic neurotransmission has been the development of L-arginine analogs that can inhibit NO synthesis such as the non-selective N^G-nitro-l-arginine methyl ester (L-NAME) and the selective Nω-propyl-L-arginine hydrochloride (nNOS inhibitor) and L-NIO dihydrochloride (eNOS inhibitor). NO cannot be stored but reaches the postjunctional target by diffusion. Drugs have been developed that act as NO donors (e.g. sodium nitroprusside and diethylamine NONOate), and these have demonstrated that exogenous NO can mimic the effects of nitrergic neurotransmission. NO reacts with hemoglobin or other reactive species such as superoxide anions, both of which inhibit neurotransmission or the response to NO donors. Once NO enters the postjunctional smooth muscle cell, it produces a response by interacting with the soluble guanylate cyclase. NO binds to its heme group, resulting in the activation of the enzyme and production of increased levels of cyclic guanosine monophosphate, which causes relaxation of smooth muscle. Once the physiologic stimulus of the action potential has ended, Ca^{2+} levels in the nerve return to low resting levels and NO synthesis stops. Therefore, the amount of NO synthesized in autonomic nerves does not reach toxic levels.[42, 43] NO, itself, is difficult to detect or measure however, the capacity to synthesize NO can be readily demonstrated

in the autonomic nervous system by immunohistochemical localization of nNOS. nNOS has been localized in both sympathetic and parasympathetic preganglionic neurons and is particularly prominent in the enteric nervous system, where it is localized in the myenteric and submucosal plexuses. nNOS has also been demonstrated in perivascular nerves supplying many blood vessels. Functional studies have demonstrated nitrergic neurotransmission throughout the cardiovascular, urogenital, respiratory, and gastrointestinal systems.[44-47] NO can function either to cause direct inhibition of smooth muscle or as a neuromodulator by inhibiting excitatory transmission.

An endothelium-derived relaxing factor has been shown to be NO. The vascular endothelium synthesizes NO from L-arginine and this action is catalyzed by the action of NO synthases, of which two forms are present in the endothelium. Endothelial eNOS is highly regulated, constitutively active and generates NO in response to shear stress and other physiological stimuli and is responsible for most of the vascular NO· produced.[48] Inducible iNOS is expressed in response to immunological stimuli, is transcriptionally regulated and, once activated, generates large amounts of NO that contribute to pathological conditions. The physiological actions of NO include the regulation of vascular tone and blood pressure, prevention of platelet aggregation and inhibition of vascular smooth muscle proliferation. Many of these actions are a result of the activation by NO of the soluble guanylate cyclase and consequent generation of cyclic guanosine monophosphate.[49] An additional target of NO is cytochrome c oxidase, the terminal enzyme in the electron transport chain, which is inhibited by NO in a manner that is reversible and competitive with oxygen. The consequent reduction of cytochrome c oxidase leads to the release of superoxide anion. This may be an NO-regulated cell signalling system which, under certain circumstances, may lead to the formation of the powerful oxidant species, such as peroxynitrite, that is associated with a variety of vascular diseases.

A functional eNOS oxidizes its substrate L-arginine to L-citrulline and NO·. This normal function of eNOS requires dimerization of the enzyme, the presence of the substrate L-arginine and the essential cofactor tetrahydrobiopterin (BH4), one of the most potent naturally occurring reducing agents. Cardiovascular risk factors such as hypertension, hypercholesterolemia, diabetes mellitus, or chronic smoking stimulate the production of reactive oxygen species in the vascular wall. NADPH oxidases represent major sources of this reactive oxygen species and have been found upregulated and activated in animal models of hypertension, diabetes, and sedentary lifestyle and in patients with cardiovascular risk factors. Superoxide ($O^{2\cdot-}$) reacts avidly with vascular NO· to form peroxynitrite ($ONOO^{-}$).[50, 51] The cofactor BH4 is highly sensitive to oxidation by ONOO−. Diminished levels of BH4 promote $O^{2\cdot-}$ production by eNOS (referred to as eNOS uncoupling). This transformation of eNOS from a protective enzyme to a contributor to

oxidative stress has been observed in several in vitro models, in animal models of cardiovascular diseases, and in patients with cardiovascular risk factors. In many cases, supplementation with BH4 has been shown to correct eNOS dysfunction in animal models and patients. In addition, folic acid and infusions of vitamin C are able to restore eNOS functionality, most probably by enhancing BH4 levels as well.

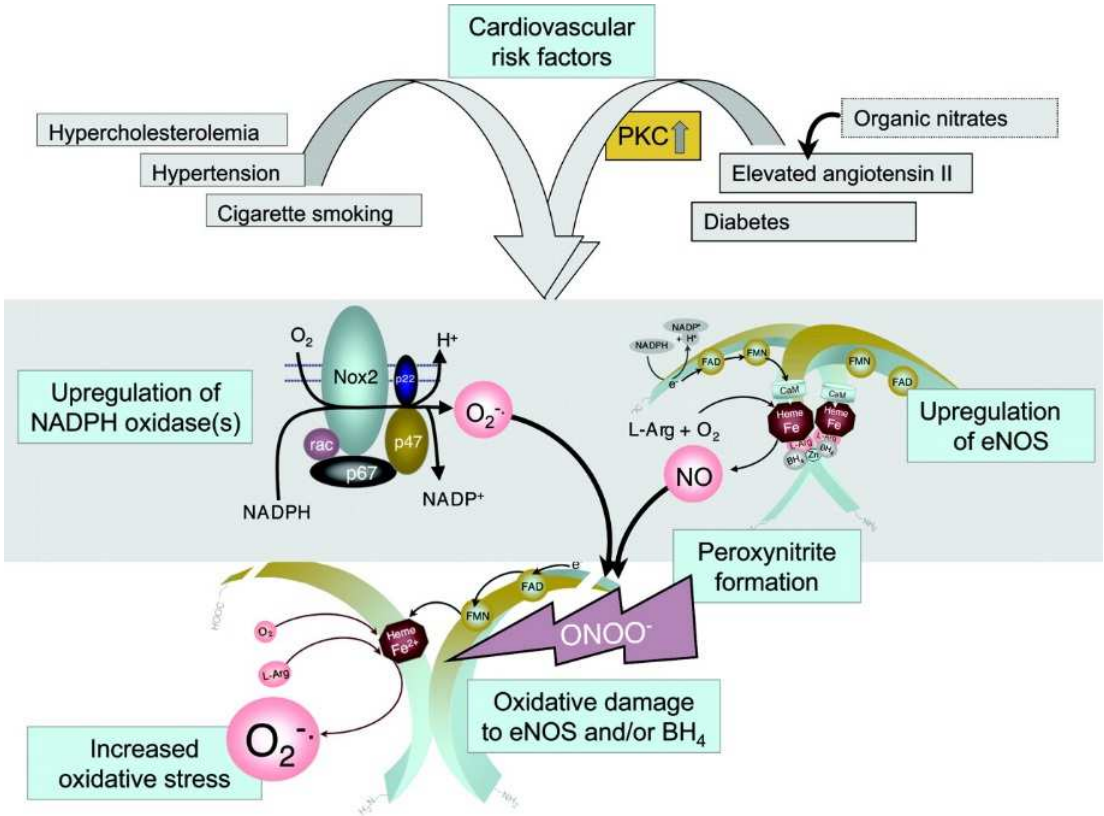

Figure 7 - Potential mechanisms by which cardiovascular risk factors lead to endothelial dysfunction. In many types of vascular disease, NADPH oxidases and eNOS are upregulated in parallel. Their respective products rapidly recombine to form $ONOO^-$. This oxidizes BH4, the essential cofactor of eNOS, and/or produces oxidative damage to the zinc-thiolate cluster of eNOS. Thus, O_2 reduction by eNOS is uncoupled from NO· formation, and a functional NOS is converted into a dysfunctional $O_2^{\cdot-}$-generating enzyme that contributes to vascular oxidative stress. (from Förstermann U , and Münzel T Circulation. 2006;113:1708-1714, Copyright © American Heart Association, Inc.)

Most cardiovascular risk factors activate molecular machinery in the endothelium that results in expression of chemokines, cytokines, and adhesion molecules designed to interact with leukocytes and platelets and target inflammation to specific tissues to clear microorganisms (Figure 7). The fundamental change involved in this process is a switch in

signaling from an NO-mediated silencing of cellular processes toward activation by redox signaling. Reactive oxygen species (ROS), in the presence of superoxide dismutase, lead to generation of hydrogen peroxide, which, like NO, can diffuse rapidly throughout the cell and react with cysteine groups in proteins to alter their function. It is intriguing that eNOS, which normally helps maintain the quiescent state of the endothelium, can switch to generate ROS in appropriate circumstances as part of endothelial activation. This is termed eNOS uncoupling, and results in superoxide formation if the key cofactor tetrahydrobiopterin is not present, or generation of hydrogen peroxide if the substrate L-arginine is deficient. Thus, the ability of eNOS to regulate both the quiescent and activated endothelial phenotype puts this enzyme at the center of endothelial homeostasis.

1.6 Hypertension

Globally cardiovascular disease accounts for approximately 17 million deaths a year, nearly one third of the total. Of these, complications of hypertension account for 9.4 million deaths worldwide every year. Hypertension is a global public health issue. Hypertension, also known as high or raised blood pressure, is a condition in which the blood vessels have persistently raised pressure. The higher the pressure in blood vessels the harder the heart has to work in order to pump blood. If left uncontrolled, hypertension can lead to a heart attack, an enlargement of the heart and eventually heart failure. Blood vessels may develop aneurysms and weak spots due to high pressure, making them more likely to clog and burst. The pressure in the blood vessels can also cause blood to leak out into the brain. This can cause a stroke. Hypertension can also lead to kidney failure, blindness, rupture of blood vessels and cognitive impairment. It contributes to the burden of heart disease, stroke and kidney failure and premature mortality and disability.

Hypertension is responsible for at least 45% of deaths due to heart disease, and 51% of deaths due to stroke. The increasing prevalence of hypertension is attributed to population growth, ageing and behavioral risk factors, such as unhealthy diet, harmful use of alcohol, lack of physical activity, excess weight and exposure to persistent stress.[52]
Enhanced activity of the sympathetic nervous system contributes to the pathogenesis and maintenance of hypertension. Sympathetic nervous system activity is increased in both the developmental and chronic stages of primary hypertension.[53, 54] Animal and human studies demonstrate that sustained sympathetic stimulation of the vasculature induces smooth muscle cell hypertrophy and hyperplasia, resulting in sustained increases in peripheral resistance. These observations indicate that sympathetic activation plays a key role in both initiating and maintaining chronic increase of blood pressure.

Vascular smooth muscle cell proliferation/hypertrophy is a consequence of the complex interaction of intravascular pressure, systemic and local hormones, genetic predispositions, and environmental factors. Animal and human studies demonstrate that sustained increases in sympathetic activation directly induce vascular remodeling. Many studies report greater circulating levels of noradrenaline in patients with hypertension than in normotensive control subjects. In normotensive subjects, increased levels of circulating noradrenaline generally induce down-regulation of noradrenergic receptors. However, in subjects with hypertension, such down-regulation appears not to occur, resulting in enhanced sensitivity to noradrenaline. The combination of enhanced sensitivity to and increased circulating levels of noradrenaline likely contributes significantly to sympathetic nervous system activity-related hypertension.

Specific animal models for studying cardiovascular diseases conditions can help support research efforts towards finding the necessary cures. The Spontaneously Hypertensive Rat (SHR) model is the most commonly used genetically hypertensive rat model and also recognized a model of endothelium dysfunction.[55-57]

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AIMS

The present study intends to clarify if endogenous adenosine and nitric oxide have a role in the modulation of sympathetic activity and if this role is preserved in hypertensive individuals. On the other hand, and since endothelium derived substances have higher impact in vascular tonus, it has been hypothesised that endothelium dysfunction may alter vascular sympathetic neurotransmission, contributing to increase vascular tone and, consequently, blood pressure.

To address this goal, it was investigated, in intact and denuded arteries, the modulatory roles of endogenous adenosine, *via* activation of adenosine A_1 or A_{2A} receptor subtypes, and NO, generated by endothelial NOS (eNOS) and neuronal NOS (nNOS), in sympathetic vascular neurotransmission. It was also evaluated if their effects could be somehow compromised in mesenteric and tail arteries from spontaneously hypertensive rats, an animal model exhibiting endothelium dysfunction and hypertension. Additionally, the regional distribution/localization and the relative amount of membrane proteins, namely adenosine receptors (A_1 and A_{2A}) and enzymes, such as neuronal nitric oxide synthase and tyrosine hydroxylase, present in adventitia layer was also determined in both mesenteric and tail arteries of the two animal strains (Wistar Kyoto, WKY and spontaneously hypertensive rats, SHR).

CHAPTER II

Experimental section

BOOK CHAPTER

IMAGING RECEPTORS WITH LASER SCANNING CONFOCAL MICROSCOPY: QUALITATIVE AND QUANTITATIVE ANALYSIS

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IMAGING RECEPTORS WITH LASER SCANNING CONFOCAL MICROSCOPY: QUALITATIVE AND QUANTITATIVE ANALYSIS

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ABSTRACT

Receptors are the molecular targets through which drugs produce their beneficial effects in a disease state. Receptors location, in the cell membrane or within the cell, contributes for the understanding of the subsequent signalling events triggered by their activation. The subcellular localization and physiological functions of receptors are closely related and thus it is crucial to precisely determine the distribution of different molecules inside the intracellular structures. This is usually studied by fluorescence microscopy and posterior evaluation of the colocalization of the molecule of interest in a definite region in the cell. Laser Scanning Confocal Microscopy (LSCM) imaging is a powerful tool allowing to distinguish a variety of unexpected locations for receptors, manly in subcellular locations such as mitochondria, lysosomes, nuclei[1] turns crucial rethinking the current knowledge of the pharmacological role ascribed to receptors. Moreover, the use of fluorescent ligands in tissues will also be addressed since it allows a multidimensional analysis and provides new insights to receptor dynamics and intracellular traffic.[2]

This chapter describes some of the the strengths, limitations and potentialities associated with receptors Laser Scanning Confocal Microscopy (LSCM) imaging, giving some practical examples concerning our current research line. Emphasis is placed on the importance of appropriate image acquisition guiding the reader through the basic and advanced knowledge of major quantitative image analysis approaches. Quantitative evaluation of receptors in tissues provides information about receptor over, subexpression or redistribution contributing for the understanding of the impairment/deregulation associated with receptors putative physiological and/or pathophysiological role.

Keywords: receptors distribution, Laser Scanning Confocal Microscopy, molecular targets, quantitative evaluation

1. INTRODUCTION

In cell biology a structure on the surface of a cell (or inside a cell) that selectively receives and binds a specific substance triggering a cellular response is called a receptor. Receptors can be divided into four main classes: ligand-gated ion channels, tyrosine kinase-coupled, intracellular steroid and G protein coupled receptors. The latter represent the largest of the several families of plasma membrane receptors, comprising more than a thousand genes and regulating virtually all known physiological processes in mammals. Because G protein coupled receptors play specific roles in human disease, they have provided useful targets for drug development, being, at present, the commonest molecular target for currently used drugs. Accordingly, receptor physiology in a specific tissue/organ can be better understood if information on the expression of receptors or other type of proteins/ligands combined with experiments that allow to establish their respective function contributes for an integrative view of ligands/receptors role both in physiology and pharmacology. The knowledege of receptors location, distribution and function has been advanced through continous improvement of methodologies and equipments. In this sense, the first approach developed to establish receptors location was based on the use of radioligands and their binding visualized by *in vitro* autoradiography in tissue sections[3-5]. However, limitations in the accuracy of this method, at the cellular level, lead to the refinement of techniques such as RT-PCR or Western-blot, classic and fluorescent immunohistochemistry microscope visualization.

1.1. Fluorescence microscopy

The application of fluorescence microscopy technologies to physiology and pharmacology is growing bringing together insights from three separate existing fields (chemistry, immunology and histology). Indeed, immunohistochemistry has grown as genomics and bioinformatics have provided unique sequences against which to raise antibodies for identifying proteins. Vital chromophores indicate localised chemical composition such as pH or Ca^{2+} concentration by changing the characteristics of their excitation or emission. Finally, highly selective ligands have been labeled covalently with fluorescent moieties to provide the potential for ligand binding studies at the subcellular level, thereby providing pharmacologically interesting insights beyond those provided by immunohistochemistry. These rejuvenated old technologies are enhanced further by the new opportunities offered by luminescent or fluorescent organic ligands found in plants or marine organisms. These can be used as conventional chromophores but, more powerfully,

can be spliced to known proteins, by genetic manipulation, to provide a covalently bonded fluorescent moiety whose chromatic properties can be exploited.

Fluorescence microscopy, therefore, allows the detailed mapping of ligands/receptors in cells/tissues by multiple fluorescent staining. Fluorescent techniques can be divided in two categories: the conventional fluorescent staining and the molecular tagging. In the context of receptor biology, these technologies offer the chance to visualise receptors, their antagonist or agonist ligands, including natural hormones and neurotransmitters, the transducing proteins with which they interact, and the indicators of the cellular response that they mediate or modify.

The majority of studies on G protein coupled receptors rely on qualitative determinations where apparent fluorescence increases or decreases in cells are taken as changes in receptor fate or activity. Other inportant information can be extracted when colocalisation occurs, i.e. two or more antigens staining patterns labeled by corresponding antibodies with different excitation spectra, visualized in different colors, overlaps. Colocalization correspond to a coexistence of molecules in a very close physical location providing valuable information to clarify their common features. Furthermore, it is of upmost importance to find a way of getting quantitative data that will help to define magnitude, concentration dependency or time-course of the processes under study. Currently, several methodologies, including laser scanning confocal microscopy (LSCM) have been used to characterize receptor dynamics: immunohistochemistry and analysis of signal transduction pathways, including visualization of protein binding to other molecules or their folding by using fluorescence ressonance energy transfer (FRET) or bioluminescence ressoance energy transfer (BRET),[6] live-cell imaging, including molecular dynamics by fluorescent protein-fusion probes,[7, 8] traffic analysis of individual proteins, such as receptors, by fluorescence recovery after photobleaching (FRAP) techniques[9] and protein turnover.

1.1.2. Laser Scanning Confocal Microscopy (LSCM)

The major application of LSCM in biomedical sciences is imaging of fixed or living tissues previously labeled with fluorescent ligands. The reader is referred to Paddock's review,[10] a chapter that will provide an excellent introduction to the principles and practices of LSCM.

The quality of an image dataset acquired by LSCM, depends mainly on two aspects, the correct processing of the sample and the microscope performance. Sample processing, in turn, depends on several factors such as, fixation, loading of fluorescent dyes or

immunostaining, and the correct mounting of the specimen. It is essential to consider the preparation of the samples (specimens) as a subject of particular relevance particularly if quantitative analysis is on our purpose: procedures, light conditions and magnification must be similar. Ideally, from the technical point of view, the specimen calibration step (performed by the computer), requires homogeneous specimens in chemical, dimension and structural features. Microscope performance is crucial for the quality of image acquisition, mainly through its ability to eliminate the "out-of-focus" from thick fluorescently labeled specimens. Since the illumination in a confocal microscopy is achieved by scanning one or more focus beams of light across the specimen using a laser, the stability of this laser, as well as efficient reflecting mirrors and sensitive low noise photodetectors, are key factors that need to be considered. Moreover, there have been considerable improvements in the digital image systems, now allowing that multiple fluorochromes to be imaged simultaneously by frame or, alternatively, sequentially on a line-by-line basis.

In modern systems such as LSCM, 'Z' axis is now usually associated to the collection of a series of images of various focal planes that can be, subsequently, used to construct 3D representations of the specimen. Sometimes, lower image stacks acquired by LSCM have lower voxel intensities when compared with the upper ones, i.e., there is intensity attenuation with depth. To reduce this, it is recommended to start the Z series at the deepest part of the specimen or, alternatively, it is also possible to correct this problem during image processing.[11] Therefore, it is possible to process images stacks to obtain both 2D or 3D reconstruction (by rotating the specimen and imaging it from several different angles) of a specimen, from a single view, using algorithms that have been in continous improvement. 4D reconstructions are also possible if time is considered. The latter is crucial for receptor signalling events or traffic studies such as visualization of receptor internalization processes (using tagged fluorescent proteins). LSCM 2D, 3D and 4D accurate reconstructions of multiple points offer better depth understanding and measurement of features located on the specimen.

2. Imaging receptors with LSCM: qualitative analysis

Fluorescent ligands bound to receptors can evidence their location and pharmacological information such as affinity or proximity between interacting molecules. Such information can be obtained, if binding is reversible, overcaming some limitations, namely low molecular concentration and diverse location of receptors. As such, relationship between receptor location and function are becoming better understood, contributing to new insights relative to drug action mechanisms and new therapeutic targets. Nevertheless,

spatial precise distribution of receptors and their function relationships are still largely unknown, making this an expanded area of study. In the last decade, studies using LSCM have been used to better understand the physiological/pathophysiological function of receptors, most of them using antibodies to identify, characterize and define the location of proteins/receptors, by immunohistochemistry, in different cells[12] and tissues,[13] including in vascular tissues.[14] In the vascular wall three layers can be identified (intima, media and adventitia), since their cells present different morphologies and different functions. In this respect, the use of fluorescent nuclear stain will enable identification of cell orientation, number, organization and cell type allowing layer vessel recognition[15, 16]. Receptors can be located in any of these cells and may interplay with other receptors, enzymes or transporters located in the same cell or in neighbouring cells. In this respect, LSCM resolution is crucial to distinguish between two points on a specimen as separate entities, which is mainly dependent on the numeric aperture of the objectives, of the substage condenser and the wavelength spectrum of light used.[10]

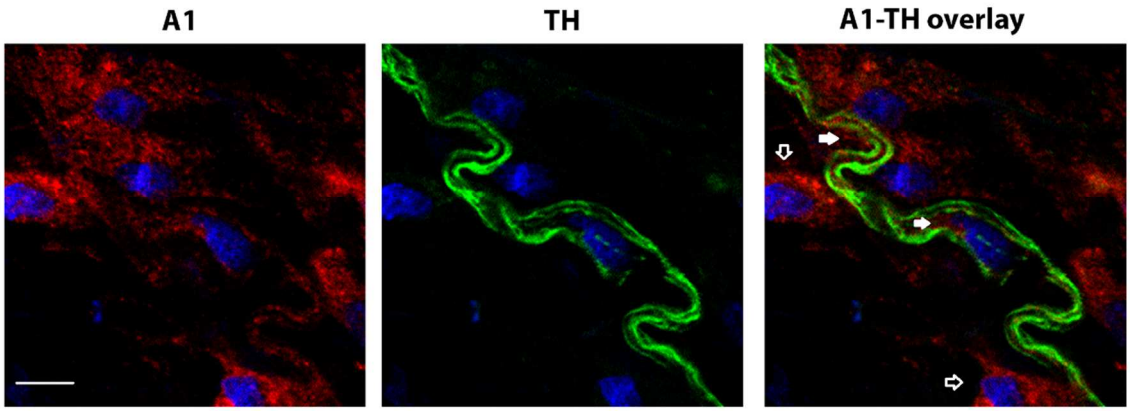

Figure 1. Laser scanning confocal immunofluorescence images of rat mesenteric artery adventitia immunostained for adenosine A_1 receptors (rabbit primary polyclonal adenosine A_1 receptor antibody and a species specific secondary Alexa 647 antibody: red), tyrosine hydroxylase (mouse primary monoclonal tyrosine-hydroxilase (TH) antibody and a species specific secondary Alexa 488 antibody: green) and DAPI (cell permeable fluorescent minor groove-binding probe for DNA, blue). Scale Bar = 20 μm. White open arrows indicates adenosine A_1 receptor population in non-neuronal cells while white filled arrows indicate adenosine A_1 receptor population on the sympathetic nerves.

