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 [³H]-noradrenaline release experiments. Purine content was determined by HPLC with fluorescence detection. Localization of adenosine A₁ or A₂A 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 A₁ 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 A₂A receptor-mediated effects were absent in arteries from both strains. Immunohistochemistry revealed an adenosine A₁ receptors redistribution from sympathetic fibers to Schwann cells, in adventitia of hypertensive mesenteric arteries which can explain, at least in part, the absence of effects observed for these receptors.

**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 A₁ receptors in hypertensive arteries may explain the latter effect.

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 from its local of origin influencing neurotransmission, inflammation and immune responses.[4] Adenosine effects occur through activation of four G-protein coupled receptors, adenosine A₁, A₂A, A₂B and A₃ 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 A₁, but not A₂A 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 hypertension.

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-established 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 (A1 and A2A 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 specific activity of adenosine receptors (A1 and A2A subtypes) in the two endogenous adenosine has a role in the modulation of sympathetic activity and if this role is preserved in hypertensive individuals.

The following antibodies were used: rabbit anti-A2A (epitope corresponding to amino acids 331-412 v, final concentration) or ultrapure water and diluted in solutions were made up in dimethylsulphoxide (DMSO: 0.01% v/v, to 1 mL). The following fluorescent probes were used: Alexa Fluor 647 goat anti-rabbit IgG (H+L) (Life Technologies, SA, Madrid, Spain). The following antibodies were used: rabbit anti-tyrosine hydroxilase (TH, 1:10 dilution, overnight, 4°C) and mouse monoclonal anti-glial fibrillary acidic protein (GFAP, 1:200 dilution, overnight, 4°C), secondary anti-mouse IgG (H+L) antibody, highly cross-adsorbed (Molecular Probes) secondary fluorescent antibodies (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.

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[3H]-Noradrenaline release experiments

Evaluation of [3H]-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 [3H]-noradrenaline (for 60 min at 37°C) and transferred into superfusion chambers, superfused with [3H]-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, S1 and S2, 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), trimet was measured in superfusate samples and solubilized arteries (sonicated 1 h 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, LLC., Lisbon, Portugal) to each sample.

Tissue labelling with [3H]-noradrenaline and the evaluation of electrically-evoked trimet overflow changes were performed as previously described.[12,13] Effects of agonists (CPA, CGS 21680), of antagonists (DPCPX, SCH 58261), and of enzyme (pentostatin, ITU, αβ-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 (α1 or anti-A2A 1:200 dilution, overnight, 4°C) and mouse monoclonal anti-tyrosine hydroxidase (TH, 1:10 dilution, overnight, 4°C) or mouse monoclonal anti-γ-ribosomal acidic protein (GFAP, 1:200 dilution, overnight, 4°C) or mouse monoclonal anti-γ-ribosomal 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 wave-length.

ε-derivatives assay in artery superfusates

1,6-Nor-ε-modified purines have been previously used for purine quantification in tissue superfusates. [3,16–19] Briefly, mesenteric artery segments were superfused (Krebs-Henseleit, 1 mL/min) and electrically stimulated twice (S1–S2; 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 80°C. From the collected samples, 910 μL were incubated with 90 μL of
chlorella acetate 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 τ-derivatives (τ-ATP, τ-ADP, τ-AMP and τ-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 (20-22°C). The mobile phase consisted of a solution of 87 mmol/L KH₂PO₄ and 10.6 mmol/L 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 1 mL/min; from 0 to 3 min of elution, an increase from 0% to 25% of buffer B at a flow rate of 1 mL/min; from 3 to 7 min of elution, 25% of buffer B at a flow rate of 1 mL/min; and from 9 to 12 min of elution an increase from 25% to 100% of buffer B at a flow rate of 1 mL/min. From 12 to 20 min of elution, 100% of buffer B at a flow rate of 1 mL/min. The run time of 20 min and the post-run time was 5 min.