Taken adenosine A_1 receptors as an example, it is possible to identify two populations of adenosine A_1 receptors in images acquired by LSCM (Figure 1A). One population is shown to be in the proximity of an enzyme involved in the generation of the neurotransmitter, noradrenaline, inside the sympathetic nerves, thyrosine hydroxilase,

whereas other receptors are located in non-neuronal cells. This type of identification is possible since qualitative image analysis is a task for which the human eye is well suited, once qualitative data is based on recognition patterns (overall arrangements of elements within the specimen).

Such information complements the knowledge that adenosine A_1 receptors are involved in sympathetic neurotransmission, as previously determined by transmitter release[17-19] and vascular reactivity studies.[20-22] On the other hand, the presence of A_1 receptors in non-neuronal cells, evidenced in these images, clearly suggest additional effects mediated by this population of A_1 receptors. This information further indicates the need to identify the type of cells where these adenosine A_1 receptors are present. Indeed, since it is well known that several types of cells can be found in adventitia, such as fibroblasts, macrophages and other immune cells or peripheral glial cells (Schwann cells),[23] it would be valuable to known the type of cell where adenosine A_1receptors are present in order to clarify their physiological/pathological role.

Other type of insights on the interplay between receptors and signalling molecules can be drawn using LSCM imaging. Indeed, this type of studies can, for instance, reveal substances that may influence receptor triggered signalling pathways/events such as reactive oxygen species (ROS), nitric oxide (NO), etc. Figure 2 shows two examples of images acquired using specific fluorescent probes for NO and ROS (DAF-FM DA and DHE, respectively), in the vicinity of adenosine A_1 receptors, suggesting the occurrence of an interplay between these receptors and those signalling molecules. This type of data can, therefore, be correlated with information in the literature to enhance knowledge. The example above mentioned correlates well with reported data indicating that adenosine receptor activation promote the increase of ROS generation[24], and can activate eNOS leading to an increase of NO production.[25]

Nevertheless, qualitative analysis may present limitations, for instance, when it is required the precise description of differences in staining patterns among sets of tissues and/or cells, comparison of staining among experimental groups, etc. In this sense, applicability of LSCM imaging observed, *per se,* is, however, limited because it is perceived differently by the eye and thus can frequently be misleading. Under ideal conditions, a person with excellent eyesight can distinguish between 30 to 35 levels in comparison to the 256 or more detected by LSCM, detecting intensity variations invisible to the human eye while for a computer, an image is just an array of values of individual pixels (for 8-bit monochrome image, this would be a sequence of numbers, with values ranging from 0 to 255). To take advantage of this ability, i.e., to achieve infomation related with this intensity variations, computer assisted methods of image analysis, particularly, quantification is required.

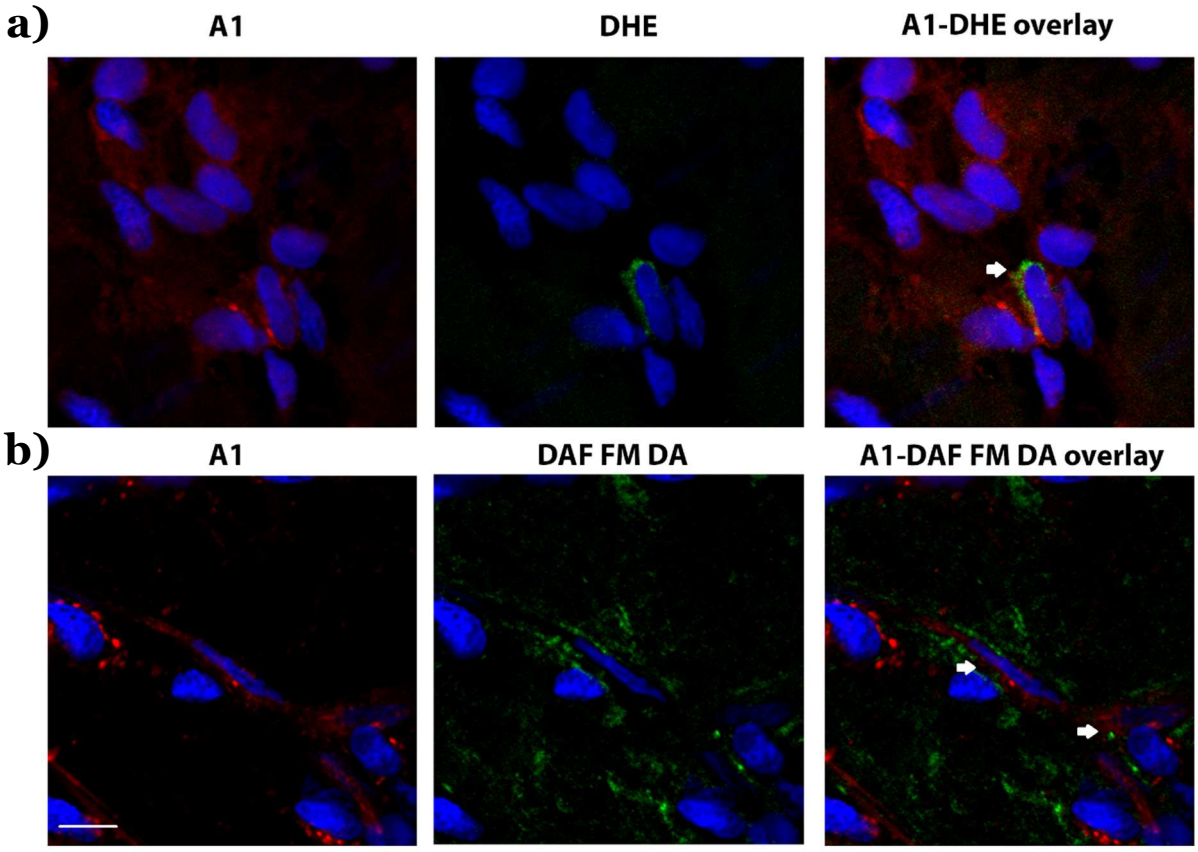

Figure 2.- Laser scanning confocal immunofluorescence images of rat mesenteric artery adventitia: a) immunostained for adenosine A_1 receptors (rabbit primary polyclonal adenosine A_1 receptor antibody and a species specific secondary Alexa 647 antibody: red), DAF-FM DA (cell-permeable fluorescent probe for the detection of nitric oxide: green) and DAPI (cell permeable fluorescent minor groove-binding probe for DNA, blue); b) immunostained for adenosine A_1 receptors (rabbit primary polyclonal adenosine A_1 receptor antibody and a species specific secondary Alexa 647 antibody: red), DHE (cell-permeable fluorescent probe for the detection of superoxide anion: green) and DAPI (cell permeable fluorescent minor groove-binding probe for DNA, blue). Scale Bar = 20 μm. White arrows indicate that NO (labeled with DAF-FM DA) or superoxide anion (labeled with DHE) in the vicinity of adenosine A_1 receptors.

3. Imaging receptors with LSCM: quantitative analysis

Notwithstanding the importance of qualitative informations, reliable quantitative data related with the expression of receptors (or other type of proteins) can be precious and open possibilities, combined with functional data, to new perspectives of study, contributing for an integrative view of receptors/proteins, both in physiology and pharmacology. Quantification is crucial for the understanding of physiopathological roles of receptors, particularly in disease states where it has been reported that specific receptors types are sub

or overexpressed.[1, 22, 26-28] Until recently, few studies have addressed the extraction of quantitative information on the expression/presence of receptors,[29-31] mostly due to methodological difficulties. In this sense, there are several image analysis software commercial or freely available (such as Metamorph, PAQI, Image J) allowing automated or semiautomated image analysis to quantify one or more receptors/protein types through particle counting, area determination and/or intensity measurement. Nevertheless, the quantification algorithm will depend on the specificities of the immunohistochemical protocols used and the aims of the study. Therefore, adjustments in the quantification procedures may be required, leading to procedures customization, to obtain valid segmentation profiles of the object of interest. Only through an adequate and criterious validation process it is possible to ensure that the methodology application is unbiased, credible and reliable[32]. Image processing can require optimization, adjustments, the use of filters, histograms, segmentation/thresholding and stack projections.

Another important aspect that should be considered is the time (duration) of the observation since this leads to fluorophores consumption which is critical when the parameter to be quantified is fluorescence intensity. Fluorescence intensity and bleaching of fluorophores can be influenced by several factors, namely mounting medium, power of excitatory light, etc. Other aspect that should be taken into consideration in LSCM is photobleaching since[33] the higher power and the focused beam induce enhanced fading of the specimen. Strategies to minimize these problems include changing experimental conditions related with fluorophores, such as increasing fluorophores concentration, use of antifade agents in mounting media or use of fluorophores resistant to bleaching.

The quality of the original data is crucial for measurement accuracy and depends on the resolution of the microscope and the amount of light detected. Digital images quality can be assessed using scatter graphs comparing the correlation of an image of a single fluorophore with a second image of the some fluorophore. Pearson's correlation coefficient can be used as a quantitative measure of quality to correct the measured colocalization of two flurophores. Depending of the type of study other correlation measurements may be needed, and are compiled in the JACoP plugin for Image J. Additionally, a proper preparation of images for coefficients calculations, and correct interpretation of obtained results will determine the sucess of a quantitative study.[34, 35]

Quantification algorithms can be increasingly complex according to the study aims, namely if the researcher wants to segment staining and threshold adjacent but not co-localized objects of interest (proteins, signaling molecules, receptors, etc). This latter aspect is directly related with the LSCM resolution (see above), but may also depend on the study features, i. e, if the researcher want to evaluate the colocalization profiles or molecules trafficking.

The complexities described above have impaired quantitative studies concerning receptors to be widespread. If we take images depicted in Figure 1A as an example, quantification procedures require an algorithm that includes instructions for the measurement of the surface area and intensity of sympathetic nerve terminals, receptors and receptors on the sympathetic nerve terminals. This algorithm allows to accurately segment and threshold adjacent but not co-localized proteins, tyrosine hydroxylase and adenosine A_1 receptors (Table 1) and was designed to run in PAQI platform (CEMUP, Porto, Portugal).

After applying this algorithm, the appropriate calculation coefficients were performed and results are (in pixels/μm^2): total sympathetic nerve fluorescence intensity ($5.6x10^5$), total adenosine A_1 receptors fluorescence intensity ($6.1x10^5$), adenosine A_1 receptors overlaid with sympathetic nerves ($4.1\ x10^5$). These data, clearly evidenced that from all receptors quantified 67% were present in sympathetic nerve terminals and 33% were found in non-neuronal cells, confirming qualitative data. Concerning disease states comparison between values obtained from normal *versus* pathological state can reveal the role of a specific receptor in the disease in study. Moreover, quantitative results concerning adenosine A_1 receptors located in non-neuronal cells may evidence a pathophysiological role attributable to these cells. Depending on the cell type involved (fibroblasts, immune or glial cells) more specific conclusions can be drawn.

Apart from the quantitative analysis of LSCM imaging other ongoing developing fluorescence methods allow to extract quantitative measurements, namely the use of fluorescent dyes that bind to effector molecules such as Ca^{2+} (Quin-2, Fura-2, Indo-1 and Fluo-3), NO (DAF-2; DAF-FM), ROS (DHE), Na^+ (SBFI), K^+ (PBFI), etc. An important advance achieved in the last decade was the engineering of fluorescent protein and its use as a tag since this approach allowed to better understand the function and location of several proteins/receptors improving intracelullar multidimensional analysis. Indeed, two families of fluorescent proteins are currently available, the green fluorescent protein (GFP), originating from the jelly fish *Aequorea victoria* and the reef coral fluorescent protein (RCFP), originating from reef corals.

Table 1. Description of the algorithm steps for the quantification of receptors, nerves and of the interception of receptors and nerves

Step 1 (analysis of sympathetic nerves) and step 2 (analysis of adenosine A_1 receptors)
1. Ensure that image is properly prepared, acquired, and processed. 2. Open Fluorescence images of sympathetic nerves (such as image from Figure 1A, TH) or of adenosine A_1 receptors (such as image from Figure 1A, A1) 3. Determining/Setting the threshold intensity reference background/noise (for all images) 4. Obtaining binary image that marks sympathetic nerves or adenosine A_1 receptors 5. Obtaining new image containing only the demarcated areas of sympathetic nerves or adenosine A_1 receptors and corrected intensity background 6. Sympathetic nerve or adenosine A_1 receptor binary images analysis and determination of the surface area of the sympathetic nerves or surface area of attachment of the adenosine A_1 receptors and the fluorescence intensity on the surface of the sympathetic nerves or adenosine A_1 receptors, corrected for background.
Step 2 (analysis of the receptors on the sympathetic nerves (overlay))
1. Measurement of the surface area and strength of adenosine A_1 receptors on the nerves 2. Results: Fluorescence images of adenosine A_1 receptors; dividing binary images of sympathetic nerves; dividing binary image adenosine A_1 receptor. 3. Obtaining image that marks the adenosine A_1 receptors on sympathetic nerves 4. Obtaining image containing only the demarcated areas of adenosine A_1 receptors on sympathetic nerves and corrected intensity background 5. The latter is analysed for determination of the surface area of attachment of the adenosine A_1 receptors on the sympathetic nerves and intensity of fluorescence of the adenosine A_1 receptors on sympathetic nerves, corrected for background

The use of fluorescence fusion proteins can be suitable, for instance, for studies where their expression is transient or expression levels change overtime (for details see [6]). These quantitative studies can be performed statically or in real time imaging. In this sense, there are plugins for Image J of upmost usefulness for this purpose. In colocalization studies when the two fluorophores colocalization may fluctuate in number or as function of time, for example, the Image J plugin "FRET and Colocalization Analyser" can be used. Moreover, many fluorescent agonists and antagonist are available for the study of receptors[36], but they need to conserve its efficacy and affinity in order to be useful for the study of the dynamic processes that include activation, translocation and cell function of receptors, so far difficult to address.

4. Conclusions

Until recently, most, if not all, pharmacological studies have been based on the development of agonist or antagonists and the targeting of the signalling cascades triggered by membrane receptor modulation. Nevertheless, several studies have raised the possibility that receptors might be located in cellular compartments other than the plasma membrane. This perspective can increase the pharmacological understanding of drugs and lead to the development of new therapeutic approaches, particularly if their physico-chemical properties enable them to cross the plasma membrane. Indeed, in the last 15 years several authors have investigated the ocurrence of receptors located inside the cell, both in cytoplasmatic organelles[37, 38] and in the nuclei,[39-41] helping in the understanding of the subsequent signalling events triggered by their activation.

Receptors should be viewed as dynamic proteins in constant traffic within the plasma membrane or among other organelle membranes where they can be stimulated by exogenous or endogenous ligands. Receptor activity is modulated by fine tunning and their steady state is balanced by a mechanism involving receptor internalization, through endocytosis. Then, receptors are routed to endosomes and they can be either recycled into its "active state" into membranes for repeated receptor activation [38] or be degradated inside lysosomes, which lead to the termination of receptor signalling mediated events.[37, 42-44]

On the other hand, there is increasing evidence of the presence and role of G protein coupled receptors such as endothelin-1, angiotensin II, bradykinin and neuropeptide Y receptors in nuclear membrane of many cell types.[40] Therefore, the subcellular location and physiological functions of receptors are closely related and justify the importance to precisely determine the distribution of different ligands and receptors

inside intracellular structures, their trafficking, dimerization or other types of receptor-protein interactions.

The combination of new techniques for fluorescent labeling and staining with advance fluorescence microscopy has offered new opportunities concerning the analysis of cellular dynamic behavior of cellular molecules, such as receptors. These methodologies renewed experimental approaches to study receptors location and/or dynamics, allowing intracellular visualization and quantification of signalling events triggered by receptor activation, through continuous determination of ions (Ca^{2+}, Na^{+}, K^{+}, etc) as well as their spatial and temporal concentration variations in single cells or in cell compartments/organelles. It is accepted that the use of dye-tagged ligands coupled to real 3D confocal microscopy is the best methodology for the research and development of new drugs and it can be performed *in vitro,* in different cell types, or *in vivo* using tissue sections of treated animals.[45, 46] A even more recent refinement of this technique consists in the cytosolic microinjection of dye-tagged ligands before real 3D confocal microscopy analysis.

In summary, available sensitive microscopy equipment, development of fluorescent antibodies, tags and probes including for agonists and antagonists, and powerful analytical computer-assisted methods is allowing increasing research advances concerning location, traffic and function of receptors in different experimental conditions including real-time scale. These advances can greatly help researchers to better understand intracellular events and receptor physiopathological roles impacting our knowledge of health and disease.

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MANUSCRIPT I

LACK OF ENDOGENOUS ADENOSINE TONUS ON SYMPATHETIC NEUROTRANSMISSION IN SPONTANEOUSLY HYPERTENSIVE RAT MESENTERIC ARTERY

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LACK OF ENDOGENOUS ADENOSINE TONUS ON SYMPATHETIC NEUROTRANSMISSION IN SPONTANEOUSLY HYPERTENSIVE RAT MESENTERIC ARTERY

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ABSTRACT

Background

Increased sympathetic activity has been implicated in hypertension. Adenosine has been shown to play a role in blood flow regulation. In the present study, the endogenous adenosine neuromodulatory role, in mesenteric arteries from normotensive and spontaneously hypertensive rats, was investigated.

Methods and Results

The role of endogenous adenosine in sympathetic neurotransmission was studied using electrically-evoked [^{3}H]-noradrenaline release experiments. Purine content was determined by HPLC with fluorescence detection. Localization of adenosine A_1 or A_{2A} receptors in adventitia of mesenteric arteries was investigated by Laser Scanning Confocal Microscopy.

Results indicate a higher electrically-evoked noradrenaline release from hypertensive mesenteric arteries. The tonic inhibitory modulation of noradrenaline release is mediated by adenosine A_1 receptors and is lacking in arteries from hypertensive animals, despite their purine levels being higher comparatively to those determined in normotensive ones. Tonic facilitatory adenosine A_{2A} receptor-mediated effects were absent arteries from both strains. Immunohistochemistry revealed an adenosine A_1 receptors redistribution from sympathetic fibers to Schwann cells, in adventitia of hypertensive mesenteric arteries which can explain, at least in part, the absence of effect of these receptors in hypertensive arteries.

Conclusion

Data highlight the role of purines in hypertension revealing that an increase in sympathetic activity in hypertensive arteries is occurring due to a higher noradrenaline/ATP release from sympathetic nerves and the loss of endogenous adenosine inhibitory tonus. The observed nerve-to-glial redistribution of inhibitory adenosine A_1 receptors in hypertensive arteries may explain the latter effect.

Keywords: mesenteric artery, adenosine, hypertension, noradrenaline

INTRODUCTION

Increased sympathetic activity has been implicated in the pathophysiology of hypertension since it drives to an enhancement of vasoconstriction.[1,2] Vascular sympathetic activity can be regulated by several endogenous substances, such as adenosine.

Extracellular adenosine can either be released as such, via nucleoside transporters, or produced from extracellular catabolism of released adenine nucleotides, namely ATP, from distinct cells including neurons. ATP is then sequentially dephosphorylated into ADP, AMP and adenosine. [3] Besides its action at the synapse, adenosine may function as a non-synaptic signalling molecule upon diffusion away from its local of origin influencing neurotransmission, inflammation and immune responses [4]. Adenosine effects occur through activation of four G-protein coupled receptors, adenosine A_1, A_{2A}, A_{2B} and A_3 receptors.[5]

In vessels, the involvement of adenosine receptors in sympathetic modulation has been described both in arteries[6-13] and in veins.[12] A reduced effect mediated by selective adenosine A_1, but not A_{2A} receptor agonists in sympathetic vascular neurotransmission in hypertensive state has been reported.[13] Nevertheless, the endogenous adenosine role in vascular sympathetic neurotransmission remains to be clarified, particularly whether the endogenous adenosine levels may have a pathophysiological impact in hypertensive states.

We postulate that the effects of endogenously generated adenosine are also impaired in hypertensive individuals leading to increased vascular sympathetic activity. The study was undertaken in mesenteric arteries from normotensive (Wistar-Kyoto, WKY) and spontaneously hypertensive rats (SHR), a well-establish model of hypertension [14,15], to determine whether endogenous adenosine has a role in the modulation of sympathetic activity and if this role is preserved in hypertensive individuals. Moreover, the regional distribution/localization and relative amount of adenosine receptors (A_1 and A_{2A} subtypes) in the two animal strains was also evaluated.

MATERIALS AND METHODS

Animals

Adult male WKY and SHR (12 weeks old, 270-350 g; Charles River, Barcelona, Spain) were used. Handling and care of animals were conducted according to the European guidelines (Directive 2010/63/EU) on the protection of animals used for scientific purposes

in agreement with the NIH guidelines. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Porto (Permit Number: 13/11/2013). Animals were sacrificed using guillotine. Two animals per experiment were used and from each mesenteric artery four segments (4-7 mg) were obtained. From each animal, no more than two tissue preparations were submitted to identical treatments.

Chemicals

The following drugs were used: levo-[ring-2,5,6-3H]-noradrenaline, specific activity 41.3 Ci/mmol, was from DuPont NEN (I.L.C., Lisboa, Portugal); desipramine hydrochloride, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine (SCH 58261), S-(4-Nitrobenzyl)-6-thioinosine (NBTI) and 5-Iodotubericidin (ITU), (8R)-3-(2-Deoxy-β-D-erythro-pentofuranosyl)-3,4,7,8-tetrahydroimidazo[4,5-d][1,3]diazepin-8-ol (pentostatin), α,β-methylene ADP, N6-cyclopentyladenosine (CPA), 2-p–(2-carboxyethyl)phenethylamino-5´-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680) were purchased from Sigma-Aldrich (Sintra, Portugal). The following antibodies were used: rabbit polyclonal anti-A1 (epitope corresponding to amino acids 287-326 mapping at the C-terminus of human adenosine A_1 receptors; sc-28995), anti-A_{2A} (epitope corresponding to amino acids 331-412 mapping at the C-terminus of human adenosine A_{2A} receptors; sc-13937) were purchased from Santa Cruz Biotechnology, Inc., CA, USA; mouse monoclonal anti-tyrosine hydroxilase antibody (TH(45): sc-136100, Santa Cruz Biotechnology, Inc., CA, USA and MAB318, Millipore Corporation, CA, USA); anti-glial fribillary acidic protein (GFAP) mouse monoclonal antibody (G6171, Sigma-Aldrich, Inc., USA) and rabbit GFAP polyclonal antibody (18-0063, Invitrogen, Life Technologies, SA, Madrid, Spain). The following fluorescent probes were used: Alexa Fluor® 488 goat anti-mouse IgG (H+L) antibody, highly cross-adsorbed and Alexa Fluor® 647 goat anti-rabbit IgG (H+L) antibody, highly cross-adsorbed (Molecular Probes®) secondary fluorescent antibodies (Invitrogen, Life Technologies, SA, Madrid, Spain); vectashield mounting medium with DAPI (Vector Laboratories, UK). Stock solutions were made up in dimethylsulphoxide (DMSO: 0.01% v/v, final concentration) or ultrapure water and diluted in superfusion medium immediately before use. DMSO was added to the superfusion medium (final concentration 0.01%), in parallel control experiments.

[^{3}H]-Noradrenaline release experiments

Evaluation of [^{3}H]-noradrenaline release experiments was carried out as previously described.[9-13] Arteries were pre-incubated in 2 ml Krebs-Henseleit solution containing 0.1 μmol/L [^{3}H]-noradrenaline (for 60 min at 37°C) and transferred into superfusion chambers, superfused with [^{3}H]-noradrenaline-free medium (1 ml/min; constant rate: Krebs-Henseleit solution with desipramine 400 nmol/L to inhibit noradrenaline's neuronal uptake). Two periods of electrical stimulation (5 Hz, 100 pulses, 1 ms, 50 mA; Hugo Sachs Elektronik, March-Hugstetten, Germany) were applied, S_1 and S_2, with 30 min intervals (t=90 min and t=120 min, respectively). The superfusate was collected each 5 min period from 85 min of superfusion onwards. At the end of the experiments (t=130 min), tritium was measured in superfusate samples and solubilized arteries (sonicated 1h with 2.5 ml perchloric acid (0.2 mol/L)) by liquid scintillation spectrometry (LS 6500, Beckman Instruments, Fullerton, USA) after adding 6 ml of a scintillation mixture (OptiPhase 'Hisafe' 3, PerkinElmer, I.L.C., Lisboa, Portugal) to each sample.

Tissue labelling with [^{3}H]-noradrenaline and evaluation of electrically-evoked tritium overflow changes was performed as previously described.[12,13] Effects of agonists (CPA, CGS 21680), of antagonists (DPCPX, SCH 58261), and of enzyme (pentostatin, ITU, α,β-methylene ADP) and nucleoside transport (NBTI) inhibitors were studied.

Laser scanning confocal microscopy (LSCM) experiments

Immunohistochemistry procedures were previously described.[13] Briefly, four tissue preparations were obtained from each artery and immediately placed in cold phosphate buffer solution (PBS; in g/L): NaCl 8.0, Na2HPO4.2H2O 0.77, KCl 0.20, KH2PO4 0.19 (pH 7.2). Each preparation was longitudinally opened and fixed (paraformaldehyde 4% PBS; 50 min; room temperature). After two 15 min PBS washing cycles, artery segments were incubated with primary antibodies raised against rabbit polyclonal individual adenosine receptor subtypes (anti-A_1 or anti-A_{2A}, 1:200 dilution, overnight, 4°C) and mouse monoclonal anti-tyrosine hydroxilase (TH, 1:10 dilution, overnight, 4°C) or mouse monoclonal anti-glial fribrilary acidic protein (GFAP, 1:200 dilution, overnight, 4°C). Thereafter, tissues were incubated with Alexa 647 anti-rabbit and Alexa 488 anti-mouse fluorescent secondary antibodies (1:1000 dilution, 1 h, room temperature). Negative controls were performed by omitting primary antibodies. After two PBS washing cycles, tissue preparations were mounted with antifading agent (vectashield mounting medium with DAPI, Vector Laboratories, UK), with the adventitial side facing up.

Preparations were visualized with Olympus FluoView FV1000 fluorescence confocal microscope system with a x60 oil immersion lens. Stacks of 1 μm thick serial optical images were captured from the entire adventitial layer, which was identified by the shape and orientation of the nuclei. Image acquisition was performed always under the same laser power, brightness and contrast conditions. Adventitia was scanned along each mesenteric artery and the resulting images were reconstructed separately for each wavelength.

ε-derivatives assay in artery superfusates

1,N^6-Etheno (ε)-modified purines have been previously used for purine quantification in tissue superfusates. [3,16-19] Briefly, mesenteric artery segments were superfused (Krebs-Henseleit, 1ml.min^{-1}) and electrically stimulated twice (S_1-S_2; 5 Hz, 100 pulses, 1 ms, 50 mA) 30-min apart (t=90 min and t=120 min). 5-min superfusates were collected and heated at 80ºC. From the collected samples, 910 μL were incubated with 90 μL of chloroacetaldehyde for 50 min at 70ºC in a dry bath incubator (Heraeus Instruments, Hanau, Germany). Reactions were stopped by placing samples on ice. Identification of the ε-derivatives (ε-ATP, ε-ADP, ε-AMP and ε-adenosine) formed in these collected samples was confirmed by HPLC using a fluorescent detector (model LS20; Perkin Elmer, Beconsfield, UK) at 230 nm excitation and 420 nm emission wavelengths. The stationary phase was 5 μm particle size packed in a 250 cm long by 4 mm internal diameter ODS- (C18) column and matching 1 cm long by 3.3 mm diameter direct-connect guard column (ACE-Advanced Chromatography technology, Aberdeen, Scotland) in a gradient HPLC system (306 and 811C Gilson, Gilson Medical Electronics, Middleton, WI, USA). The column was kept at room temperature (20-22ºC). The mobile phase consisted of a solution of 87 mmol/L KH_2PO_4 and 10.6 mmol/L Na_2HPO_4 (pH 6.0) as buffer A; buffer B was made up 25% methanol and 75% buffer A. Gradient elution was used according to the following linear program: from 0 to 20 min of elution, a convex gradient from 0 % to 100% of Buffer B at a flow rate of 1mL/min; from 0 to 3 min of elution, an increase from 0% to 25% of buffer B at a flow rate of 1mL/min; from 3 to 7 min of elution, 25% of buffer B at a flow rate of 1mL/min; and from 8 to 12 min of elution an increase from 25% to 100% of buffer B at a flow rate of 1mL/min; from 12 to 20 min of elution, 100% of buffer B at a flow rate of 1mL/min. The run time was 20 min and the post-run time 5 min.

Data Analysis

Measurement of drug effects on electrically-evoked tritium overflow.

Electrically-evoked tritium overflow from artery segments incubated with [^{3}H]-noradrenaline has been shown to reflect action potential-evoked neuronal noradrenaline release and drug-induced changes in evoked tritium overflow are assumed to reflect changes in neuronal noradrenaline release. Effects of drugs added after S_1 on electrically-evoked tritium overflow were evaluated as ratios of the overflow elicited by S_2 and the overflow elicited by S_1 (S_2/S_1). S_2/S_1 ratios obtained in individual experiments in which a test compound A was added after S_1 were calculated as a percentage of the respective mean ratio in the appropriate control group (solvent instead of A). When the interaction of A, added after S_1, and a drug B added 5 min before S_2, was studied, the "appropriate control" was a group in which A alone was used.

Laser Scanning Confocal Microscopy images quantification

Quantitative analysis of confocal z-stacks images was performed using image analysis software (PAQI, CEMUP, Porto, Portugal). Briefly, a sequential routine was designed and developed to analyse each fluorescent signal used. PAQI software measured the surface area and strength of the fluorescence signal marking the postganglionic nerves, the surface area and strength of the fluorescence signal marking the receptors and determined the surface area of attachment of the receptors on the nerves as well as the intensity of fluorescence of the receptors on nerves (corrected for background).

Quantification of the ε-derivatives formed in collected artery superfusates

The amount of adenine nucleotides and adenosine (pmol/mg of tissue) in each collected sample was estimated from calibration curves of purine standards (derivatized as described above for the samples), run with every set of 20 samples.

Statistics

Results are expressed as mean±s.e.m. and *n* denotes the number of tissue preparations. Differences of means were compared for significance using one- or two-way ANOVA, followed by *post-hoc* Holm-Sidak's multicomparison *t* test. A P value lower than 0.05 was considered to denote statistically significant differences.

RESULTS

Noradrenaline release from mesenteric sympathetic nerve terminals

Stimulation (100 pulses/5 Hz) significantly increased tritium outflow from mesenteric artery of both WKY and SHR. In the absence of drugs other than desipramine (400 nmol/L), the average basal overflow (b_1, fractional release), immediately before S_1, was similar in WKY (0.070±0.008; n=15) and SHR (0.071±0.003; n=11) mesenteric arteries, whereas electrically-evoked tritium overflow (S_1, ratio of the total tritium content of the tissue) was more pronounced in SHR mesenteric arteries (0.358±0.05; n=11; $p<0.05$) comparatively to those from WKY (0.229±0.037; n=15). In control conditions, basal outflow and electrically-evoked tritium overflow remained constant throughout experiments, with b_n/b_1 and S_n/S_1 average values close to unity (data not shown).

Role of endogenous adenosine in vascular sympathetic neurotransmission

Endogenous adenosine-mediated effects in mesenteric artery sympathetic neurotransmission were evaluated by blocking the high affinity adenosine receptor subtypes (A1 and A2A) with the selective antagonists, DPCPX and SCH 58261, respectively. In the presence of DPCPX (100 nmol/L), a facilitation of electrically-evoked tritium overflow was observed (Figure 1). This finding is compatible with the occurrence of a tonic inhibition mediated by endogenous adenosine via adenosine A1 receptor activation. However, this effect was only observed in WKY mesenteric arteries. SCH 58261 (20 nmol/L) did not modify tritium overflow in mesenteric arteries from both strains, discarding the occurrence of a putative tonic adenosine A2A receptor-mediated facilitation. Upon reducing endogenous adenosine levels by preventing AMP (formed from dephosphorylation of ATP released after nerve stimulation) conversion into adenosine, through inhibition of ecto-5'-nucleotidase (with α,β-methylene ADP, 10 μmol/L), we observed a facilitation of similar magnitude of that detected in the absence of tonic adenosine A1 receptor inhibition (revealed by DPCPX treatment). Likewise, this effect was only observed in WKY mesenteric arteries.

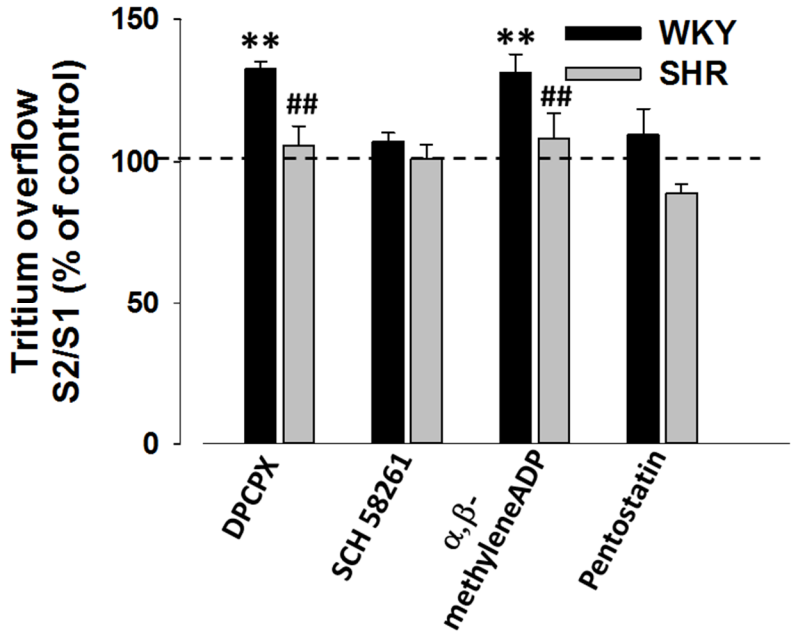

Figure 1 - Influence of endogenous adenosine on tritium overflow modulation from WKY and SHR MA: interaction with DPCPX, SCH 58261, α,β-methylene ADP or pentostatin. Tissues were electrically stimulated (S_1-S_2: 100 pulses, 5 Hz). Drugs were added after S_1 and kept until the end of the experiment. Values are mean±s.e.m. from 4-12 segments. Significant differences from the appropriate control: *P<0.05; from WKY: ## P<0.001.

Pentostatin (10 μmol/L), an adenosine deaminase inhibitor, did not alter the electrically-evoked tritium overflow (Figure 2). However, NBTI (5 μmol/L), a bidirectional equilibrative nucleoside transporter inhibitor and ITU (100 nmol/L), an adenosine kinase inhibitor were able to increase tritium overflow, but only in WKY mesenteric arteries (Figure 2). Moreover, the facilitatory effects observed in the presence of ITU or NBTI were completely antagonised by SCH 58261 (Figure 2). In contrast, electrically-evoked tritium overflow from SHR mesenteric arteries was unaffected by pharmacological manipulation of endogenous adenosine levels (Figures 1 and 2).

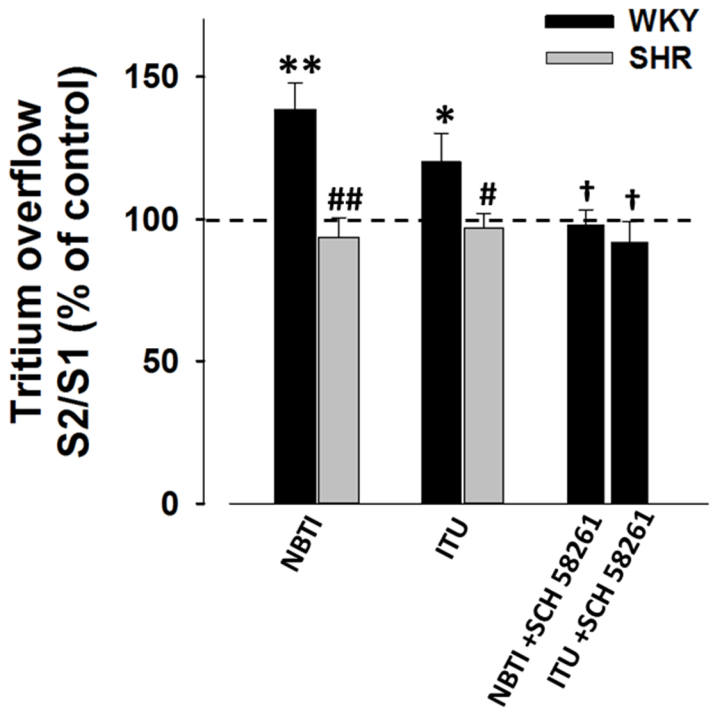

Figure 2 - Effects of NBTI or ITU in the absence or presence of SCH 58261 on the electrically-evoked tritium overflow from WKY and SHR MA. Tissues were electrically stimulated (S_1-S_2: 100 pulses, 5 Hz). Drugs were added after S_1 and kept until the end of the experiment. Values are mean±s.e.m. from 4-12 segments. Significant differences from the appropriate control: * $P<0.05$, * $P<0.05$; from WKY: ## $P<0.001$; from the inhibitor alone: † $P<0.05$.

ATP and adenosine levels in WKY and SHR mesenteric arteries

Noradrenaline/ATP co-transmission has been demonstrated to occur in mesenteric artery.[20,21] Endogenous levels of ATP and adenosine were significantly higher in superfusates from SHR mesenteric arteries, comparatively to those of WKY (Figure 3), both under basal conditions and after electrical stimulation (5 Hz/100 pulses). Only minute amounts of ADP and AMP were found (data not shown). Moreover, stimulation increased ATP released amounts comparatively to the amounts observed in basal conditions, but only in SHR tissues. Surprisingly, adenosine levels, before and after stimulation, were similar in arteries from both strains, although still higher than those of ATP.

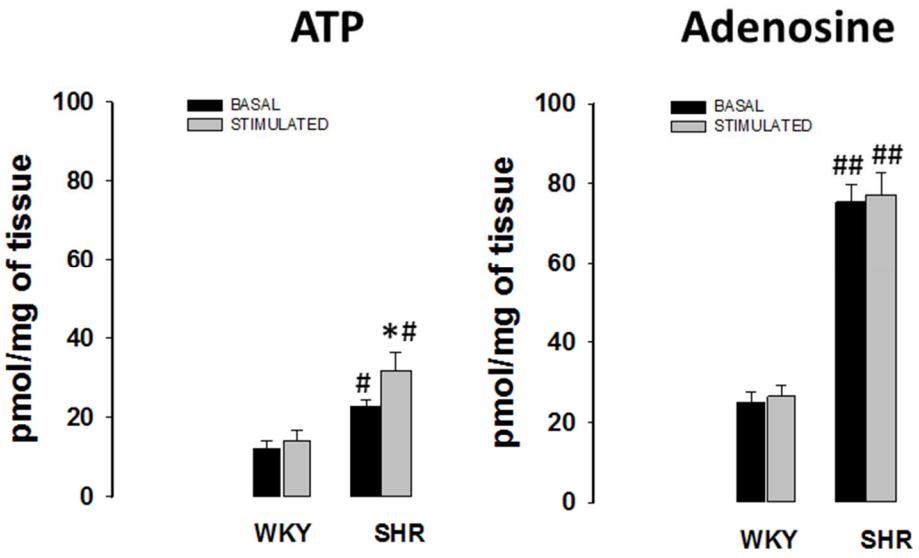

Figure 3 - Basal and electrically-evoked ATP (A) and adenosine (B) release in MA of WKY and SHR. Tissues were electrically stimulated (S_1-S_2: 100 pulses, 5 Hz). Significant differences from basal conditions: *P<0.05; from WKY: # P<0.05; ##P<0.001.

Effects of A_1 and A_{2A} ARs agonists *on vascular sympathetic neurotransmission*

CPA (a selective adenosine A1 receptor agonist, 100 nmol/L) inhibited electrically-evoked tritium overflow in mesenteric arteries from both strains. This inhibition was more pronounced in WKY (63.28±3.9%; n=6; $p<0.05$) than in SHR (74.70±2.86%; n=14; $p<0.05$). Conversely, the selective adenosine A2A receptor agonist, CGS 21680 (100 nmol/L), facilitated tritium overflow to similar extent: 123.64±5.18% (n=4; $p<0.05$) and 121.62±7.73% (n=4; $p<0.05$) in WKY and SHR mesenteric arteries, respectively. Effects elicited by adenosine receptor agonists were supressed by pre-incubation with the corresponding selective adenosine receptor antagonists, DPCPX (100 nM) and SCH 58261 (20 nM). These results demonstrate that mesenteric arteries from WKY and SHR exhibit functional adenosine A1 and A2A receptors, which can be selectively activated by stable adenosine analogues. These results suggest that activation of adenosine A2A receptos is largely preserved in hypertensive rats but the adenosine A1 receptor-mediated inhibition is somehow compromised in these animals.

Localization A_1 and A_{2A} ARs in vascular sympathetic neurons in the adventitia of WKY and SHR mesenteric arteries

In WKY and SHR mesenteric arteries, adenosine A1 receptor immunoreactivity showed considerable overlay, but did not co-localize, with the sympathetic neuronal marker (TH; Figure 4A), indicating that they might be localized on the same cellular structure (postganglionic sympathetic nerves). This agrees with the fact that adenosine A1 receptors are membrane receptors while tyrosine hydroxylase (TH) is localized inside sympathetic neurotransmitter storage vesicles.[22]

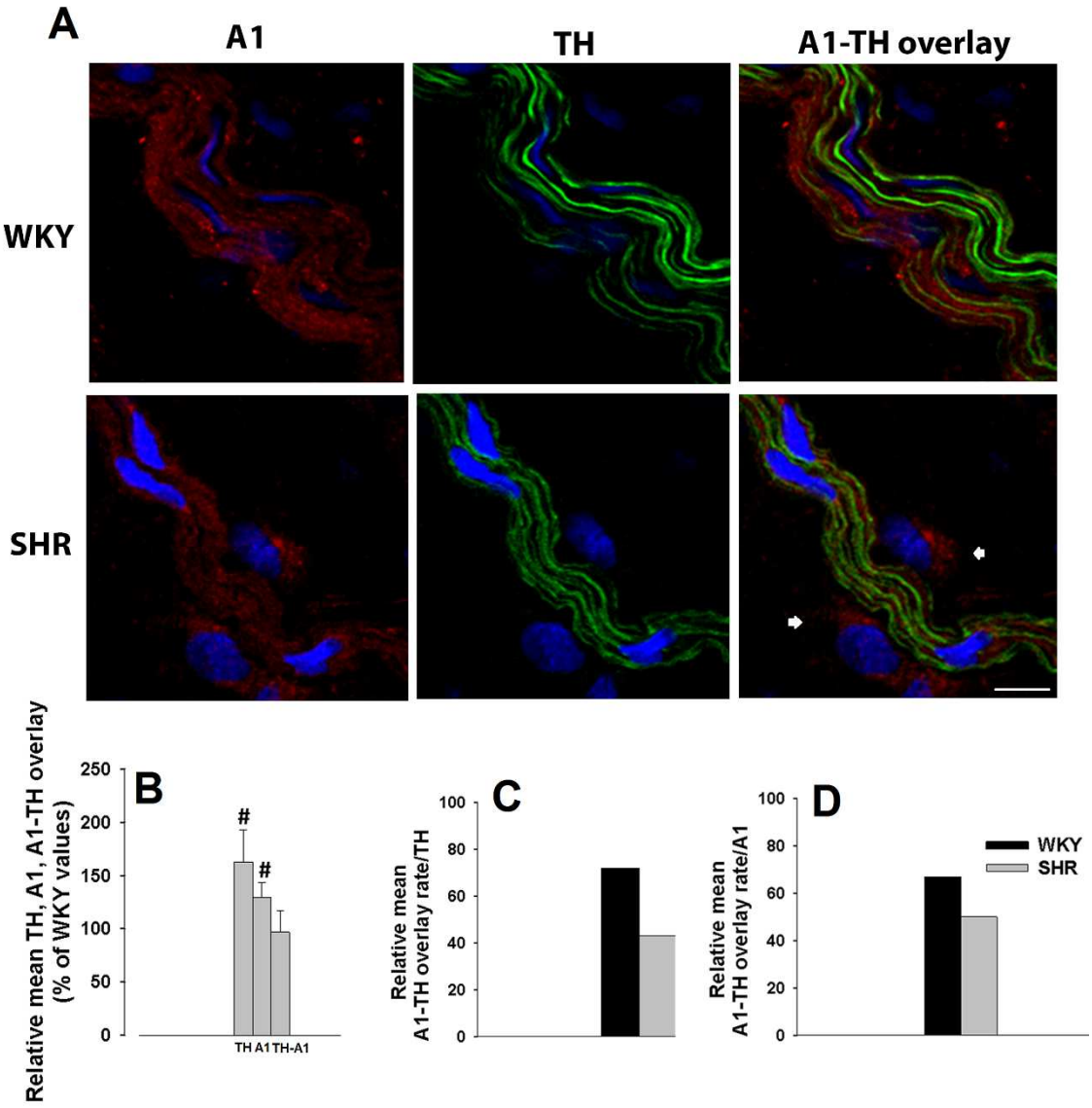

Figure 4 - LSCM representative images of WKY and SHR MA exhibiting (A) A_1ARs (red), TH (green) and overlay of A_1-TH immunoreactivities, nuclei (blue); (B) Relative means of TH, A_1 and TH-A_1 overlay expressed as percentage of WKY values. (C) Mean percentage of overlay rate with TH (D) and mean percentage of overlay rate with A_1 are depicted. Arrows highlight non-neuronal cells. Images are reconstructions from 9-28 serial optical sections analyzed using PAQI software. Values are mean±s.e.m.; 3-4 animals. Significant differences from WKY: $^{\#}$ $P<0.05$, $^{\#\#}$ $P<0.001$. Scale bar = 20 μm.