Data Analysis

Measurement of drug effects on electrically-evoked tritium overflow. Electrically-evoked tritium overflow from artery segments incubated with [³H]-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 S₁ on electrically-evoked tritium overflow were evaluated as ratios of the overflow elicited by S₂ and compared for significance using one- or two-way ANOVA, the overflow elicited by S₁ (S₂/S₁). S₂/S₁ ratios obtained in after S₁ were calculated as a percentage of the respective mean on nerves (corrected for background).

Following 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 9,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.

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. However, 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.

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).

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
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.

Effects of adenosine A1 and A2A receptor 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 (63.29±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 suppressed 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 receptors is largely preserved in hypertensive rats but the adenosine A1 receptor-mediated inhibition is somehow compromised in these animals.

Localization of adenosine A1 and A2A receptors 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 an enzyme localized inside sympathetic neurotransmitter storage vesicles.[22] 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 immuno-reactivities 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).

Localization of adenosine A1 and A2A receptors 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.

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 arteries (Figure 5). TH immunoreactivity in SHR exceeds that observed in WKY (Figure 5B), while A2A-TH overlaid immuno-reactivities 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).

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cells. This scenario may change towards putative activation of P2 receptors, along with adenosine receptors, in hypertensive arteries[2] a possibility 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, NBTH was able to increase noradrenaline release, suggesting that adenosine uptake may be the dominant adenosine inactivation pathway in mesenteric arteries. NBTH 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 $K_d$ 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 NBTH-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

Figure 3. Basal and electrically-evoked ATP (A) and adenosine (B) release in mesenteric arteries from WKY and SHR. Tissues were electrically stimulated (S1-S2: 100 pulses, 5 Hz). Significant differences from basal conditions: *P < 0.05; from WKY: #P < 0.05; ##P < 0.001. doi:10.1371/journal.pone.0105540.g003
(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 hyperten-

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Figure 4. Laser scanning confocal microscopy representative images of WKY and SHR mesenteric arteries exhibiting (A) adenosine A1 receptor (red), TH (green) and overlay of A1-TH immunoreactivities, nuclei (blue); (B) Relative means of TH, A1 and TH-A1 overlay expressed as percentage of WKY values. (C) Mean percentage of overlay rate with TH (D) and mean percentage of overlay rate with A1 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.
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sive rats.[35] Accordingly, decreases in adenosine $A_1$ 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]

Figure 5. Laser scanning confocal microscopy representative images of WKY and SHR mesenteric arteries exhibiting (A) adenosine $A_{2A}$ receptor (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. doi:10.1371/journal.pone.0105540.g005
Figure 6. Laser scanning confocal microscopy images of adenosine A₁ (panel A) and A₂A receptor (panel B) immunoreactivities in GFAP-immunoreactive (Schwann) cells localized in adventitia from WKY and SHR mesenteric arteries; 3–4 animals. Scale bar = 20 μm.
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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 fibers 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.

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Author Contributions

Conceived and designed the experiments: JBS PCS PF CD. Performed the experiments: JBS MSVR FF. Analyzed the data: JBS PCS PF CD. Contributed reagents/materials/analysis tools: CS PCS CD. Contributed to the writing of the manuscript: JBS PCS PF CD.