Adenosine A1 receptor immunoreactivity in non-neuronal cells was also observed, particularly in mesenteric arteries from SHR. TH and A1 immunoreactivities in SHR exceeds those observed in WKY arteries (Figure 4B). However, overlaid TH and A1 immunoreactivities were roughly similar in arteries from both strains (Figure 4B). Figure 4C shows that adenosine A1 receptor and TH overlaid immunoreactivities are markedly reduced in SHR comparatively to WKY tissues, when data was normalized by total TH immunoreactivity: 70% of sympathetic neurons exhibit adenosine A1 receptor immunoreactivity in WKY versus only 40% observed in SHR. Adenosine A1 receptor immunoreactivity was also observed in cells other than sympathetic neurons: 33% in WKY and 50% in SHR mesenteric arteries (Figure 4A and 4D).

Similar analysis of adenosine A2A receptor and tyrosine hydroxylase (TH) was performed in WKY and SHR mesenteric arteries (Figure 5). TH immunoreactivity in SHR exceeds that observed in WKY (Figure 5B), while A2A-TH overlaid immunoreactivities are lower in SHR arteries. Figure 5C shows that A2A-TH, normalized by total amount TH immunoreactivity, is significantly reduced in SHR (38%) comparatively to WKY tissues, where almost all sympathetic neurons (99% of TH-positive immunoreactivity) also exhibit adenosine A2A receptor immunoreactivity. Moreover, the percentage of cells exhibiting adenosine A2A receptor immunoreactivity other than sympathetic neurons was similar in WKY (77%) and SHR arteries (73%) (Figure 5D).

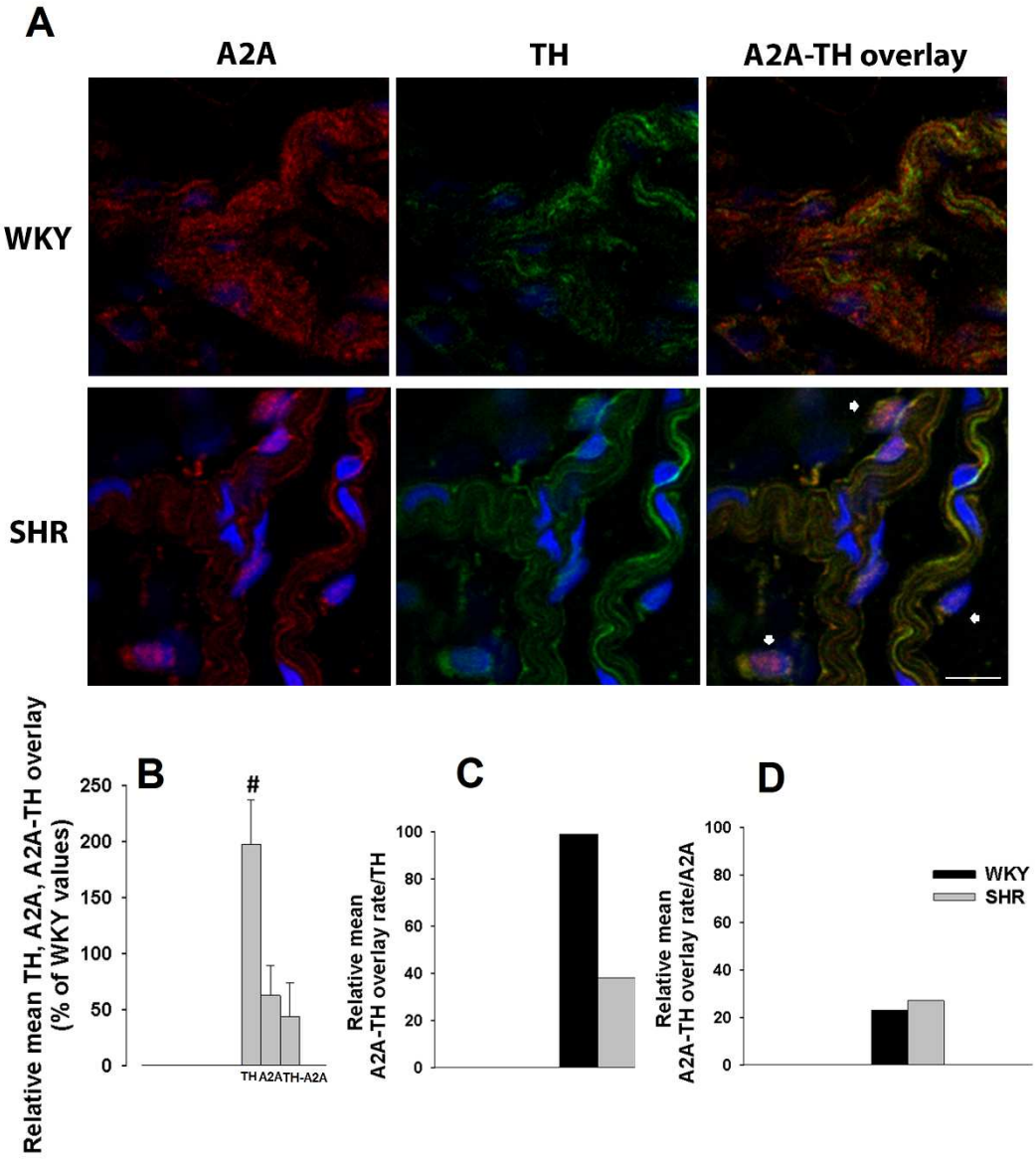

Figure 5 - LSCM representative images of WKY and SHR MA exhibiting (A) A_{2A}ARs (red), TH (green) and overlay of A_{2A}-TH immunoreactivities, nuclei (blue); (B) Relative means of TH, A_{2A} and TH-A_{2A} overlay expressed as percentage of WKY values. (C) Mean percentage of overlay rate with TH (D) and mean percentage of overlay rate with A_{2A} are depicted. Arrows highlight non-neuronal cells. Images are reconstructions from 11-20 serial optical sections analyzed using PAQI software. Values are mean±s.e.m.; 3-4 animals. Significant differences from WKY: $^{\#}P<0.05$, $^{\#\#}P<0.001$. Scale bar = 20 μm.

Localization of A_1 and A_{2A}ARs in Schwann cells of the adventitia of WKY and SHR mesenteric arteries

In the adventitia of mesenteric artery several cell types can co-exist.[23,24] Since we have observed adenosine A1 (Figure 4D) and A2A receptor (Figure 5D) immunoreactivities outside sympathetic neurons and since these neurons are wrapped with Schwann cells, we hypothesized that the non-neuronal cells exhibiting adenosine A1 or A2A receptor immunoreactivities were Schwann cells. GFAP (peripheral glial cell marker)[25] immunoreactivity overlaid with adenosine A1 (Figure 6A) or A2A (Figure 6B) receptor immunoreactivities in the adventitia of both strains. Therefore, in addition to sympathetic nerves, Schwann cells also present adenosine A1 and A2A receptors.

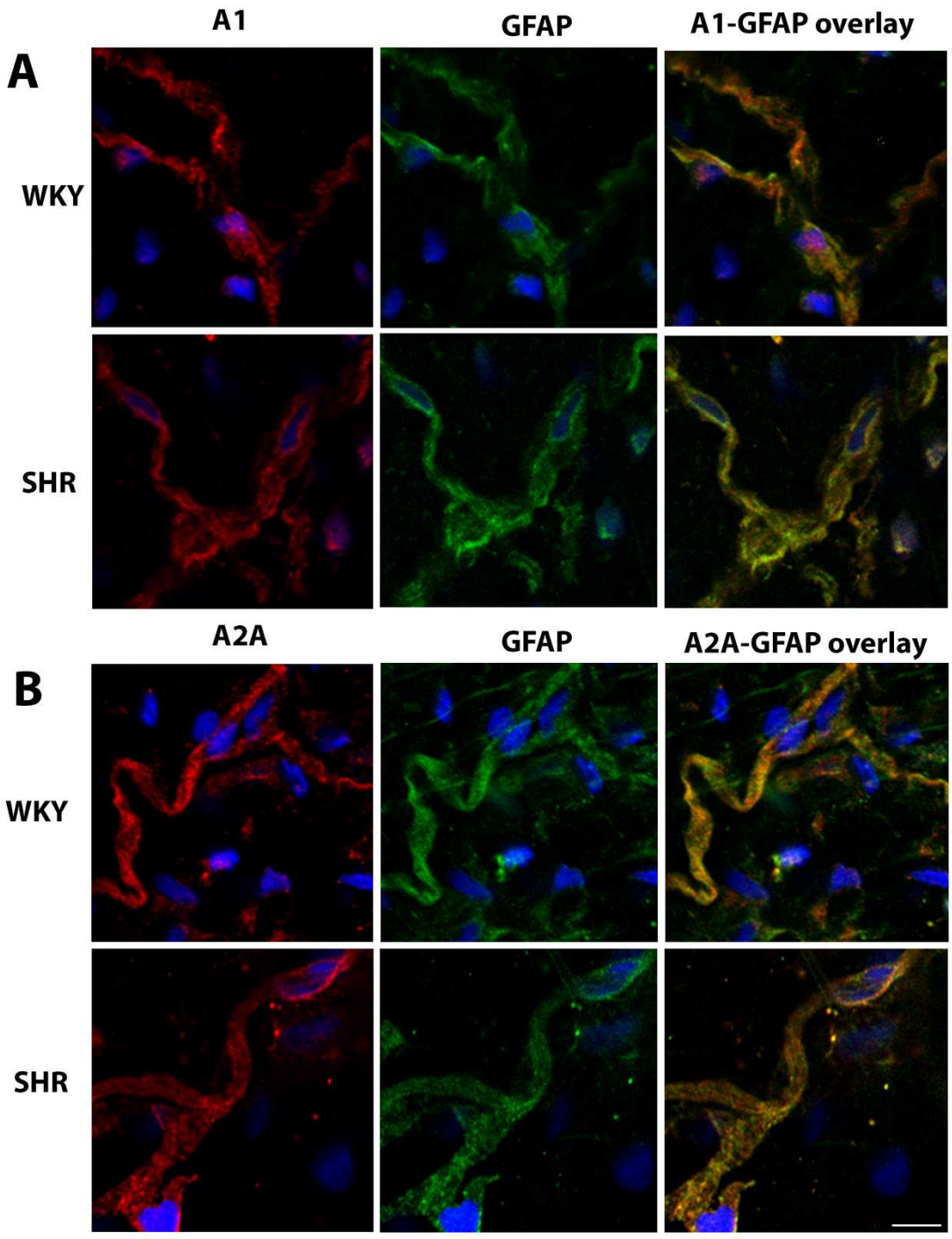

Figure 6. LSCM images of A_1ARs (panel A) and A_{2A}ARs (panel B) immunoreactivities in GFAP-immunoreactive (Schwann) cells located in adventitia of WKY and SHR MA; 3-4 animals. Scale bar = 20 μm.

DISCUSSION

There is a gap in the knowledge regarding the complex interplay between receptor expression and the role of the endogenous neuromodulator, adenosine, on vascular sympathetic neurons. This study shows that endogenous adenosine contributes to down-modulate noradrenaline release from sympathetic neurons through activation of adenosine A1 receptors in normotensive mesenteric arteries, but this effect is completely lost in hypertensive arteries justifying the observed increase of noradrenaline release.

In normotensive mesenteric arteries removal of the inhibitory tone of adenosine either by inhibiting ecto-5'-nucleotidase or by selectively blocking adenosine A1 receptors caused a facilitation of noradrenaline release from stimulated sympathetic neurons up to 32%. Therefore, it seems that, under physiological conditions, endogenous adenosine contributes to restrain transmitter release from stimulated mesenteric arteries via activation of inhibitory adenosine A1 receptors, as previously demonstrated.[26] Data also indicate that the adenosine A1 receptor inhibitory tonus is mediated predominantly by adenosine originated from metabolism of released adenine nucleotides. These results along with the observation that the amount of adenosine in superfusates was unaffected upon stimulation were rather surprising. Nevertheless, our findings gain physiological significance if one considers that inhibitory adenosine A1 receptors operate to restrain noradrenaline release under basal conditions, as a consequence of adenosine accumulation from hydrolysis of ATP released from neighbouring cells. This scenario may change towards putative activation of P2 receptors, along with adenosine receptors, in hypertensive arteries[2] a possible scenario if one takes into consideration the increased ATP levels found in stimulated SHR superfusates.

Transmitters release modulation ascribed to extracellular adenosine accumulation depends both on its formation and on cellular uptake and deamination.[27-29] We showed that adenosine deaminase inhibition failed to modify noradrenaline release, causing minor changes in extracellular adenosine accumulation. Interestingly, NBTI was able to increase noradrenaline release, suggesting that adenosine uptake may be the dominant adenosine inactivation pathway in mesenteric arteries. NBTI enhancement of noradrenaline release was antagonised by SCH 58261, indicating that this effect is mediated by adenosine A2A receptors, activated by endogenous adenosine (Figure 2). These apparent contradictory results, showing that SCH 58261 was unable to modify transmitter release from stimulated sympathetic neurons (Figure 1), may be explained due to differences in Kd ascribed to inhibitory adenosine A1 and facilitatory A2A receptors, the latter requiring concentrations two-fold higher than those required to activate adenosine A1 receptors.[30] Nucleoside transporter inhibition seems to increase extracellular adenosine to levels high enough to

activate adenosine A2A receptors, while in its absence the amount of adenosine may be insufficient to activate these receptors. Therefore, in conditions that favour extracellular adenosine accumulation, this nucleoside may reach concentrations higher enough to activate adenosine A2A receptors, as described in other tissues.[27-29]

To our knowledge, this is the first study demonstrating that the endogenous adenosine neuromodulatory role of sympathetic transmission is significantly impaired in mesenteric arteries from hypertensive rats. In SHR mesenteric arteries we failed to detect both adenosine A1 and A2A receptor mediated effects (after NBTI-induced extracellular adenosine accumulation). This is occurring despite the extracellular levels of both ATP and adenosine were significantly higher in hypertensive mesenteric arteries than those measured in normotensive artery superfusates. These differences on adenosine neuromodulation may be explained by changes in adenosine A1 and/or A2A receptors: i) activity/desensitization, ii) downregulation or iii) redistribution into other organelle or cells. In this regard, we showed, using enzymatically stable and selective adenosine receptor agonists, that neuromodulatory activity of adenosine A1 but not of A2A receptors is significantly impaired in hypertensive mesenteric arteries.

Laser scanning confocal microscopy data confirmed previous reports of a sympathetic hyperinnervation in hypertensive mesenteric arteries.[31-34] Interestingly, increases in the number and thickness of sympathetic nerve fibres observed in SHR mesenteric arteries was not accompanied by a correspondent enhancement of adenosine A1 and/or A2A receptors overlaying these neurons: a decrease of both adenosine A1 (from 70% to 40%) and A2A receptors (from 99% to 40%) in sympathetic neurons in hypertensive versus normotensive mesenteric arteries was observed. These lower amounts can explain, at least in part, the lack of adenosine inhibitory (and facilitatory) tone regulating noradrenaline release from stimulated SHR mesenteric arteries, leading to higher extracellular noradrenaline levels.

Data also show, for the first time, that 33% of adenosine A1 receptors and 77% of adenosine A2A receptors, in WKY mesenteric artery adventitia, are present in other cells than sympathetic neurons (TH-immunoreactive cells). While the amount of adenosine A1 receptors localized in these cells (Schwann cells) increased to roughly 50% in SHR arteries, we observed no changes in the amount of adenosine A2A receptors in arteries from both strains. This finding might explain the small (~20%) relative increase in the amount of adenosine A1 receptors in hypertensive mesenteric arteries, suggesting a neuron-to-glia redistribution of adenosine A1 receptors in hypertensive arteries. To our knowledge, this is also the first time where distribution/redistribution of adenosine A1 or A2A receptors between neurons and Schwann cells in hypertensive arteries has been reported. Changes in the localization of adenosine receptors and the increased amount of extracellular adenosine

observed in SHR mesenteric arteries suggest that receptor desensitization may be the main reason for adenosine receptor activity impairment observed in hypertensive arteries. Indeed, the lack of tonic adenosine A1 receptor-mediated inhibition in these arteries may contribute to increase noradrenaline release from stimulated sympathetic nerves. This provides a convincing explanation for the dominance of noradrenaline as neurotransmitter in hypertensive rats.[35] Accordingly, decreases in adenosine A1 receptor-mediated inhibitory tonus may cause a profound impact in vascular reactivity, contributing to hypertension. Moreover, the amount of ATP released from sympathetic nerves was higher in SHR than in WKY mesenteric arteries which correlates well with a previous work where an enhanced purinergic function was described, revealing ATP as a vasoconstrictor in SHR arteries.[2]

CONCLUSIONS

Results from this work highlight the role of purines in hypertension. Data showed that the increase in sympathetic activity in hypertensive arteries may be partially due to an higher ATP release from sympathetic postganglionic nerves and the lack of endogenous adenosine inhibitory tonus. The latter might be explained by the nerve-to-glial redistribution of inhibitory adenosine A1 receptors found to occur in hypertensive arteries. These mechanisms would lead to an increase in noradrenaline and ATP release from stimulated sympathetic nerves. Taken this into account one can predict that, in the synaptic cleft, in addition to higher levels of noradrenaline, increased amounts of ATP are likely to accumulate with subsequent vasoconstriction of vascular smooth muscle cells by α1 adrenoceptors and P2 receptors activation, respectively. Such changes may have impact in vascular reactivity, contributing to hypertension, renewing the interest of the purinergic system as a target for novel therapeutic strategies.

Conflict(s) of Interest/Disclosure(s)

All authors declare no conflict of interest related to the present work.

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MANUSCRIPT II

ENDOTHELIAL DYSFUNCTION IMPAIRS VASCULAR NEUROTRANSMISSION IN TAIL ARTERIES

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ENDOTHELIAL DYSFUNCTION IMPAIRS VASCULAR NEUROTRANSMISSION IN TAIL ARTERIES

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ABSTRACT

Objectives

The present study intends to clarify if endothelium dysfunction impairs vascular sympathetic neurotransmission.

Methods

Electrically-evoked tritium overflow (100 pulses/5 Hz) was evaluated in arteries (intact and denuded) or exhibiting some degree of endothelium dysfunction (spontaneously hypertensive arteries), pre-incubated with [^{3}H]-noradrenaline in the presence of enzymes (nitric oxide synthase (NOS); nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; xanthine oxidase; cyclooxygenase; adenosine kinase) inhibitors and a nucleoside transporter inhibitor.

Results

Inhibition of endothelial nitric oxide synthase with L-NIO dihydrochloride reduced tritium overflow in intact arteries whereas inhibition of neuronal nitric oxide synthase with Nω-Propyl-L-arginine hydrochloride was devoid of effect showing that only endothelial nitric oxide synthase is involved in vascular sympathetic neuromodulation. Inhibition of enzymes involved in reactive oxygen species or prostaglandins production with apocynin and allopurinol or indomethacin, respectively, failed to alter tritium overflow. A facilitation or reduction of tritium overflow was observed in the presence of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) or of 5-iodotubericidin, respectively, but only in intact arteries. These effects can be ascribed to a tonic inhibitory effect mediated by A_1 receptors. In denuded and hypertensive arteries, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine (SCH 58261) reduced tritium overflow, suggesting the occurrence of a tonic activation of A_{2A} receptors. When endogenous adenosine bioavailability was increased by the nucleoside transporter inhibitor, S-(4-Nitrobenzyl)-6-thioinosine, tritium overflow increased in intact, denuded and hypertensive arteries.

Conclusions

Among the endothelium-derived substances studied that could alter vascular sympathetic transmission only adenosine/adenosine receptor mediated mechanisms were clearly impaired by endothelium injury/dysfunction.

Keywords: endothelium dysfunction, nitric oxide, adenosine, noradrenaline, SHR.

INTRODUCTION

It is well established that blood vessel tone can be modulated by the release of various endothelium-derived endogenous substances[1] namely contracting (endothelin, prostaglandin F2a and thromboxane A2) and/or relaxing (prostaglandin I2 and nitric oxide, NO) factors.[2, 3] These substances can modify vascular smooth muscle tone directly, acting on smooth muscle cells, or indirectly, by altering sympathetic transmission.[4] Such regulation can be impaired if integrity and/or function of endothelium is compromised. Increasing evidence suggest that endothelial dysfunction, with altered NO production and oxidative stress, may contribute to the pathogenesis of hypertension driving to increased peripheral vascular resistance, as endothelium removal and endothelium dysfunction resulted in the enhancement of contractile responses to vasoconstrictor agents.[5-9] Moreover, several studies revealed that endothelial cells may be dynamically involved in the vascular response to receptor stimulation,[5] namely α- or β-adrenoceptors[10] or adenosine receptors.[11-14]

In addition, some studies reported that endothelium may alter sympathetic transmission, known to be implicated in cardiovascular diseases. This is true for central noradrenergic neurons associated with cardiovascular control[15] and also for the peripheral sympathetic innervation of vessels.[16] However, to our knowledge, the role of endothelium dysfunction in sympathetic transmission has never been studied, although some studies had shown that endothelium might influence the levels of noradrenaline released from sympathetic terminals.[11]

The present investigation was designed to address the hypothesis that endothelium dysfunction alters vascular sympathetic neurotransmission and contributes to increase vascular tone and, consequently, blood pressure. To achieve this goal, we have investigated, in intact and denuded arteries, the modulatory roles exerted by i) endogenous NO, generated by endothelial NOS (eNOS) and neuronal NOS (nNOS); ii) oxidative stress, through evaluation of enzymes involved in reactive oxygen species production such as NADPH oxidase and xanthine oxidase; iii) prostaglandins production, through cyclooxygenase activity and iv) endogenous adenosine *via* activation of adenosine A_1 or A_{2A} receptor subtypes, in the sympathetic vascular neurotransmission. We have also evaluated if these pathways are compromised in tail arteries from spontaneously hypertensive rats, an animal model exhibiting endothelium dysfunction.[17, 18]

MATERIALS AND METHODS

Chemicals

The following drugs were used: levo-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 41.3 Ci/mmol, was from DuPont NEN (I.L.C., Lisboa, Portugal); desipramine hydrochloride, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), Indomethacin (cyclooxygenase (COX) inhibitor), Allopurinol, 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine (SCH 58261), S-(4-Nitrobenzyl)-6-thioinosine (NBTI), 5-Iodotubericidin (ITU) and saponin were purchased from Sigma-Aldrich (Sintra, Portugal); Nω-Propyl-L-arginine hydrochloride, L-NIO dihydrochloride and Apocynin (NADPH oxidase inhibitor) were purchased from TOCRIS Bioscience.

All other reagents were of analytical grade. Stock solutions were made up in dimethylsulphoxide (DMSO: 0.01% v/v, final concentration) or ultrapure water and diluted in superfusion medium immediately before use. DMSO was added to the superfusion medium (final concentration 0.01%), in parallel control experiments.

Animals

Adult male Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) (12 weeks old, 270-350 g) purchased from Charles River (Barcelona, Spain) were used. Animals were kept under light/dark cycles of 12/12 h, 20-22ºC, and had free access to water and pellet food. Handling and care of animals were conducted according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes; (in agreement with the NIH guidelines) and granted by the Faculty of Pharmacy of University of Porto Ethics Committee. In accordance with good practice, in studies involving the response of the sympathetic nervous system, the use of anesthetics is inadvisable once it may compromise the validity and reproducibility of results. Thus, the animals are sacrificed using a guillotine, the method being reported as the sacrificial advisable in studies involving the nervous system. The tissue samples were collected immediately after sacrifice. Two animals per experiment were used and tail arteries were dissected. Seven tissue preparations (5 to 9 mg) were obtained from each artery.

Experimental Protocol

[^{3}H]-Noradrenaline release experiments

The protocol used to label tissue preparations with [^{3}H]-noradrenaline and to evaluate changes on electrically-evoked tritium overflow was performed according to previous studies.[19-23] Briefly, tail arteries were dissected and, for experiments with denuded preparations, endothelium was mechanically removed with a thin stainless steel wire. In a set of experiments endothelium was chemically removed using saponin (0.1 mg/ml). After dissection, arteries were immediately placed in cold Krebs-Henseleit solution of the following composition (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03 (pH 7.4).

Arteries were then incubated in 2 ml Krebs-Henseleit solution containing 0.1 □M [^{3}H]-noradrenaline (for 60 min at 37ºC) and transferred into superfusion chambers, superfused with [^{3}H]-noradrenaline-free medium (1ml.min^{-1}; constant rate: Krebs-Henseleit solution with desipramine 400 nM to inhibit noradrenaline's neuronal uptake). Up to three periods of electrical stimulation (5 Hz, 100 pulses, 1 ms, 50 mA; Hugo Sachs Elektronik, March-Hugstetten, Germany) were applied: the first stimulation period, t = 45 min (S_0), served to test the viability of the preparation and medium was not collected during this period; the subsequent two stimulation periods (S_1 and S_2) were applied with 30 min intervals (t=90 min and t=120 min, respectively). The superfusate was collected in 5 min periods from 85 min of superfusion onwards (t=0 min being the onset of superfusion). At the end of the experiments (t=130 min), tritium was measured in superfusate samples and solubilized arteries (sonicated 1h with 2.5 ml perchloric acid (0.2 M)) by liquid scintillation spectrometry (LS 6500, Beckman Instruments, Fullerton, USA) after adding 6 ml of scintillation mixture (OptiPhase 'Hisafe' 3, PerkinElmer, I.L.C., Lisboa, Portugal) to each sample.

From each animal, no more than three tissue preparations were submitted to identical treatments. Effects of antagonists (DPCPX and SCH 58261), and of several enzyme inhibitors (L-NAME, Nω-Propyl-L-arginine hydrochloride and L-NIO dihydrochloride, ITU, Apocynin, Indomethacin and Allopurinol) on NA release (estimated as tritium overflow) were studied.

ε-adenosine assay in artery superfusates

1,N^6-Etheno (ε)-modified purines (adenine nucleotides and adenosine) have been previously prepared with collected samples from tissue superfusates by several authors.[24-26] Briefly, tail artery segments were transferred into superfusion chambers, superfused at a constant rate of 1ml.min^{-1} with Krebs-Henseleit solution. Up to three periods of electrical stimulation (5 Hz, 100 pulses, 1 ms, 50 mA; Hugo Sachs Elektronik, March-Hugstetten, Germany) were applied: the first stimulation period, t = 45 min (S_o), served to test the viability of the preparation and the medium was not collected during this period; the subsequent two stimulation periods (S_1 and S_2) were applied with 30 min intervals (t=90 min and t=120 min, respectively). The superfusate was collected in 5 min periods before and after stimulation and each sample was then heated at 80°C (to denature enzymes that might be co-released). The collected samples (910 μl) were then incubated with chloroacetaldyde (90 μl) for 50 min at 70°C in a dry bath incubator (Heraeus Instruments, Hanau, Germany). Reactions were stopped by placing samples on ice. Identification of the ε-adenosine formed in this collected samples was confirmed by HPLC using a fluorescent detector (model LS20; Perkin Elmer, Beconsfield, UK) at 230 nm excitation and 420 nm emission wavelengths. The stationary phase was 5 μm particle size packed in a 25 cm long by 4 mm internal diameter ODS- (C18) column and matching 1 cm long by 3.3 mm diameter direct-connect guard column (ACE-Advanced Chromatography technology, Aberdeen, Scotland) in a gradient HPLC system (306 and 811C Gilson, Gilson Medical Electronics, Middleton, WI, USA). The column was kept at room temperature (20-22°C). The mobile phase consisted of a solution of 87 mM KH2PO4 and 10.6 mM Na2HPO4 (pH 6.0) as buffer A; buffer B was made up 25% methanol and 75% buffer A. Gradient elution was used according to the following linear program: from 0 to 20 min of elution, a convex gradient from 0 % to 100% of Buffer B at a flow rate of 1ml/min; from 0 to 3 min of elution, an increase from 0% to 25% of buffer B at a flow rate of 1ml/min; from 3 to 7 min of elution, 25% of buffer B at a flow rate of 1ml/min; and from 8 to 12 min of elution an increase from 25% to 100% of buffer B at a flow rate of 1ml/min; from 12 to 20 min of elution, 100% of buffer B at a flow rate of 1ml/min. The run time of 20 min and the post-run time was 5 min.

Data analysis

[^{3}H]-Noradrenaline release experiments

Evaluation of [^{3}H]-noradrenaline release experiments were carried out as previously described. Briefly, effects of drugs added after S_1 on electrically-evoked tritium overflow were evaluated as ratios of the overflow elicited by S_2 and the overflow elicited by S_1 (S_2/S_1). S_2/S_1 ratios obtained in individual experiments in which a test compound A was added after S_1 were calculated as a percentage of the appropriate control group (solvent instead of A).

Quantification of the ε-adenosine formed in collected artery superfusates

The amount of adenosine (pmol/mg of tissue) in each collected sample was estimated from calibration curves of adenosine standards, derivatized as described, run with every set of 20 samples.

Statistics

Results are expressed as mean±s.e.m. and *n* denotes the number of tissue preparations. Differences of means were compared for significance using one- or two-way ANOVA followed by *post-hoc* Holm-Sidak's multicomparisons *t* test. A P value less than 0.05 was considered to denote statistically significant differences.

RESULTS

Noradrenaline release from tail sympathetic nerve terminals

Electrically-evoked tritium overflow from tissue preparations incubated with [^{3}H]-noradrenaline has been shown to reflect action potential-evoked neuronal noradrenaline release and drug-induced changes in evoked tritium overflow can be assumed to reflect changes in neuronal noradrenaline release, as observed in previous studies.[19-23]

The outflow of tritium was markedly increased in tail arteries, pre-incubated with [^{3}H]-noradrenaline, when stimulated with 100 pulses/5 Hz (*Figure 1*).

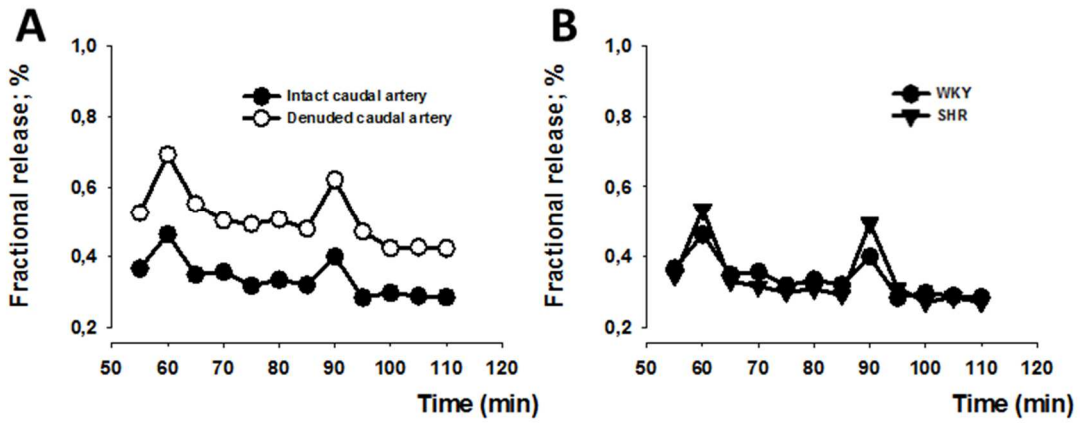

Figure 1. Time course of tritium outflow from: (A) intact (filled circles) and denuded (open circles); (B) SHR (triangles) and WKY (circles) tail artery segments from typical experiments. After pre-incubation with [^{3}H]-noradrenaline, tissues were superfused with [^{3}H]-noradrenaline free medium containing desipramine (400 nM). Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period and was measured in samples collected every 5 min. Artery segments were stimulated twice by 100 pulses/5 Hz, (S_1, S_2). Each line represents the outflow of tritium from a single superfusion chamber.

The fractional rate of efflux immediately before S_1 (b_1) and electrically-evoked tritium overflow (S_1), in the absence of drugs other than desipramine (400 nM), are shown in Table 1.

In the present study, endothelium integrity was compromised by mechanical removal or chemical injury (that induces degenerative changes in the endothelial cells).[27]

Nevertheless, b_1 and S_1 values obtained using the two different procedures were similar; therefore further experiments were carried out with mechanical removal of endothelium. Endothelium (mechanical or chemically compromised) did not modify the basal outflow (b_1) but increased the electrically-evoked tritium overflow (S_1) (Table 1). In tail arteries from SHR, the basal outflow (b_1) was smaller than those observed in WKY arteries and S_1 values were intermediate between those of intact and denuded arteries (Table 1; *Figure 1*). In control conditions, basal outflow and electrically-evoked tritium overflow remained constant throughout experiments, with b_n/b_1 and S_n/S_1 average values close to unity (data not shown).

Table 1. Basal tritium outflow (b_1), electrically-evoked tritium overflow (S_1) and S_2/S_1ratios from WKY and SHR segments of tail artery

	Basal Outflow (b_1) (fractional rate of outflow; min^{-1})	**Evoked Overflow (S_1)** (% of tissue tritium content)	***n***
WKY endothelium			
Intact	0.095±0.004	0.185±0.014	20
Mechanically removed	0.077±0.003*	0.278±0.013*	16
Chemically removed	0.071±0.013*	0.264±0.025*	5
SHR endothelium			
Intact	0.060±0.004*	0.250±0.016*	15

Tail artery segments were pre-incubated with [^{3}H]-noradrenaline for 40 min. After pre-incubation with [^{3}H]-noradrenaline, arteries were superfused with [^{3}H]-noradrenaline free medium containing desipramine (400 nM). Arteries were stimulated twice at 30-min intervals (S_1-S_2; 100 pulses, 5 Hz, 1 ms, 50 mA): b_1 refers to the 5-min period immediately before S_1. The electrically-evoked tritium overflow was calculated by subtracting the estimated basal outflow from total outflow observed during and in the 25-min period subsequent to S_1 and expressed as a percentage of the tissue tritium content at the onset of stimulation. Values presented are means±SEM and *n* denotes the number of artery segments. Significant differences from WKY intact endothelium: *P<0.05 (one-way ANOVA followed by *post-hoc* Holm-Sidak's multicomparisons *t*-test)

Influence of endothelium upon vascular sympathetic neurotransmission

Sympathetic transmission can be altered by substances produced in endothelial cells, such as NO,[28, 29] reactive and/or nitrogen oxygen species (ROS and/or RNS), prostaglandins.[30, 31]

NO was documented as having influence on vascular reactivity namely by altering contractions elicited by electrical field stimulation.[32] To evaluate the role of NO in vascular sympathetic neurotransmission, a set of experiments was carried out using NOS inhibitors: L-NAME, a non-specific NOS inhibitor (100 μM), Nω-Propyl-L-arginine hydrochloride, a specific neuronal NOS (nNOS) inhibitor (100 nM) and L-NIO dihydrochloride a specific endothelial NOS (eNOS) inhibitor (500 nM). Results shown in *Figure 2* revealed that, in intact tail arteries, both L-NAME and L-NIO dihydrochloride were

unable to inhibit tritium release in 27 and 35%, respectively, while Nω-Propyl-L-arginine hydrochloride was ineffective to modify it. In denuded arteries all tested compounds failed to change tritium overflow. Taken together, these data suggests that nNOS is not contributing to alter sympathetic transmission in tail arteries. In SHR arteries, all NOS inhibitors used were devoid of effect.

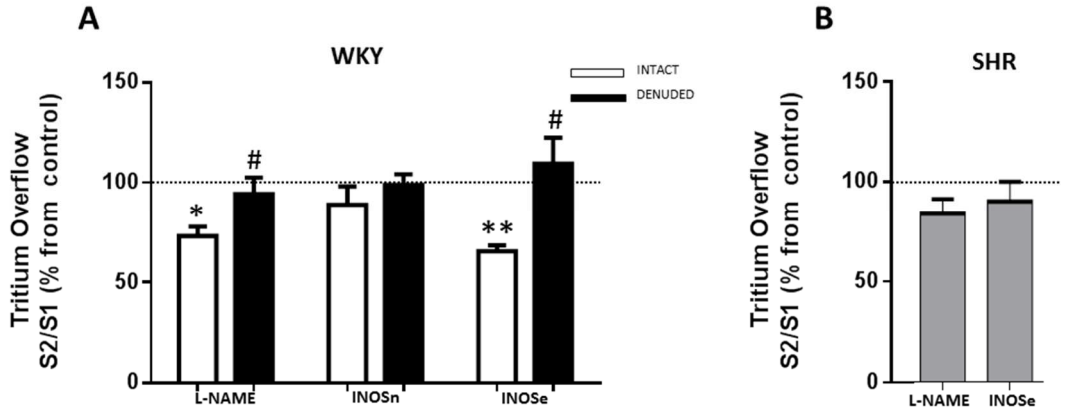

Figure 2. Influence of NOS inhibitors on vascular sympathetic transmission in: (A) WKY intact and denuded and (B) SHR tail arteries. Interaction with L-NAME, a non-selective NOS inhibitor (100 μM), Nω-Propyl-L-arginine hydrochloride, a specific neuronal NOS (nNOS) inhibitor (INOSn: 100 nM) and L-NIO dihydrochloride, a specific endothelial NOS (eNOS) inhibitor (INOSe: 500 nM), on the electrically-evoked tritium overflow. Tissues were electrically stimulated (S_1-S_2: 100 pulses/5 Hz). Drugs were added immediately after S_1 and kept until the end of the experiment. *Ordinates:* S_2/S_1 values obtained in individual artery segments, expressed as a percentage of the appropriate S_2/S_1 control value. Values are mean±s.e.m. from 4 to 15 artery segments (5 WKY and 3 SHR). Significant differences from the appropriate control: *P<0.05, **P<0.001; from denuded arteries: #P<0.05 (two-way ANOVA followed by *post-hoc* Holm-Sidak's multicomparisons *t*-test)

An increase in NO availability has been linked with an increase in intracellular calcium levels.[33] In intact arteries, the calcium ionophore A23187 (100 nM), described to promote a massive increase in intracellular calcium levels, failed to change noradrenaline release (1.10±0.13; n=4; P>0.05).

Oxidative stress may generate ROS that can alter NO-mediated effects, by altering NO bioavailability.[34] Therefore, we tested the effects, on the electrically-evoked tritium overflow, elicited by inhibitors of enzymes involved in the formation of ROS. Vascular NADPH oxidase, an important source of vascular radical formation,[35] was inhibited using

apocynin (10 μM) and xanthine oxidase, an enzyme with particularly high expression in vascular endothelium, known as a source of ROS,[36] was inhibited using allopurinol (10 μM). These inhibitors were unable to modify tritium release in intact, denuded and SHR tail arteries (*Figure 3*).

In the endothelium, prostaglandins are generated after cyclooxygenase (type I and II) activation, and were described to influence sympathetic transmission[37] through the activation of different pathways, some of them involving NO-mediated effects.[38] To challenge this possibility, indomethacin, a non-selective cyclooxygenase inhibitor, was used. Indomethacin failed to change tritium overflow in intact, denuded and SHR tail arteries (*Figure 3*).

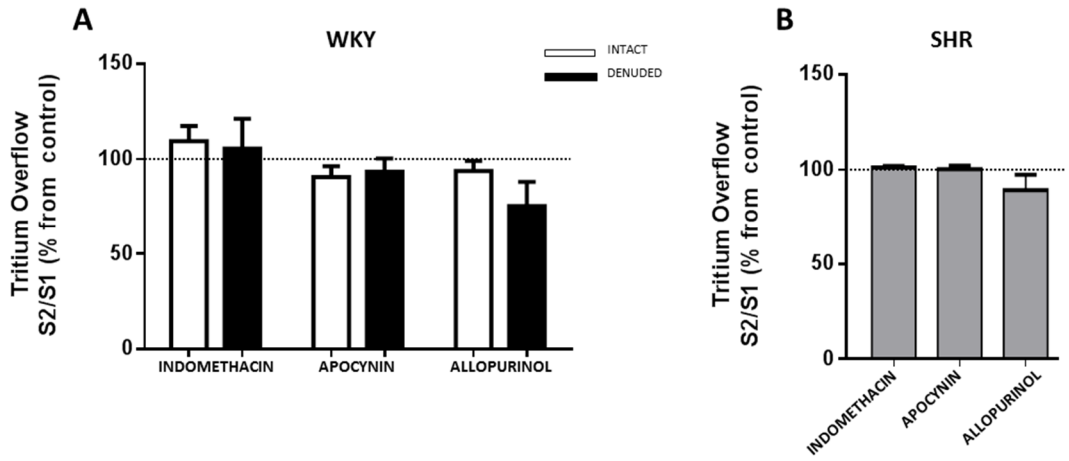

Figure 3. Prostaglandins and ROS involvement on vascular sympathetic transmission in: (A) WKY intact and denuded and (B) SHR tail arteries. Effects of indomethacin (10 μM, non-selective COX inhibitor), apocynin (10 μM, NADPH oxidase inhibitor) and allopurinol (10 μM, xanthine oxidase inhibitor) on the electrically-evoked tritium overflow. Tissues were electrically stimulated (S_1-S_2: 100 pulses/5 Hz). Drugs were added immediately after S_1 and kept until the end of the experiment. *Ordinates:* S_2/S_1 values obtained in individual artery segments, expressed as a percentage of the appropriate S_2/S_1 control value. Values are mean±s.e.m. from 5 to 14 artery segments (5 WKY and 3 SHR). Non-Significant differences from the appropriate control were found (two-way or one-way ANOVA).

Role of endogenous adenosine upon vascular sympathetic transmission

An ubiquitous mediator described to be involved in neurotransmission both in central and peripheral nervous system is adenosine.[39, 40] To evaluate if endogenous adenosine is modulating vascular noradrenaline release pathways, selective antagonists for A_1 and A_{2A} receptors, DPCPX (100 nM) and SCH 58261 (20 nM) were used (*Figure 4*). In the presence of DPCPX, a facilitation (up to 20%) occurred, compatible with a tonic inhibitory effect mediated by A_1 receptors observed only in intact arteries. By opposition, SCH 58261 revealed an inhibition that is, most likely, due to a tonic effect mediated by A_{2A} receptors but only in denuded (up to -35%) and hypertensive (up to 20%) arteries (with endothelium integrity compromised). To evaluate the impact of endogenous adenosine increase on neurotransmission dynamics, ITU (100 nM), an adenosine kinase inhibitor that prevents the phosphorylation of adenosine into AMP, was used. An inhibition of tritium release was observed in intact tissues while in endothelium free tissues (denuded arteries); a facilitation of tritium release was observed (*Figure 4*). These effects correlate well with data obtained in the presence of DPCPX and SCH in intact and denuded arteries, respectively (*Figure 4*).

These findings suggest that adenosine availability may be higher in denuded arteries comparatively to intact ones. In endothelium dysfunctional arteries (SHR arteries) however, noradrenaline release was not altered by ITU treatment, probably because there is already a constitutively higher amount of adenosine in hypertensive/endothelium dysfunctional arteries. Since transmitters release modulation ascribed to extracellular adenosine availability depends not only on its formation but also on its cellular uptake (by nucleoside transporters), experiments were carried out in the presence of NBTI (a nucleoside transporter inhibitor, 5 μM) that facilitated tritium overflow in all artery preparations (intact, denuded and SHR). However, facilitation was more pronounced in intact arteries. This difference can be related to the facilitation induced by NO, as shown in *Figure 2*, and confirmed by experiments where intact arteries were treated with NBTI plus L-NAME that showed increased levels of noradrenaline release (149.8±12.8; n=5) similar to those observed in denuded arteries (150.3±4.7; n=5).

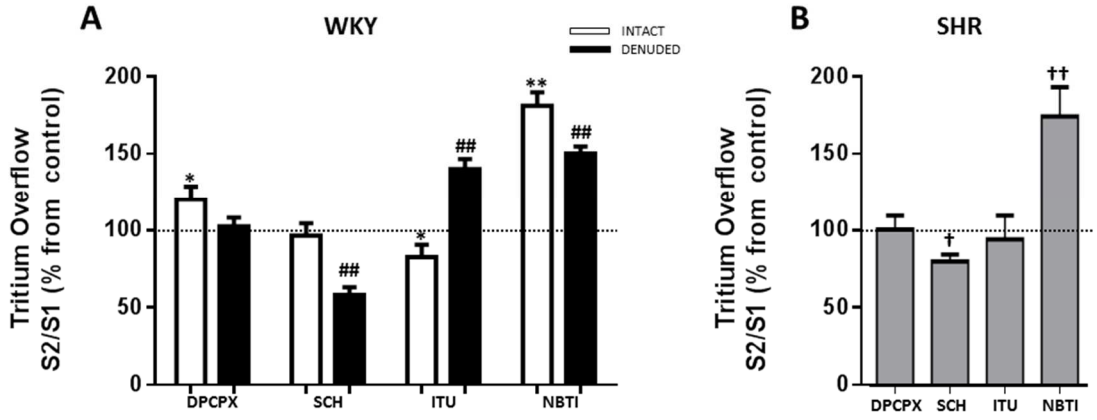

Figure 4. Influence of endogenous adenosine on vascular sympathetic transmission in: (A) WKY intact and denuded and (B) SHR tail arteries. Interaction with selective adenosine receptor antagonists DPCPX (100 nM; A_1 subtype antagonist), SCH 58261 (20 nM; A_{2A} subtype antagonist) and ITU (100 nM adenosine kinase inhibitor) and/or NBTI (5 μM; a nucleoside transporter inhibitor) on the electrically-evoked tritium overflow. Tissues were electrically stimulated (S_1-S_2: 100 pulses/5 Hz). Drugs were added immediately after S_1 and kept until the end of the experiment. *Ordinates:* S_2/S_1 values obtained in individual artery segments, expressed as a percentage of the appropriate S_2/S_1 control value. Values are mean±s.e.m. from 5 to 15 artery segments (7 WKY and 4 SHR). Significant differences from the appropriate control: *P<0.05, **P<0.001; from denuded arteries: ##P<0.001; from WKY arteries: †P<0.05; ††P<0.001 (two-way ANOVA followed by *post-hoc* Holm-Sidak's multicomparisons *t*-test).