References

1. Anam MN, Bolli P, Kioski W, Bulhier FR (1981) Enhanced alpha-adrenoreceptor-mediated vasoconstriction in essential hypertension. Hypertension 5: 119–123.
2. Gomotileke L, Kacelic V, Dunn WR (2013) Influence of pressure on adenosine triphosphate function as a sympathetic neurotransmitter in small mesenteric arteries from the spontaneously hypertensive rat. J Hypertens 31: 312–320.
3. Diniz C, Fresco P, Goncalves J (2005) Regional differences in extracellular purine degradation in the prosthetic and epididymal portions of the rat vas deferens. Clin Exp Pharmacol Physiol 32: 721–727.
4. Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dargel PC (2006) Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol Ther 112: 356–404.
5. Feurid BA, Johansson S, Wang YQ (2011) Adenosine and the regulation of metabolism and body temperature. Adv Pharmacol 61: 77–94.
6. Iles P, Rickham H, Brod I, Bucher B, Stoelet JC (1989) Subsensitivity of presynaptic adenosine A₁-receptors in caudal arteries of spontaneously hypertensive rats. Eur J Pharmacol 174: 237–251.
7. Ralevic V (2000) Suppression inhibition by adenosine A₁ receptors, but not P2 receptors, in the hamster mesenteric arterial bed. Eur J Pharmacol 367: 287–293.
8. Donoso MV, Aedo E, Huidobro-Toro JP (2006) The role of adenosine A2A and A3 receptors on the differential modulation of norpinephrine and noradrenaline release from peripheral sympathetic nerve terminals. J Neurochem 96: 1680–1693.
9. Diniz C, Fresco P, Leal S, Goncalves J (2004) Adenosine receptors involved in modulation of noradrenaline release in isolated rat tail artery. Eur J Pharmacol 504: 17–25.
10. Fresco P, Diniz C, Queiroz G, Goncalves J (2002) Release inhibitory receptors activation favours the A2A-adenosine receptor-mediated facilitation of noradrenaline release in isolated rat tail artery. Br J Pharmacol 136: 230–236.
11. Fresco P, Diniz C, Goncalves J (2004) Facilitation of noradrenaline release by activation of adenosine A(2A) receptors triggers both phospholipase C and adenylyl cyclase pathways in rat tail artery. Cardiovasc Res 65: 739–746.
12. Rocha-Pereira C, Sousa JB, Vieira-Rocha MS, Fresco P, Goncalves J, et al. (2013) Differential inhibition of noradrenaline release mediated by inhibitory A(1)-adenosine receptors in the mesenteric vein and artery from normotensive and hypertensive rats. Neurochem Int 62: 399–405.
13. Rocha-Pereira G, Arribas SM, Fresco P, Gonzalez MC, Goncalves J, et al. (2013) Impaired inhibitory function of presynaptic A1-adenosine receptors in SHR mesenteric arteries. J Pharmacol Sci 122: 59–70.
14. Okamoto K, Aski K (1963) Development of a strain of spontaneously hypertensive rats. Jpn Circ J 27: 282–293.
15. Trippodo NC, Frohlich ED (1981) Similarities of genetic (spontaneous) hypertension, Man and rat. Circ Res 48: 309–319.
16. Bobalova J, Mutafova-Yambolieva VN (2001) Co-release of endogenous ATP and noradrenaline from guinea-pig mesenteric veins exceeds co-release from mesenteric arteries. Clin Exp Pharmacol Physiol 28: 397–401.
17. Bobalova J, Bobal P, Mutafova-Yambolieva VN (2002) High-performance liquid chromatographic technique for detection of a fluorescent analogue of ADP-ribose in isolated blood vessel preparations. Anal Biochem 305: 269–276.
18. Levi T, Head RJ, Westfall DP (1984) High-pressure liquid chromatographic-fluorometric detection of adenosine and adenosine nucleotides: application to endogenous content and electrically induced release of adenyyl purines in guinea pig vas deferens. Anal Biochem 137: 93–100.
19. Todorov LD, Mihaylova-Todorova S, Craviso GL, Bjar RA, Westfall DP (1996) Evidence for the differential release of the co-transmitters ATP and noradrenaline from sympathetic nerves of the guinea-pig vas deferens. J Physiol 496 (Pt 3): 731–746.
20. Sneddon P, Burnstock G (1984) ATP as a co-transmitter in rat tail artery. Eur J Pharmacol 106: 149–152.
21. Burnstock G (2009) Purinergic co-transmission. Exp Physiol 94: 20–24.
22. De Fougantland D, Wachtchow DA, Costa M, Brooks SJ (2008) Immunohistochemical characterization of the innervation of human colonic mesenteric and submucosal blood vessels. Neurogastroenterol Motil 20: 1212–1226.