Adenosine levels in WKY and SHR tail arteries

Extracellular adenosine can either be released as such, via nucleoside transporters, or produced from extracellular catabolism of released adenine nucleotides, namely ATP, from distinct cells including neurons.[41] ATP is then sequentially dephosphorylated into ADP, AMP and adenosine. Endogenous levels of adenosine were significantly higher in superfusates from SHR tail arteries, comparatively to those of WKY (*Figure 5*), both under basal conditions and after stimulation (5 Hz/100 pulses). Surprisingly, adenosine levels, before and after stimulation, were similar in both strains.

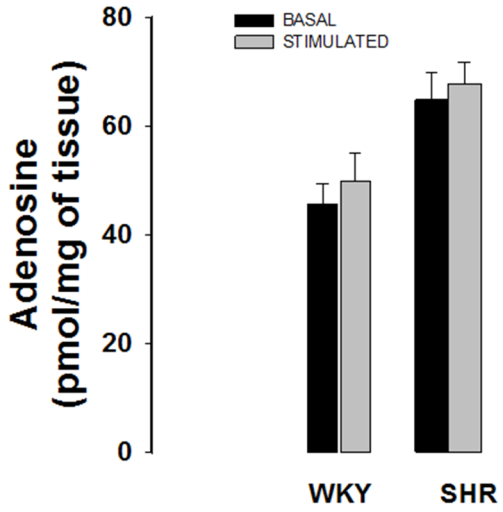

Figure 5. Basal and electrically-evoked adenosine release from tail arteries of WKY and SHR. Tissues were electrically stimulated (S_1-S_2: 100 pulses/5 Hz). Values are mean±s.e.m. from 8-10 artery segments (3 WKY and 3 SHR). Significant differences from WKY arteries: # $P<0.05$ (one-way ANOVA followed by *post-hoc* Holm-Sidak's multicomparisons *t*-test).

DISCUSSION

In the present study endothelium was removed (mechanical or chemically) from tail arteries to mimic endothelium injury/dysfunction, and this strategy was found to induce an increase in noradrenaline release from vascular sympathetic nerves. This observation is in line with previous reports that described endothelium dependent effects in vascular responsiveness, including sympathetic transmission.[2, 10-14] Nevertheless, data revealed, for the first time, that this increase of noradrenaline release is still occurring in vessels exhibiting endothelial dysfunction (SHR tail arteries). Surprisingly, endothelium-derived substances such as ROS and prostaglandins, did not alter the neuromodulatory mechanisms, whereas adenosine-mediated effects in vascular neurotransmission were clearly impaired by endothelium injury/dysfunction.

The increase in sympathetic transmission (elicited by endothelial dysfunction) observed can not be explained by an increase in oxidative stress, since NADPH oxidase and/or xanthine oxidase inhibitors did not alter noradrenaline release levels. Therefore, ROS can be discarded as i) a putative effector in this imbalance, contrary to ROS-mediated effects in other vascular physiological functions[42] or as ii) being involved in NO inactivation in arteries which would decrease NO bioavailability.[43-45] NO bioavailability

is also a consequence of its production status and might influence NO-mediated effects, including those related to sympathetic transmission. Indeed, NO production was found to increase noradrenaline release but only in intact arteries which can be explained by the interplay of NO with the adrenergic system pathway, namely with facilitatory β-adrenoceptors, as previously described.[46, 47] In intact arteries, the non-selective NOS (L-NAME) and eNOS inhibitors, inhibited noradrenaline release whereas nNOS inhibitor did not alter noradrenaline release. In denuded and SHR arteries, however, all NOS inhibitors failed to alter sympathetic transmission and, as such, NO cannot be considered the key element involved in altering sympathetic transmission when integrity of endothelium is compromised. It has been proposed, in previous studies, that NO did not alter sympathetic neurotransmission in tail arteries,[48] which is in line with the absence of nNOS activity observed in the present study. However, our data clearly showed an eNOS-mediated effect in sympathetic transmission, which represents, to our knowledge, a new role for eNOS in vessels.

It has been previously described that adenosine A_1 and A_{2A} receptor activation in endothelial cells lead to an increase in intracellular Ca^{2+}, a middle step pathway in the cascade of events that, consequently, triggers eNOS.[33] As we have observed that adenosine and NO are most likely operating independently to influence noradrenaline release and since alteration in intracellular calcium levels was devoid of effect, the interplay between signaling cascades involving these signaling mediators in the modulation of sympathetic neurotransmission can be discarded. Moreover, indomethacin was also devoid of effects reinforcing the idea that the signaling pathway triggered by endothelial A_1 receptors activation that would lead to activation of K^+_{ATP} channels-PLA2-COX-PGI2-PKA-NOS pathway[33] is not involved in the modulation of noradrenaline release in tail arteries. These observations indicate, therefore, that the high affinity adenosine receptor subtypes, A_1 and A_{2A}, are most likely located in presynaptic terminals and not in endothelial cells. In this regard, adenosine A_1 and A_{2A} receptors are distinctly involved in neurotransmission modulation when endothelium integrity is altered: in denuded endogenous adenosine is tonically activating A_{2A} receptors (causing a marked increase in sympathetic activity comparatively to the activity observed in intact arteries), an effect that is still observed, with similar magnitude, in SHR arteries. An additional contribution to the increased sympathetic activity observed in these tissues can be ascribed to the absence of inhibitory A_1 receptor-mediated effects (in both denuded and SHR arteries). These data strongly contrast with results obtained in intact arteries where modulation of noradrenaline release by endogenous adenosine involves inhibitory but not facilitatory adenosine receptors (see *Figure 4*). Since adenosine A_{2A} receptors require concentrations two-fold higher than those needed to activate adenosine A_1 receptors[49] endogenous adenosine availability seems to

be crucial to discriminate the subtype of adenosine receptor that is activated. Therefore, the sympathetic activity impairment observed in arteries exhibiting some degree of endothelium dysfunction can be ascribed to alterations in the dynamics of the adenosinergic system. Adenosine receptors, nucleoside transporters and adenosine availability are known to be the key effectors in adenosinergic system dynamics and any change in one of these items may cause profound impact in the function of the others.[50]

When adenosine bioavailability increased by inhibiting adenosine kinase (by ITU) a dual effect was observed, depending on artery endothelium integrity state: in intact arteries an inhibition of noradrenaline release was observed while in arteries where endothelium was removed a facilitation of noradrenaline occurred (*Figure 4*). In SHR arteries, ITU did not alter noradrenaline release, which it is conceivable to happen if there is already a constitutively higher amount of adenosine in hypertensive/endothelium dysfunctional arteries (*Figure 5*). Therefore, although ITU could increase even more adenosine levels, this increase was unable to potentiate further A_{2A} activation (that is likely already in its maximum activity state). Since these effects are due to a tonic activation of adenosine receptors subtypes (A_1 and A_{2A}), a possible nucleoside transport dynamic change can be behind the increased availability of adenosine observed in SHR arteries.

Blockade of nucleoside transport (by NBTI) caused facilitation of noradrenaline release in intact, denuded and SHR arteries due to the increase in extracellular adenosine levels, able to activate facilitatory A_{2A} receptors.

An additional source of adenosine could be attributable to electrically-evoked ATP release from sympathetic neurons however the amount of adenosine observed before and after stimulation was similar, in SHR arteries, challenging this possibility.

Taken together, the facilitatory effects observed in the presence of ITU and NBTI in denuded arteries suggest that the main mechanism that regulates adenosine extracellular levels involves nucleoside uptake to endothelial cells. Thus, when endothelium is somehow compromised this transport seems to be deregulated causing accumulation of extracellular adenosine.

In conclusion, the present study revealed, for the first time, that endothelium dysfunction causes an increase in sympathetic neurotransmission constituting, an additional aggravating factor in pathologies secondary to hypertension or hypertension itself. Surprisingly, the involvement of endothelium-derived substances (ROS and prostaglandins) in mechanisms leading to altered neuromodulation was ruled out, whereas adenosine-mediated neuromodulatory mechanisms were clearly impaired by endothelium injury/dysfunction.

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MANUSCRIPT III

ENDOTHELIAL NITRIC OXIDE MODULATORY ROLE ON VASCULAR SYMPATHETIC REACTIVITY

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ENDOTHELIAL NITRIC OXIDE MODULATORY ROLE ON VASCULAR SYMPATHETIC REACTIVITY

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ABSTRACT

Conflicting data suggests that NO may influence noradrenaline release from vascular postganglionic sympathetic nerves. Some studies indicate that NO promote an inhibition while others indicate the involvement of NO in the facilitation of noradrenaline release. We aim at investigating the role of NO in vascular sympathetic neurotransmission in the mesenteric and tail arteries. The influence of NO on noradrenaline release was found to be distinct between mesenteric and tail arteries with a pronounced enhancement of noradrenaline release caused by NO mediated activation in intact tail arteries: DEA NONOate (10 μM), a NO donor, facilitated tritium overflow up to 18,80±0.10% or by the inhibition (up to -25,79±0,045% obtained in the presence of L-NAME (100 μM), a NO synthase inhibitor. In denuded arteries L-Name (100 μM) failed to modify noradrenaline release. DEA NONOate (10 μM) and SNP (100 μM), two different NO donors caused inhibition of noradrenaline release in denuded arteries, -18,92±0,15% and -24,42±0,061%, respectively. Data indicate that the signalling pathway activated by exogenous NO, ultimately causes a reduction in noradrenaline release in denuded tail arteries, in opposition to the effects observed in intact tail arteries. Endogenous NO, produced most probably in the endothelium, in turn, mediate an enhancement of noradrenaline release. These indicate that NO from different sources can induce opposite effects probably through activation/influence of distinct pathways.

Keywords: Endothelium, nitric oxide, vascular sympathetic nerves, noradrenaline.

INTRODUCTION

Endothelium is an important regulator of vascular tonus via release of various endothelium-derived substances[1] such as nitric oxide (NO).[2] Several studies have reported that endothelium may decrease noradrenaline release from vascular postganglionic sympathetic nerves [rabbit and canine vessels: 3, 4, 5] and neurogenic vasoconstriction [rabbit carotid artery: 6]. However, there is no consensus in the literature regarding the modulatory role of NO on vascular sympathetic reactivity: it has been described that an inhibitor of NO production did not modify noradrenaline release [dog temporal artery: 7] but rather caused a reduction in adrenaline release *in vivo* [rabbit: 8, 9]. The source of the NO involved in this putative modulatory mechanism is still unknown: some studies ascribe to exogenous NO a reduction in neurotransmitter release [frog neuromuscular junction: 10] while others have reported the involvement of endogenous NO in sympathetic vasoconstriction [rat: 11].

The aim of the present work was to study whether or not NO (from endothelial or extra-endothelial origin) is able to modify noradrenaline release from vascular postganglionic sympathetic nerve endings in a vascular bed, the isolated ventral rat tail artery.

METHODS

Chemicals

The following drugs were used: levo-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 41.3 Ci/mmol, was from DuPont NEN (I.L.C., Lisboa, Portugal); desipramine hydrochloride, sodium nitroprusside, L-Arginine and Nω-Nitro-L-arginine methyl ester hydrochloride were purchased from Sigma-Aldrich (Sintra, Portugal). DEA-NONOate was purchased from MERCK Milipore. Stock solutions were made up in dimethylsulphoxide (DMSO: 0.01% v/v, final concentration) or ultrapure water and diluted in superfusion medium immediately before use. DMSO was added to the superfusion medium (final concentration 0.01%), in parallel control experiments.

Animals

Adult male Wistar rats (270-360 g; Charles River, Barcelona, Spain) were used. Animals were kept under light/dark cycles of 12/12 h, 20-22°C, and free access to water and pellet food. Handling and care of animals were conducted according to the European Union guidelines for animal research (86/609/EEC; in agreement with the NIH guidelines) and Portuguese law (Portarias n°1005/92 and n°1131/97). Animals were killed by stunning followed by exsanguination. Two animals per experiment were used and the mesenteric/caudal (endothelium intact or denuded) arteries were dissected out. Endothelium was removed by gentle rubbing of the inner lumen with a stainless steel wire. Five to six tissue preparations (5 to 9 mg) were obtained from each artery.

[^{3}H]-Noradrenaline release experiments

After dissection, arteries were immediately placed in cold Krebs-Henseleit solution of the following composition (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3 and dissodium EDTA 0.03 (pH 7.4). The protocol used to label tissue preparations with [^{3}H]-noradrenaline and to evaluate changes on electrically-evoked tritium overflow was performed according to previous studies [12, 13, 14, 15], with minor modifications. Briefly, arteries were pre-incubated in 2 ml Krebs-Henseleit solution containing 0.1 μM [^{3}H]-noradrenaline (for 40 min at 37°C) and transferred into superfusion chambers, superfused with [^{3}H]-noradrenaline-free medium (1ml min^{-1}; constant rate: Krebs-Henseleit solution with desipramine 400 nM to inhibit noradrenaline's neuronal uptake). Up to three periods of electrical stimulation (5 Hz, 100 pulses, 1 ms, 50 mA; Hugo Sachs Elektronik, March-Hugstetten, Germany) were applied: the first stimulation period, t = 45 min (S_0), served to test the viability of the preparation and medium was not collected during this period; the subsequent two stimulation periods (S_1 to S_2), started at t=90 min, were applied with 20 min intervals. The superfusate was collected in 5 min periods from 85 min of superfusion onwards (t=0 min being the onset of superfusion). At the end of the experiments (t=130 min), tritium was measured in superfusate samples and solubilized arteries and veins by liquid scintillation spectrometry (LS 6500, Beckman Instruments, Fullerton, USA) after adding 6 ml of scintillation mixture (OptiPhase 'Hisafe' 3, PerkinElmer, I.L.C., Lisboa, Portugal) to each superfusate sample. At the end of experiments 2.5 ml perchloric acid (0.2 M) were added to each tissue and these flasks were sonicated for 1h. After tissue removal, 6 ml of scintillation mixture were also

added. From each animal, no more than two tissue preparations were submitted to identical treatments.

Data analysis

Evaluation of [^{3}H]-noradrenaline release experiments was carried out as previously described.[12, 13, 14, 15] Briefly, effects of drugs added after S_1 on electrically-evoked tritium overflow were evaluated as ratios of the overflow elicited by S_n and the overflow elicited by S_1 (S_n/S_1). S_n/S_1 ratios obtained in individual experiments in which a test compound A was added after S_1 were calculated as a percentage of the respective mean ratio in the appropriate control group (solvent instead of A). When the interaction of A, added after S_1, and a drug B added 5 min before S_n, was studied, the "appropriate control" was a group in which A alone was used.

Results are expressed as mean±s.e.m. and *n* denotes the number of tissue preparations. Differences between means were compared for significance using the unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by post-hoc Dunnet's multiple-comparison *t*-test. A value of $P<0.05$ was considered to denote statistically significant differences.

RESULTS

NO induced changes in evoked-tritium release: mesenteric and tail arteries

Electrically-evoked tritium overflow from tissue preparations incubated with [^{3}H]-noradrenaline has been shown to reflect action potential-evoked neuronal noradrenaline release[16] and the drug-induced changes in evoked tritium overflow may be assumed to reflect changes in neuronal noradrenaline release, as observed in previous studies.[13, 14]

In the absence of drugs (except 400 nM desipramine, which was present in the superfusion medium, in all experiments, to inhibit the neuronal uptake of noradrenaline), the fractional rate of outflow immediately before S_1 (b_1) was 0.0864±0.0065 min^{-1} (*n*=15) for intact mesenteric arteries. For tail arteries the fractional rate of outflow immediately before S_1 (b_1) was 0.0984±0.0056 min^{-1} (*n*=17) or 0.09660±0.0066 min^{-1} (*n*=15) for intact and denuded tail arteries, respectively. Tritium overflow elicited by S_1 was 1.03±0.09%

(n=15) of the mesenteric arteries tritium content. In tail arteries, tritium overflow elicited by S_1 was 0.28±0.04% (n=17) or 0.38±0.08% (n=15) of total tissue content for intact and denuded tail arteries, respectively. Differences with S_1 values ($P<0.05$) were observed between S_1 values in mesenteric and tail arteries. Basal outflow and electrically-evoked tritium overflow remained constant throughout experiments, with b_2/b_1 and S_2/S_1 values close to unity.

The influence exerted by NO on noradrenaline release from vascular sympathetic nerve endings was tested using NO donors as well as a NO synthase inhibitor in two different vascular beds: mesenteric and tail artery (Figure 1).

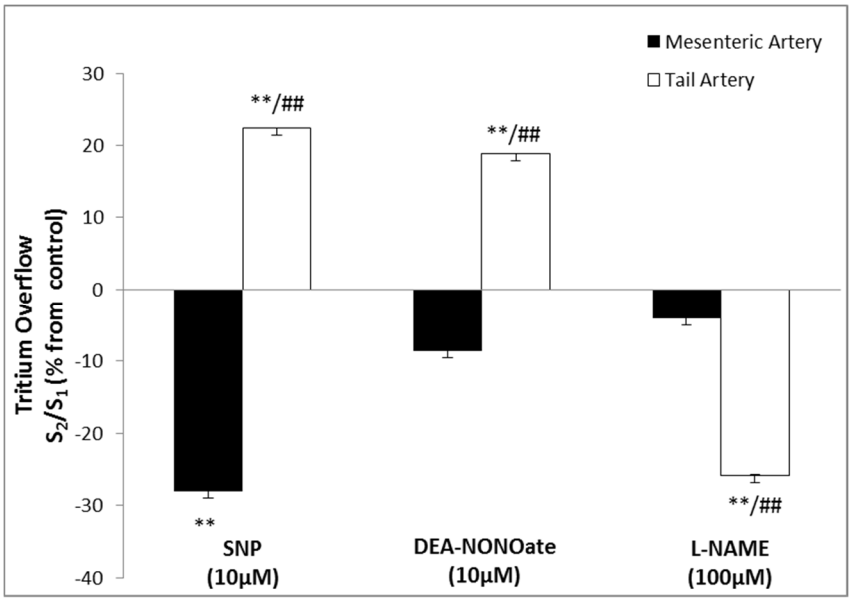

Fig. 1 Effects of NO synthase inhibitor (L-NAME, 100 μM) and NO donors (SNP, 10 μM; DEA NONOate, 10 μM) on noradrenaline release from rat mesenteric (black bars) and tail (white bars) arteries. Ordinates: S_2/S_1 values obtained in individual tissues preparations corrected from the appropriate S_2/S_1 control value. Values are mean±s.e.m from 6-20 tissue preparations; statistically significant differences (ANOVA followed by Dunnet's or Student's *t*-test: *p<0.05 or **p<0.001, from the respective control and #p<0.05 or ##p<0.001, from the mesenteric arteries preparations).

Results indicate a clear difference in NO mediated effects in these two vessels: in the tail artery NO induce an enhancement in [^{3}H]-noradrenaline release while in mesenteric artery the effects observed with DEA NONOate (10 μM) and L-NAME (100 μM) indicate a poor

involvement of NO in sympathetic neurotransmission. SNP (10 μM) was able to reduce noradrenaline release, an effect that was not mimicked by the other NO donor DEA NONOate. This might be explained has previously described by a possible decomposition of SNP by tissues attributable to interaction of its ion with sulfhydryl groups.[17]

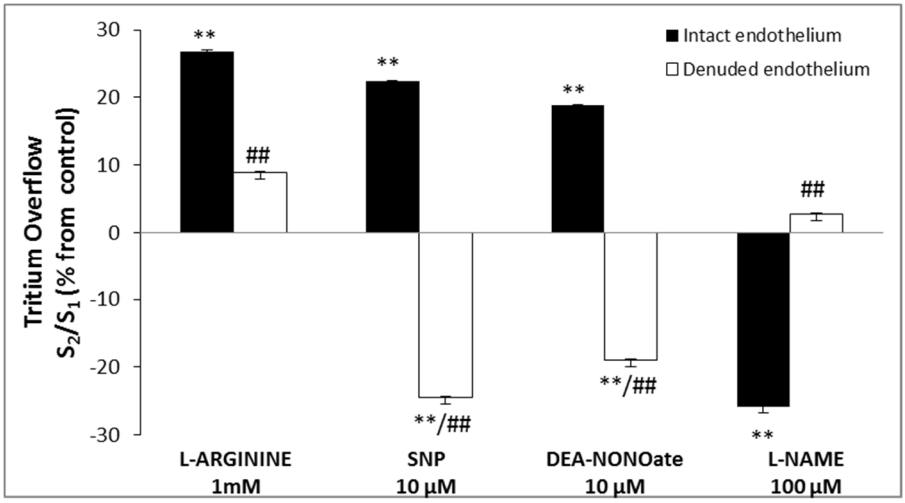

Fig. 2 Effects of NO synthase substrate (L-Arginine, 1mM) and NO synthase inhibitor (L-NAME, 100 μM) and NO donors (SNP, 10 μM; DEA NONOate, 10 μM) on noradrenaline release from rat intact (black bars) and denuded (white bars) tail arteries. Ordinates: S_2/S_1 values obtained in individual tissues preparations corrected from the appropriate S_2/S_1 control value. Values are mean±s.e.m from 6-20 tissue preparations; statistically significant differences (ANOVA followed by Dunnet´s or Student's t test: *p<0.05 or **p<0.001, from the respective control and #p<0.05 or ##p<0.001, from the endothelium intact artery preparations).

Role of tail artery endothelium NO in evoked-tritium release

NO was found to be involved in the enhancement of [^{3}H]-noradrenaline release from postganglionic sympathetic nerve endings in rat tail arteries (Fig. 1). In order to investigate the source of NO involved in this facilitation, experiments with intact and denuded tail arteries were carried out. L-NAME (100 μM) failed to modify [^{3}H]-noradrenaline release in denuded tail arteries while in intact arteries it reduces [^{3}H]-noradrenaline release (Fig. 2). This effect agrees well with a tonic effect mediated by an NO produced most probably in the endothelium. Data obtained with NO donors indicate that the signalling pathway activated

by exogenous NO (NO donors mediated effects), ultimately causes a reduction in [^{3}H]-noradrenaline release in denuded arteries, in opposition to the effects observed in intact arteries. Clearly, this indicates that distinct pathways are being influenced by NO from different sources.

CONCLUSIONS

In rat mesenteric artery, the availability of nitric oxide appears to have a poor or no effect on sympathetic transmission. Rather, in tail artery, the results support the occurrence of a modulating effect on vascular sympathetic transsynaptic transmission mediated by NO. Endothelial NO signalling pathways seem to promote noradrenaline release but there is no tonic effect mediated by neuronal NO. Data indicate that the signalling pathway activated by exogenous NO, ultimately causes a reduction in noradrenaline release in denuded tail arteries, in opposition to the effects observed in intact tail arteries. Endogenous NO, produced most probably in the endothelium, in turn, mediate an enhancement of noradrenaline release. These indicate that NO from different sources can induce opposite effects probably through activation/influence of distinct pathways.

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MANUSCRIPT IV

NITRIC OXIDE PRODUCTION IN MESENTERIC AND TAIL ARTERIES: DIFFERENTIAL ROLE OF eNOS AND nNOS IN SYMPATHETIC NEUROTRANSMISSION

Submitted for publication in Nitric Oxide: Biology and Chemistry

NITRIC OXIDE PRODUCTION IN MESENTERIC AND TAIL ARTERIES: DIFFERENTIAL ROLE OF eNOS AND nNOS IN SYMPATHETIC NEUROTRANSMISSION

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ABSTRACT

Endothelium is widely recognized as an important regulator of blood vessel tone *via* release of various endothelium-derived endogenous substances, such as nitric oxide (NO). The aim of the present study was to clarify the modulatory role exerted by endogenous NO (generated by eNOS and nNOS) in sympathetic neurotransmission and to characterize the distribution profile of enzyme isoforms involved in NO generation, eNOS and nNOS.

The role of NO in sympathetic neurotransmission was studied using electrically-evoked [^{3}H]-noradrenaline release experiments. Localization of NOS isoform in adventitia of mesenteric arteries was investigated by Laser Scanning Confocal Microscopy.

NO donors reduced noradrenaline release in mesenteric arteries while, in tail arteries, NO donors caused opposite effects. In mesenteric arteries, L-NAME was devoid of effect while both Nω-Propyl-L-arginine hydrochloride, a nNOS inhibitor, and L-NIO dihydrochloride, eNOS inhibitor, increased noradrenaline release. In tail arteries, L-NAME and L-NIO dihydrochloride reduced noradrenaline release whereas nNOS inhibitor, failed to change neurotransmistter release. Immunohistochemistry revealed that nNOS was present in Schwann cells but also in other type of cells in the adventitia of mesenteric and tail arteries. It further showed lower amounts of nNOS in tail arteries comparatively to values observed in mesenteric arteries.

In conclusion, the NO modulatory role on sympathetic neurotransmission differs in the mesenteric and tail arteries depending on the NOS isoform, eNOS and/or nNOS, involved in its production. Furthermore, nNOS locatization may also be crucial to this effect, since nNOS present in Schawnn cells seem to be the main source of NO to perivascular sympathetic nerves.

Keywords: NO; sympathetic neurotransmission; nNOS; Schwann cells

INTRODUCTION

Endothelium is widely recognized as an important regulator of blood vessel tone *via* release of various endothelium-derived endogenous substances[1, 2] with vasoconstriction and/or vasodilation properties.[3, 4] One of such substances is nitric oxide (NO). NO contributes to maintain vascular homeostasis by modulating the vascular dilator tone and regulating local cell growth. NO is not a locally-acting messenger and the spatial distribution of NO in tissue is large. Indeed, since NO is uncharged and highly soluble in hydrophobic environments, it can diffuse freely across biological membranes and signal many cell sites from its site of generation.[5] Therefore, NO can modify vascular smooth muscle tone directly, acting on smooth muscle cells, or indirectly, by altering sympathetic neurotransmission.[6-9] Indeed, NO is considered a physiological modulator of sympathetic neurotransmission by deactivating noradrenaline in the neurovascular junction.[10-14]

Several studies have suggested that endothelial dysfunction due to decreased nitric oxide (NO) production and/or destruction by reactive oxygen species (ROS) contributes to hypertension, increasing peripheral vascular resistance.[4, 15-21]. Alterations in NO modulation in sympathetic neurotransmission have also been implicated in hypertension.[22] NO production occurs via NO synthases (NOS) activation: NOS (endothelial NOS, eNOS, and neuronal NOS, nNOS), may generate small amounts of NO (picomolar levels) and contribute to homeostasis.[23-25].

Recently it has been reported that eNOS expression is paradoxically increased rather than decreased in endothelial dysfunction[26-30] and eNOS can lead to the production of superoxide rather than NO.[31-36] Moreover, studies concerning NO and its role in endothelial regulation of vasomotor tone[37] revealed that endothelial cells may be dynamically involved in the vascular response to receptor stimulation, namely α- or β-adrenoceptors[38] and adenosine receptors.[39-41]. However, the role of nNOS inhibition on cardiovascular system is still unclear. Some studies indicated that the inhibition of nNOS does not significantly affect blood pressure of both normotensive[42-46] and spontaneously hypertensive rats[47] while others have reported that modulation of the L-Arg/NO system may be used in therapeutics of hypertension, regulating NO production.[35, 48, 49]

The aim of the present study was to clarify the modulatory role exerted by endogenous NO (generated by eNOS and nNOS) in sympathetic neurotransmission and to characterize the distribution profile of enzyme isoforms involved in NO generation, eNOS and nNOS, in the adventitia layer of two arteries, mesenteric and caudal, arteries currently used as models to study neurotransmission.

MATERIALS AND METHODS

Animals

Adult male WKY (12 weeks old, 270-350 g; Charles River, Barcelona, Spain) were used. Handling and care of animals were conducted according to the European guidelines (Directive 2010/63/EU) on the protection of animals used for scientific purposes in agreement with the NIH guidelines. Two animals per experiment were used. Seven tissue segments (5 to 9 mg) were obtained from each tail artery and four tissue segments (4-7 mg) were obtained from each mesenteric artery. Briefly, tail arteries were dissected and, for experiments with denuded preparations, endothelium was mechanically removed with a thin stainless steel wire

Chemicals

The following drugs were used: levo-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 41.3 Ci/mmol, was from DuPont NEN (I.L.C., Lisboa, Portugal); desipramine hydrochloride, (S)-2-Amino-5-guanidinopentanoic acid (L-Arginine), Sodium nitroprusside (SNP), Diethylamine NONOate diethylammonium salt (DEA-NONOate), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), Nω-Propyl-L-arginine hydrochloride and L-NIO dihydrochloride were purchased from Sigma-Aldrich (Sintra, Portugal). The following antibodies used: mouse monoclonal anti-NOS1 (sc-5302), were purchased from Santa Cruz Biotechnology, Inc., CA, USA; rabbit GFAP polyclonal antibody (18-0063) was purchased from Invitrogen, Life Technologies, SA, Madrid, Spain). The following fluorescent probes were used: Alexa Fluor® 488 goat anti-mouse IgG (H+L) antibody, highly cross-adsorbed and Alexa Fluor® 647 goat anti-rabbit IgG (H+L) antibody, highly cross-adsorbed (Molecular Probes®) secondary fluorescent antibodies (Invitrogen, Life Technologies, SA, Madrid, Spain); vectashield mounting medium with DAPI (Vector Laboratories, UK). Stock solutions were made up in dimethylsulphoxide (DMSO: 0.01% v/v, final concentration) or distilled water and diluted in superfusion medium immediately before use. DMSO was added to the superfusion medium (final concentration 0.01%), in parallel control experiments.

[^{3}H]-Noradrenaline release experiments

Evaluation of [^{3}H]-noradrenaline release experiments was carried out as previously described.[50-54] Arteries were pre-incubated in 2 ml Krebs-Henseleit solution containing 0.1 μmol/L [^{3}H]-noradrenaline (for 60 min at 37^{0}C) and transferred into superfusion chambers, superfused with [^{3}H]-noradrenaline-free medium (1 ml min-1; constant rate: Krebs-Henseleit solution with desipramine 400 nmol/L to inhibit noradrenaline's neuronal uptake). Up to three periods of electrical stimulation (5 Hz, 100 pulses, 1 ms, 50 mA; Hugo Sachs Elektronik, March-Hugstetten, Germany): two stimulation periods (S_1 and S_2) were applied with 30 min intervals, t=90 min and t=120 min, respectively. The superfusate was collected each 5 min period from 85 min of superfusion onwards. At the end of the experiments (t=130 min), tritium was measured in superfusate samples and solubilized arteries (sonicated 1h with 2.5 ml perchloric acid (0.2 mol/L)) by liquid scintillation spectrometry (LS 6500, Beckman Instruments, Fullerton, USA) after adding 6 ml of a scintillation mixture (OptiPhase 'Hisafe' 3, PerkinElmer, I.L.C., Lisboa, Portugal) to each sample.

Tissue labelling with [^{3}H]-noradrenaline and evaluation of electrically-evoked tritium overflow changes was performed as previously described.[53, 54] Effects of NOS inhibitors and donors, in tail and mesenteric arteries, were studied.

Laser scanning confocal microscopy (LSCM) experiments

Immunohistochemistry procedures were as described previously.[54] Four tissue preparations were obtained from each artery and immediately placed in cold phosphate buffer solution (PBS; in g/L): NaCl 8.0, Na2HPO4.2H2O 0.77, KCl 0.20, KH2PO4 0.19 (pH 7.2). Each preparation was longitudinally opened and fixed (paraformaldehyde 4% PBS; 50 min; room temperature, RT). After two 15 min PBS washing cycles, artery segments were incubated with primary antibodies raised against nNOS, an mouse monoclonal anti-NOS1 (1:200 dilution, overnight, 4^{0}C) or mouse monoclonal anti-glial fribrilary acidic protein (GFAP, 1:200 dilution, overnight, 4^{0}C). Thereafter, tissues were incubated with Alexa 647 anti-rabbit and Alexa 488 anti-mouse fluorescent secondary antibodies (1:1000 dilution, 1 h, RT). Negative controls were performed by omitting primary antibodies. After two PBS washing cycles, tissue preparations were mounted with antifading agent (vectashield mounting medium with DAPI, Vector Laboratories, UK), with the adventitial side facing up. Preparations were visualized with a Leica SP5 LSCM system (Leica Microsystems, Germany) fitted with an inverted microscope (x63 oil immersion lens). Stacks of 1-μm-thick

serial optical images were captured from the entire adventitial layer, which was identified by the shape and orientation of the nuclei. Image acquisition was performed always under the same laser power, brightness and contrast conditions. Adventitia was scanned along each mesenteric and tail arteries and the resulting images were reconstructed separately for each wavelength.

Data Analysis

Measurement of drug effects on electrically-evoked tritium overflow.

Electrically-evoked tritium overflow from artery segments incubated with [^{3}H]-noradrenaline has been shown to reflect action potential-evoked neuronal noradrenaline release and drug-induced changes in evoked tritium overflow are assumed to reflect changes in neuronal noradrenaline release. Effects of drugs added after S_1 on electrically-evoked tritium overflow were evaluated as ratios of the overflow elicited by S_2 and the overflow elicited by S_1 (S_2/S_1). S_2/S_1 ratios obtained in individual experiments in which a test compound A was added after S_1 were calculated as a percentage of the respective mean ratio in the appropriate control group (solvent instead of A).

Laser Scanning Confocal Microscopy images quantification

Quantitative analysis of confocal z-stacks images was performed using image analysis software (PAQI, CEMUP, OPorto, Portugal). Briefly, a sequential routine was designed and developed to analyse each fluorescent signal used. PAQI software measured the surface area and strength of the receptors, the surface area and strength of the Schwann cells and determined the surface area of attachment of the receptors on the Schwann cells.

Statistics

Results are expressed as mean±s.e.m. and n denotes the number of tissue preparations. Differences of means were compared for significance using one- or two-way ANOVA followed by post-hoc Holm-Sidak's multicomparisons. A P value lower than 0.05 was considered to denote statistically significant differences.

RESULTS

The fractional rate of basal tritium outflow (b_1) and electrically-evoked tritium overflow (S_1) of mesenteric and tail arteries are shown in Table 1 and Figure 1. Electrically-evoked tritium overflow (S_1) is similar in mesenteric and tail arteries but is more pronounced in denuded arteries evidencing, clearly, the importance of endothelium in sympathetic neurotransmission. Moreover, in mesenteric denuded-arteries S_1 value is even more pronounced (almost the double) than that exhibited by tail arteries. Basal outflow and electrically-evoked tritium overflow remained constant throughout the experiments, with b_n/b_1 and S_n/S_1 values of control samples close to unity (data not shown).

Table 1. Basal tritium outflow (b_1), electrically-evoked tritium overflow (S_1) and S_2/S_1 ratios from mesenteric and caudal arteries

	Basal Outflow (b_1) (fractional rate of outflow; min^{-1})	**Evoked Overflow (S_1)** (% of tissue tritium content)	**S_2/S_1**	***n***
Mesenteric artery	0.070±0.008	0.229±0.037	0.9801±0.048	15
Caudal artery	0.075±0.003	0.185±0.015	1.0275±0.081	25

Tissue preparations of mesenteric and caudal arteries were pre-incubated with [^{3}H]-noradrenaline for 40 min. After pre-incubation with [^{3}H]-noradrenaline, tissues were superfused with [^{3}H]-noradrenaline free medium containing desipramine (400 nM). Tissues were stimulated twice at 30-min intervals (S_1-S_2; 100 pulses, 5 Hz, 1 ms, 50 mA): b_1 refers to the 5-min period immediately before S_1. The electrically-evoked tritium overflow was calculated by subtracting the estimated basal outflow from total outflow observed during and in the 25-min period subsequent to S_1 and expressed as a percentage of the tissue tritium content at the onset of stimulation. Values presented are means±SEM and *n* denotes the number of tissue preparations. Differences of means were compared for significance using Student´s t test): *P<0.05

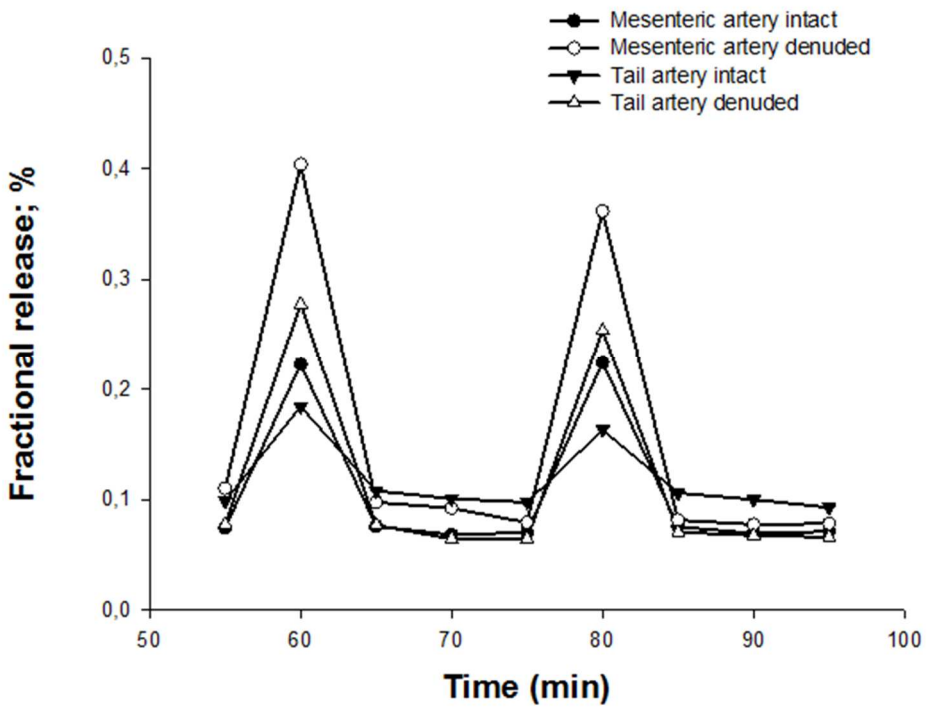

Figure 1. Time course of tritium outflow from intact (filled circles) and denuded (open circles) tail artery segments, and from intact (filled triangles) and denuded (open triangles) mesenteric artery segments, taken from a typical experiment. After pre-incubation with [^{3}H]-noradrenaline, tissues were superfused with [^{3}H]-noradrenaline free medium containing desipramine (400 nM). Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period and was measured in samples collected every 5 min. Artery segments were stimulated twice(S_1, S_2) by 100 pulses/5 Hz, 1 ms, 50 mA. Each line represents the outflow of tritium from a single superfusion chamber.

Nitric oxide in sympathetic neurotransmission in mesenteric and tail artery

The nitric oxide donor DEA/NONOate (10 μM) reduced the electrically-evoked tritium overflow from mesenteric arteries. Similar results were obtained in the presence of another NO donor, SNP (10 μM; Fig 2A). In contrast, in tail arteries both NO donors induced an increase of electrically-evoked tritium overflow, as depicted in Fig 2A. L-arginine, a NOS substrate, facilitated tritium release both in mesenteric and tail arteries.

In a set of experiments, endothelium denuded mesenteric and tail arteries were used to determine the role of NO in sympathetic postganglionic terminals. In these conditions, the two NO donors, DEA/NONOate and SNP, did not affect tritium overflow in mesenteric arteries but inhibited tritium overflow in tail arteries (Fig 2B). L-arginine induced a

facilitation in tritium release in mesenteric artery while in tail artery, it did not change electrically-evoked tritium release (Fig 2B) suggesting the inactivation, or absence, of NOS signaling machinery in tail artery. In addition, L-arginine induced facilitation was more pronounced in endothelium denuded compared to that observed in intact mesenteric arteries ($P<0.05$) indicating a putative occurrence of different NOS-signalling events or the involvement of different NOS subtypes.

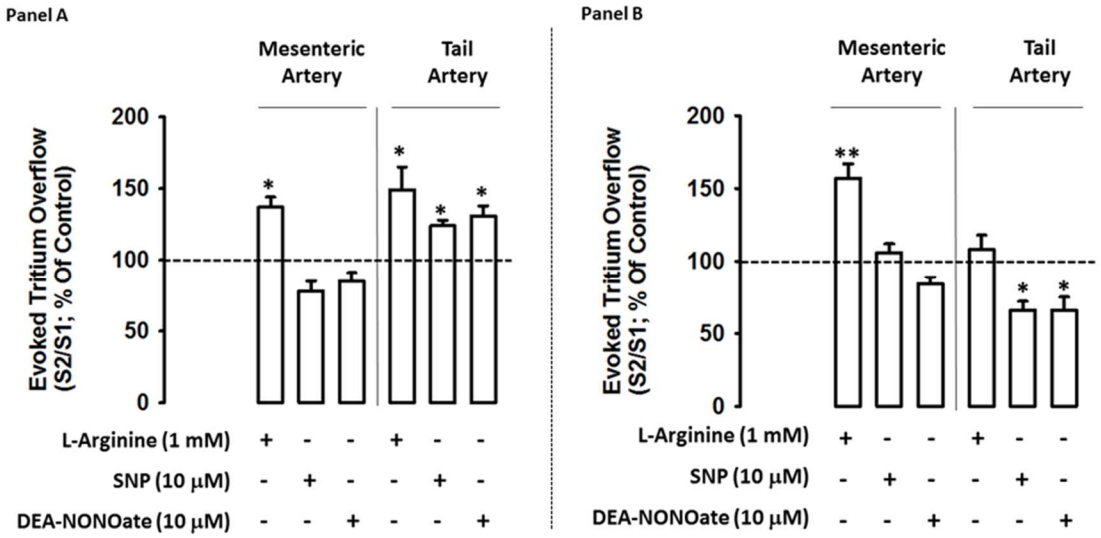

Figure 2. Influence of nitric oxide donors on the modulation of electrically-evoked tritium overflow in intact (panel A) and denuded (panel B) tail and mesenteric arteries: interaction with L-Arginine (1 mM, a substrate for NOS) and nitric oxide donors, SNP (10 μM) and DEA-NONOate (10 μM). Arteries were electrically stimulated (S_1-S_2: 100 pulses, 5 Hz, 1 ms, 50 mA). Drugs were added immediately after S_1 and kept until the end of the experiment. *Ordinates:* S_2/S_1 values obtained in individual tissue preparations, expressed as a percentage of the appropriate S_2/S_1 control value. Values are mean±s.e.m. from 7-24 tissue segments. Significant differences from the appropriate control: *P<0.05; **P<0.001 (two-way ANOVA followed by *post-hoc* Holm-Sidak's multicomparisons *t*-test).

Endogenous nitric oxide production in mesenteric and tail arteries: role of eNOS and nNOS

L-NAME, described as a non-selective NOS inhibitor, L-NIO dihydrochloride, considered a more selective eNOS inhibitor, and Nω-Propyl-L-arginine hydrochloride, a more selective nNOS inhibitor, were used to challenge this later possibility (Fig.3).

In tail artery, L-NAME and L-NIO dihydrochloride reduced electrically-evoked tritium overflow, an effect compatible with the facilitatory role of NO described above in this artery whereas nNOS inhibitor, failed to change tritium release (Fig 3). In endothelium denuded tail arteries nNOS and eNOS inhbitors also failed to modify tritium release (98.90±6.81%; n=9 and 109.56±13.02%; n=4, respectively; values corrected from S_2/S_1 of the appropriate control samples).

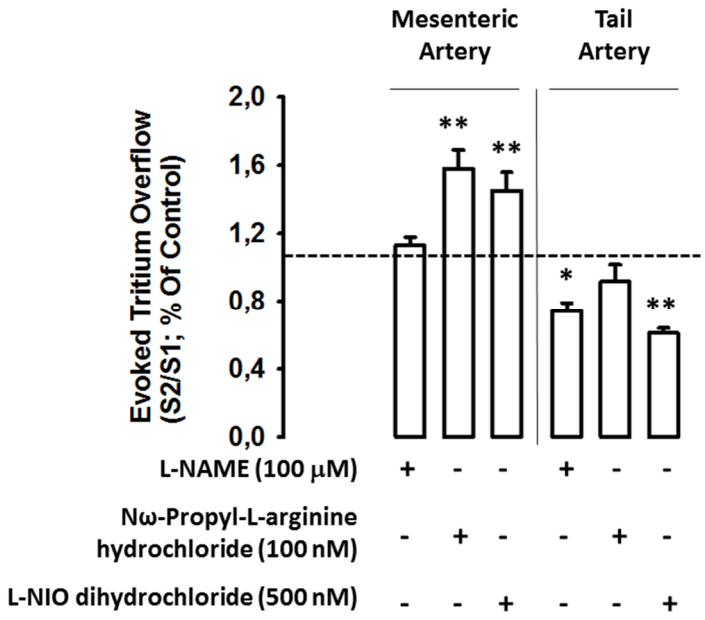

Figure 3. Influence of nitric oxide inhibitors on the modulation of electrically-evoked tritium overflow in tail and mesenteric arteries: interaction with nitric oxide inhibitors L-NAME (100 µM, a non-selective nNOS inhibitor), Nω-Propyl-L-arginine hydrochloride (100 nM, a selective nNOS inhibitor) and L-NIO dihydrochloride (500 nM, a selective eNOS inhibitor). Arteries were electrically stimulated (S_1-S_2: 100 pulses, 5 Hz, 1 ms, 50 mA). Drugs were added immediately after S_1 and kept until the end of the experiment. *Ordinates:* S_2/S_1 values obtained in individual tissue preparations, expressed as a percentage of the appropriate S_2/S_1 control value. Values are mean±s.e.m. from 7-24 tissue segments. Significant differences from the appropriate control: *P<0.05; **P<0.001 (two-way ANOVA followed by *post-hoc* Holm-Sidak's multicomparisons *t*-test).

When we evaluate the effects elicited by these NOS inhibitors on the evoked tritium release in the mesenteric arteries we detected a different profile: L-NAME was devoid of effect while Nω-Propyl-L-arginine hydrochloride, a nNOS inhibitor, was able to increase tritium release up to 49% and L-NIO dihydrochloride, eNOS inhibitor, up to 45%.

Results are suggestive of different NOS subtypes producing NO in locations where the subsequent signaling cascade drives to different neuromodulatory roles or, alternatively, the amount of NO produced may differ and, consequently, differently influence neurotransmission.

Characterization of nNOS distribution in the adventitia of mesenteric and tail arteries

In mesenteric and tail arteries immunoreactivity for nNOS (anti-NOS1-immunoreactive) was found in the adventitia layer, but the relative amount of nNOS present in tail arteries is much lower (~27%) than that exhibited in mesenteric arteries (Figure 4). Previously we have showed that sympathetic nerves are surrounded by Schwann cells (anti-GFAP-immunoreactive) and, therefore, we tested the possibility that nNOS could be expressed in these cells since they have a trophic role to sympathetic neurons: NOS1 and GFAP staining overlay might indicate that they could be located on the same cellular structure.

Figure 4C shows that NOS1 and GFAP overlaid immunoreactivities markedly decrease in tail as compared with mesenteric arteries when data was normalized by the total GFAP immunoreactivity: 50% of Schwann cells exhibit NOS1 immunoreactivity in mesenteric arteries versus the 4% observed in tail arteries. Similar results were also observed in Figure 4D (upon normalization of NOS1-GFAP overlay with the total NOS1 immunostaining). NOS1 immunoreactivity was also observed in cells other than Schwann cells in both mesenteric and tail artery images: 62.5% in mesenteric and 88% in tail artery images (Figure 4 A e D).

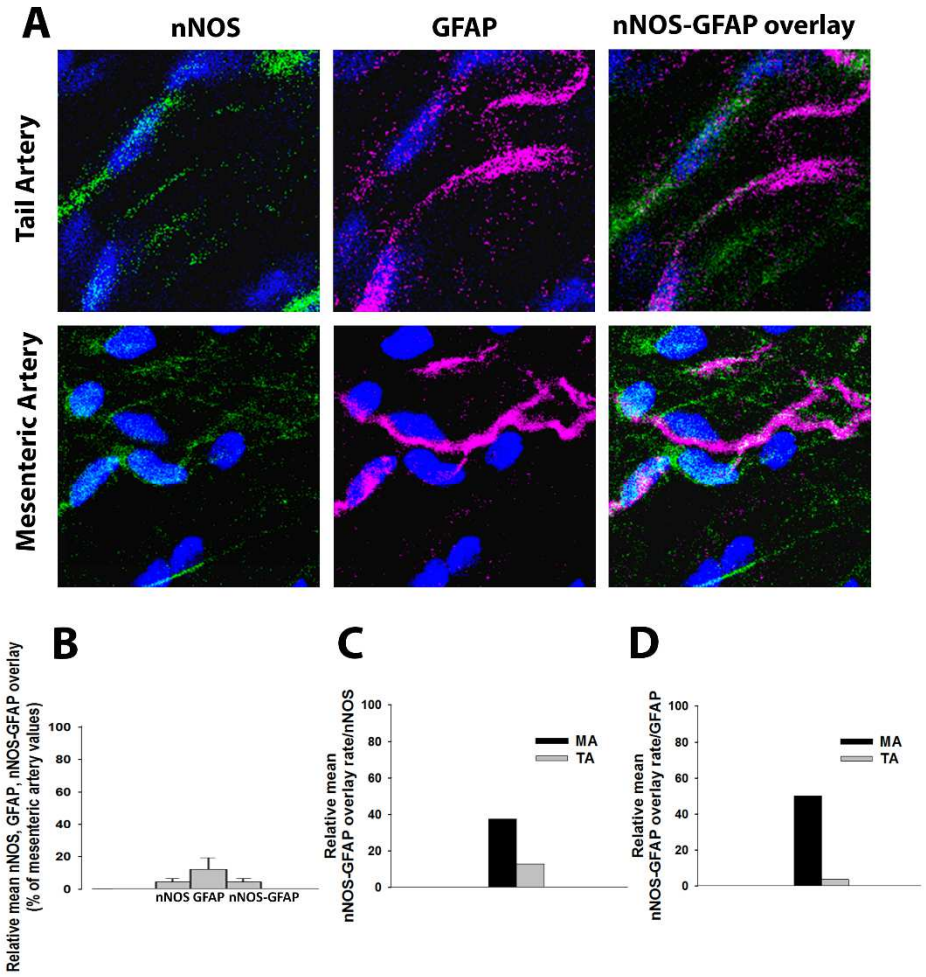

Figure 4. nNOS in Schwann cells in the adventitia of tail and mesenteric arteries. Confocal immunofluorescence representative images of adventitial tail and mesenteric arteries (A) immunostained for nNOS (a primary mouse monoclonal anti-NOS1 and a species specific secondary Alexa 488 antibody: green), or GFAP (a primary rabbit anti-GFAP polyclonal antibody and a species specific secondary Alexa 647 antibody: magenta) and DAPI (nuclear fluorescence stain, blue) were obtained using LSCM (all images are reconstructions from 11-28 serial optical sections). (B) Relative means of nNOS, GFAP and nNOS-GFAP overlay expressed as percentage of mesenteric artery values. (C) Mean percentage of overlay rate with GFAP (D) and mean percentage of overlay rate with nNOS are depicted. Values are mean±s.e.m.; 3-4 animals. Significant differences from mesenteric artery: #P<0.05, ## P<0.001. Scale bar = 20 μm.

DISCUSSION

In the present study we have demonstrated that neuromodulation exerted by NO in vascular sympathetic postganglionic nerves is largely dependent on: i) the NOS isoform that generates it, probably due to ii) the different distribution of NOS isoforms, eNOS and nNOS, or to iii) differences in the relative amount of nNOS isoform in the two arteries.

In this study we used two different arteries, mesenteric and tail, that have been used as model for the study of the neuromodulatory role of many substances in the vasculature.[6, 8, 9, 55-57] In mesenteric arteries NO-mediated effects in neurotransmission has been largely studied.[12] Our data with selective isoform NOS inhibitors, in intact mesenteric arteries, showed facilitation of noradrenaline release of similar magnitude, an effect that correlates well with previous studies in rat[8, 10-12] and rabbit[58] mesenteric arteries describing NO as a mediator able to reduce noradrenaline release. However, the non-selective NOS inhibitor, L-NAME, which inhibits both isoforms from producing NO, failed to modify noradrenaline release. These results suggest that in mesenteric arteries both nNOS and eNOS contribute to NO production that, ultimately, leads to a decrease in noradrenaline release. However, NO donors failed to modify noradrenaline release, in our experimental conditions, in both intact and denuded arteries, which may indicate that endogenous NO neuromodulation have achieved its maximum effect. Curiously, the non-selective substrate for NOS, L-arginine, facilitated noradrenaline release, in intact and denuded mesenteric arteries, contrary to what was observed in tail arteries where endothelium removal eliminated the facilitatory effect. This data indicate that in mesenteric arteries, NOS seem to produce other substance than NO, active in changing neurotransmission as well. L-citrulline could be such mediator since other authors have previously described its involvement as a neuromodulator in rat motor nerve endings.[59, 60]

In intact tail arteries, however, we found a different neuromodulatory role of NO in neurovascular junction since increase in NO amount, *via* addition of NO donors, increased noradrenaline levels. Curiously, in denuded tail arteries, and by opposition, NO increase inhibited noradrenaline release. This may indicate that different signalling mechanisms would be triggered in the presence or in the absence of endothelium. Information gathered recently may explain these data through the occurrence of nitrite production and nitrite-mediated effects.[61] Indeed, the nitrite reductase activity was described to be located in the oxygenase domain of eNOS[62] and that inhibition with L-NAME would attenuate the production of NO from nitrite.[63] eNOS would have a center role in this type of effect and its activity, either as nitrite reductase or as NOS synthase, in intact tissues would explain the facilitatory effects observed. In denuded arteries, the lack of, or diminished amount, of

eNOS would explain the opposite results. Moreover, this possibility agrees well with the results obtained using several NOS inhibitors. In intact tissues, both the selective eNOS inhibitor, L-NIO dihydrochloride, as well as the non-selective NOS inhibitor, L-NAME, reduced noradrenaline release, whereas in denuded arteries they did not change noradrenaline release. Furthermore, the nNOS inhibitor, Nω-Propyl-L-arginine hydrochloride, failed to modify noradrenaline release both in intact and denuded tail arteries suggesting a crucial role for eNOS in the increase of noradrenaline release detected.

Confocal microscopy studies showed the presence of nNOS isoform in the adventitia layer of both mesenteric and tail arteries, confirming previous reports.[64] Confocal microscopy data agrees well with the absence of nNOS activity found in the tail artery since a considerable lower amount (a reduction of 27%) of this isoform was detected in the adventitia of tail comparatively to mesenteric arteries. Our data also show, for the first time, that nNOS is distributed in two main locations: one expressed in Schwann cells and another, more abundant in other cells: about 63% and 88% in the adventitia of mesenteric and tail arteries, respectively. Therefore, it is fair to speculate that nNOS, expressed in Schwann cells, might be producing NO that can be causing the inhibition of noradrenaline release from sympathetic nerves. Our findings are in agreement with this possibility since the relative amount of nNOS expressed in Schwann cells in the two arteries are markedly different: 50% in mesenteric vs 4% in tail arteries. These data also correlates with the functional results obtained that showed a lack of neuromodulatory role of nNOS in tail arteries whereas, in mesenteric arteries, nNOS contributed to reduce by half noradrenaline release.

Another important finding concerns the changes in sympathetic neurotransmission observed in denuded arteries suggesting that when arterial endothelium integrity is somehow compromised the levels released of noradrenaline in neurovascular junction increase, contributing to vasoconstriction. Indeed, in addition to NO, as explained above, the absence of other endothelium derived substances such as adenosine, endothelin-1, etc might be contributing to the increase of noradrenaline release observed.

In summary, the present results suggest that the NO modulatory role on sympathetic neurotransmission differs in the mesenteric and tail arteries depending on the NOS isoform, eNOS and/or nNOS, involved in its production. Moreover nNOS location may also be crucial to this effect, since nNOS present in Schawnn cells seem to be the main source of NO to perivascular sympathetic nerves.

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CHAPTER III

General Discussion and Conclusions

DISCUSSION

Evidence has highlighted the role of vascular sympathetic neurotransmission in the regulation of vascular tonus.[1-3] Indeed, modification of adenosine receptor-mediated effects has been recently suggested to contribute to hypertension pathoetiology.[4-7] Combined application of different methodologies provided complementary information allowing a better understanding of how sympathetic neurotransmission is affected in pathological conditions such as hypertension and endothelium dysfunction. Electrically-evoked [^{3}H]-noradrenaline release experiments allow to analyze the effects caused by endogenous adenosine and nitric oxide on the amount of noradrenaline released from sympathetic nerves of the vessels, and how their action could be changed in the hypertensive state. The mechanisms by which these mediators could be altering neurotransmitters release and be influenced by endothelial dysfunction were also evaluated. Measurement of the amounts of ATP and adenosine released in these experiments were also carried out clarifying their role as neurotransmitter or neuromediator, respectively. Immunohistochemical studies revealed how distribution (redistribution) of both membrane proteins, such as adenosine receptors, or enzymes could occur in pathological conditions influencing, therefore, the ability of these proteins to be activated.

The work carried out in this thesis allowed to identify and understand some of the changes in vessel physiology during hypertension, particularly those concerning the levels of neurotransmitter release and the dynamics of adenosinergic system. The latter is related with the amounts of nucleosides and/or nucleotides, which depend both on nucleotide metabolism and nucleoside transporter activity, and individual adenosine receptor subtype activation. Other important mediator in vessels is NO and its effects were found to be largely dependent on the isoform that produced it, nNOS or eNOS. These isoforms presence and distribution can condition the amount of NO available and, therefore, NO-mediated effects. Two different arteries, mesenteric and tail arteries, which have been extensively used as models to study the neuromodulatory role of many substances in the vasculature, were used.

In hypertensive conditions, sympathetic neurotransmission was found to be altered occurring an increase in the release of both co-transmitters, noradrenaline and ATP.[8] The increase in the concentration of these neurotransmitters in the synaptic cleft may lead, ultimately, to an increase in vascular reactivity and vasoconstriction by activation of α_2 adrenoceptors (by noradrenaline) or P2 receptors (by ATP). Moreover, we showed that the increased levels of noradrenaline release found in hypertensive state can be justified by i) the absence of tonic inhibitory adenosine A_1 receptor-mediated effects which, in normotensive conditions inhibited sympathetic transmission, ii) the redistribution of

adenosine A_1 receptors to Schwann cells, which may hinder adenosine A_1 receptor activation, iii) the preserved facilitatory adenosine A_{2A} receptor-mediated effects, a *via* only activated with higher adenosine amounts than those needed to activate adenosine A_1 receptors, iii) an enhancement of adenosine levels as a consequence of the metabolism of the higher levels of electrically-evoked ATP released in the hypertensive state and iv) the fact that NO availability may be compromised since eNOS seem to be inactive.

In normotensive mesenteric and tail arteries, tonic adenosine A_1 receptor mediated effect inhibited noradrenaline released, an effect mediated predominantly by adenosine originated from metabolism of released adenine nucleotides. This indicates that, under basal conditions, inhibitory adenosine A_1 receptors operate to restrain noradrenaline release, as a consequence of adenosine accumulation from hydrolysis of ATP released from neighbouring cells. Moreover, the amount of ATP released from sympathetic nerves was higher in SHR than in WKY mesenteric arteries which may lead to P2 receptors activation, revealing ATP as an important vasoconstrictor in hypertensive arteries, a role recently described by Goonetilleke and co-workers (2013).[8]

Neurotransmitter release modulation ascribed to extracellular adenosine accumulation depends both on its formation and on cellular uptake and deamination. Adenosine uptake seems to be the dominant adenosine inactivation pathway in arteries. Nucleoside transporter inhibition can increase extracellular adenosine to levels high enough to activate adenosine A_{2A} receptors, while in its absence the amount of adenosine is only sufficient to activate adenosine A_1 receptors, which can be explained by the difference in their Kd´s.[9] Since adenosine A_{2A} receptors require concentrations two-fold higher than those needed to activate adenosine A_1 receptors, endogenous adenosine availability is crucial to discriminate the subtype of adenosine receptor that is activated. In conditions that favour extracellular adenosine accumulation, this nucleoside may also reach concentrations high enough to activate adenosine A_{2A} receptors, leading to the increase of noradrenaline release and, consequently, of sympathetic activity.

Laser scanning confocal microscopy data confirmed previous reports of a sympathetic hyperinnervation in hypertensive arteries. However, increases in the number and thickness of sympathetic nerve fibres observed in SHR arteries was not accompanied by a correspondent enhancement of adenosine A_1 and/or A_{2A} receptors overlaying these neurons. These lower adenosine A1 receptors amount can explain, at least in part, the lack of adenosine inhibitory tone regulating noradrenaline release from stimulated SHR arteries, leading to higher extracellular noradrenaline levels.

Adenosine A_1 and A_{2A} receptors are present in other cells than sympathetic neurons. These cells were identified as being glial cells, more specifically, Schwann cells, which have a trophic role for nerve cells in the adventitial layer of these arteries. In hypertensive

arteries, an increase amount of receptors can be observed in non-neuronal cells suggesting a neuron-to-glia redistribution of adenosine A_1 receptors. Changes in the localization of adenosine receptors and the increased amount of extracellular adenosine observed in SHR arteries suggest that receptor desensitization may be the main reason for adenosine receptor activity impairment observed in hypertensive arteries. These results can help to clarify current unknown aspects of the complex interplay between receptor expression and the role of adenosine.

It is clear from the literature that, in hypertension, endothelium function is compromised.[10-12] It is also accepted that endothelium derived substances alter vascular reactivity.[13, 14] To evaluate if endothelium could also alter vascular sympathetic neurotransmission, arteries were denuded (mechanical or chemically). In these conditions an increase in noradrenaline release from vascular sympathetic nerves was found. In SHR arteries (vessels that exhibit endothelial dysfunction)[15-18] this increase of noradrenaline release is also occurring. In denuded arteries, endogenous adenosine tonically activates adenosine A_{2A} receptors leading to a marked increase in noradrenaline release comparatively to the activity observed in intact arteries, an effect also observed, with similar magnitude, in SHR arteries. Moreover, an additional contribution to the increased sympathetic activity observed in these tissues (both denuded and SHR arteries) can be ascribed to the absence of inhibitory A_1 receptor-mediated effects. Therefore, the sympathetic activity impairment observed in arteries exhibiting some degree of endothelium dysfunction can be ascribed to alterations in the dynamics of the adenosinergic system.

Adenosine receptors, nucleoside transporters and adenosine availability are accepted to be key effectors in the dynamics of adenosinergic system and any change in one of these items may cause profound impact in the function of the others. In denuded arteries, a facilitation of noradrenaline release was observed when adenosine availability was increased. This effect may be due to the absence of nucleoside uptake to endothelial cells that seems to be the main mechanism that regulates adenosine extracellular levels. Thus, when endothelium is somehow compromised this transport seems to be deregulated causing accumulation of extracellular adenosine. Indeed, it seems that endothelium dysfunction causes an increase in noradrenaline released levels, contributing to vasoconstriction, an additional aggravating factor to hypertension.

Our results ruled out the involvement of endothelium-derived substances (ROS and prostaglandins) in mechanisms leading to altered neuromodulation, whereas adenosine-mediated neuromodulatory mechanisms were found to be impaired by endothelium injury/dysfunction. Evidence in the literature indicated that NO-induced vasodilation may be compromised in hypertension.[13, 18-23] NO mediated effects in vascular sympathetic

neuromodulation were found to be dependent on the NOS isoform that generates it, probably due to the different distribution of NOS isoforms, eNOS and nNOS in arteries, or to differences in the relative amount of nNOS isoform in the two arteries studied.

In mesenteric arteries, selective isoform NOS (nNOS and eNOS) inhibitors caused a facilitation of noradrenaline release of similar magnitude. Therefore, both nNOS and eNOS contribute to NO production reducing noradrenaline release. However, in the presence of exogenous L-arginine a facilitation of noradrenaline release was observed suggesting the activation of alternative pathways: NOS seem to produce other substance than NO, which can be L-citrulline, an active neuromediator.[24, 25]

In intact tail arteries we found a different neuromodulatory role of NO since it increased neurovascular sympathetic activity, contrary to what is described to occur in most arteries.[26] Curiously, and by opposition, in denuded tail arteries, NO inhibited noradrenaline release. Findings indicate that different signalling mechanisms would be triggered in the presence or in the absence of endothelium, probably due to the occurrence of nitrite and nitrite-mediated effects.[27] In the oxygenase domain of eNOS, a nitrite reductase activity was described[28] and its inhibition would attenuate the NO production from nitrite.[29, 30] Data indicate that eNOS would produce the predominant effect, and its activity either as nitrite reductase or as nitric oxide synthase, in intact tissues would explain the facilitatory effects observed. In denuded arteries, the lack of, or diminished amount of eNOS, observed in our work, would explain the observed opposite results.

The absence of effects mediated by nNOS in tail arteries revealed this additional feature of eNOS, once NO availability was found to be dependent only of eNOS activity. In mesenteric arteries, however, nNOS is active and produces enough NO to mask the nitrite reductase activity exhibited by eNOS. This possibility is in agreement with the results obtained using several NOS inhibitors and also with laser scanning confocal microscopy experiments. In intact tail arteries, the selective eNOS inhibitor reduced noradrenaline release, whereas in denuded tail arteries it failed to modify noradrenaline release. Furthermore, the nNOS inhibitor did not modify noradrenaline release both in intact and denuded tail arteries, suggesting a crucial role for eNOS in the increase of noradrenaline release detected.

Distribution of nNOS isoform in the adventitia layer of both mesenteric and tail arteries revealed the existence of considerable lower amounts of this isoform in tail arteries compared to mesenteric arteries. Other important aspect, that should be highlighted, concerns the expression of nNOS in Schwann cells in the adventitia layer of mesenteric and tail arteries. The hypothesis that nNOS, expressed in Schwann cells, might produce NO that causes noradrenaline release inhibition from sympathetic nerves can be raised. Our findings are in agreement with this possibility: the relative amount of nNOS expressed in Schwann

cells in the two arteries is markedly different. Additionally, the functional results obtained also support this hypothesis since they showed a lack of neuromodulatory role of nNOS in tail arteries whereas, in mesenteric arteries, nNOS contributed to reduce, by half, noradrenaline release.

CONCLUSIONS

Results from this work clarified some aspects related with vascular sympathetic neurotransmission, particularly when endothelium dysfunction and/or hypertension is present. Our experimental approach allowed to clarify the role of endogenous adenosine and nitric oxide in the modulation of sympathetic neurotransmission and verified that they were impaired in hypertension. Data showed that an increase in the sympathetic activity in hypertensive arteries may be partially due to a higher content in ATP release from sympathetic postganglionic nerves and the lack of endogenous adenosine inhibitory tonus. The latter might be explained by the nerve-to-glial redistribution of inhibitory adenosine A_1 receptors found to occur in hypertensive arteries. These mechanisms would lead to an increase in noradrenaline and ATP release from stimulated sympathetic nerves. Taken this into account one can predict that, in the synaptic cleft, in addition to the higher levels of noradrenaline, increased amounts of ATP are likely to accumulate, both leading to subsequent vasoconstriction of vascular smooth muscle cells. Moreover, endogenous adenosine neuromodulatory role of sympathetic transmission is significantly impaired in mesenteric and tail arteries from hypertensive rats. In SHR mesenteric arteries we failed to detect both adenosine A_1 and A_{2A} receptor mediated effects. This is occurring despite the extracellular levels of both ATP and adenosine being significantly higher in hypertensive mesenteric arteries than those measured in normotensive artery superfusates. Additionally, using selective adenosine receptor agonists, it was found that the neuromodulatory activity of adenosine A_1 receptor, but not of adenosine A_{2A} receptors, is significantly impaired in hypertensive mesenteric arteries. In this regard, these differences on adenosine neuromodulation may be explained by the occurrence of adenosine A_1 receptors desensitization and redistribution to Schwann cells.

Our data also allowed to confirm the hypothesis that endothelial dysfunction is altering vascular sympathetic neurotransmission contributing to increase vascular tone. In mesenteric arteries both nNOS and eNOS contribute equally to NO production conducing to a reduction of noradrenaline release. However, in tail arteries, the absence of nNOS seems to be conditioning NO levels. Moreover, the fact that eNOS can present nitrite

reductase activity may lead to a reduction of NO levels. This can explain the facilitation of noradrenaline release observed in tail arteries, where the inhibitory effect of NO is absent or reduced in these vessels.

The presence of nNOS isoform in Schwann cells and the occurrence of redistribution of adenosine receptors to Schwann cells in pathological conditions reinforce the importance of these cells in the peripheral nervous system. These findings can help us to understand the impact of those alterations in vascular reactivity, contributing to hypertension, renewing the interest of purinergic system as a target for novel therapeutic strategies.

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