Adenosine enhances long-term the contractile response to angiotensin II in afferent arterioles

Authors
Andreas Patzak¹²*, En Yin Lai²*, Michael Fähling¹, Mauricio Sendeski¹, Peter Martinka¹, Pontus B. Persson¹, A. Erik G. Persson²

¹Institute of Vegetative Physiology, University Hospital Charité, Humboldt-University of Berlin, ²Department of Medical Cell Biology, Division of Physiology, University of Uppsala, Uppsala, Sweden,

* authors contributed equally to the work

Address for correspondence:
PD Dr. A. Patzak, Johannes-Müller-Institut für Physiologie, Humboldt-Universität zu Berlin, Universitätsklinikum Charité, Tucholskystr. 2, 10117 Berlin
Tel.: +49 450 528238, Fax.: +49 450 528972
e-mail: andreas.patzak@charite.de

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Abstract

Adenosine (Ado) enhances angiotensin II (Ang II) induced constrictions of afferent arterioles (Af) by receptor dependent and independent pathways. Here we test the hypothesis that transient Ado treatment has a sustained effect of Af contractility resulting in increased Ang II responses after longer absence of Ado. Treatment with Ado (cumulative from $10^{-11}$ to $10^{-4}$ mol/l) and consecutive washout for 10 or 30 min increased constrictions upon Ang II in isolated perfused Af. Cytosolic calcium transients upon Ang II were not enhanced in Ado treated vessels. Selective or global inhibition of A1- and A2-adenosine receptors did not inhibit the Ado effect. Nitrobenzylthioinosine (NBTI, Ado transport inhibitor), clearly reduced the Ado mediated responses. Selective inhibition of p38 mitogen activated protein kinase (MAPK) with SB203580 also prevented the Ado effect. Inosine treatment did not influence arteriolar reactivity to Ang II. Contractile responses of Af upon norepinephrine (NE) and endothelin 1 (ET-1) were not influenced by Ado. Phosphorylation of the p38 MAPK and of the regulatory unit of myosin light chain (MLC20) was enhanced after Ado treatment and Ang II in Af. However, phosphorylation of p38 MAPK induced by NE or ET-1 was reduced in vessels treated with Ado, while MLC20 was unchanged. The results suggest an intracellular, long lasting mechanism including p38 MAPK activation responsible for the increase of Ang II induced contractions by Ado. The effect is not calcium dependent and specific for Ang II. The prolonged enhancement of the Ang II sensitivity of Af may be important for the tubuloglomerular feedback.

Keywords

adenosine, angiotensin II, cytosolic calcium, p38 MAPK, afferent arterioles, tubuloglomerular feedback, kidney
Introduction

The role of adenosine (Ado) in the control of renal perfusion and filtration is incompletely understood. While Ado exerts only vasodilatory effects in most of vascular beds mediated by the subtypes $A_{2A}AR$ and $A_{2B}AR$ (8), constriction can also be found in the renal vasculature. This renal vasoconstrictor effect is mediated by Ado type 1 receptors ($A_1AR$). Recent studies hint at a dose dependent activation of both receptor types in the kidney, which results in either predominant constrictor or vasodilator effect of exogenously applied adenosine in different experimental models (18; 21). It was also shown that an $A_1AR$ mediated constrictor effect of Ado is probably restricted to afferent arterioles (10; 12; 13).

Ado is a candidate for mediation of the tubuloglomerular feedback in the kidney. Assumed involvement in the tubuloglomerular feedback basis on Ado’s ability to constrict afferent arterioles and finds further support by observing no tubuloglomerular feedback in $A_1AR$ deficient mice (3; 30). Angiotensin II (Ang II) acts as a modulating factor for the tubuloglomerular feedback. Angiotensin II type 1 receptor inhibition, lack of these receptors, or inhibition of the angiotensin converting enzyme reduce the tubuloglomerular feedback (for review see (27)). On the other hand, Ado influences the Ang II sensitivity of the renal microvasculature acting on $A_1AR$ and $A_2AR$ (18), suggesting an interplay of Ado and Ang II in the control of renal vascular resistance (9; 11; 23; 33).

In a recent study we showed a restoration of desensitized Ang II induced contractions by Ado in isolated perfused afferent arterioles (17). Ado treatment in between successive Ang II applications restored contraction up to the initial level of the Ang II application. Ado restored the Ang II response of afferent arterioles by a receptor independent mechanism which is caused by enhanced calcium sensitivity in these vessels.

In the present study, we tested the hypothesis of a prolonged action of Ado on the Ang II induced constriction in afferent arterioles. We show that transient Ado treatment induces a
significant amplification of the Ang II response of afferent arterioles for as long as 30 min after end of treatment. This effect of Ado is not mediated by Ado receptors, depends on Ado transportation into the cell, and includes p38 MAPK activation. Such a prolonged influence of Ang II induced contractions in arterioles by transient extracellular increase of Ado may help control renal filtration via the tubuloglomerular feedback.

**Methods**

**Animals**

Mice of the C57BL6 strain (male, body mass between 22 g and 28 g, Scanbur BK AB, Sollentuna, Sweden) were included. A1AR receptor deficient mice (A1(-/-)) and their controls (A1(+/+)), were included to test the effect of chronic lack of the A1AR receptor. These mice were female with a body mass between 23 g and 29 g. A1(-/-) were generated as described by Johansson et al. (15). A1(-/-) and A1(+/+) were siblings from mating between A1(+/-) mice. Animals were fed with standard mouse chow and allowed free access to tap water. All procedures conformed to the Guide for Care and Use of Laboratory Animals prepared by Institute for Laboratory Animal Research (ILAR). The local ethics committee for Uppsala University approved the procedures for this study.

**Dissection and perfusion of afferent arterioles**

Dissection and perfusion procedures have been described before (24). In brief: Outer cortical afferent arterioles of mice were dissected at 4°C in albumin-enriched Dulbecco’s modified Eagle’s medium (DMEM, 0.1%). Arterioles with their glomeruli were perfused in a thermo-regulated chamber (37°C) fixed on the stage attached to an inverted microscope (Nikon, Badhoevedorp, The Netherlands) using a perfusion system, which allows the adjustment of outer holding and inner perfusion pipettes (Vestavia Scientific, Vestavia Hills, AL, USA). The perfusion pipette, with a diameter at the tip of exactly 5µm, was connected to a reservoir
containing the perfusion solution. A second holding pipette was used to maintain the attached glomerular tuft. The pressure in the pressure head was 100mmHg, which corresponds to physiological pressure and flow (about 50nl/min) in the connected afferent arteriole (24). Criteria for using an arteriole consisted of a satisfactory, remaining basal tone and no vasodilatation. Both criteria were tested by rapidly increasing the perfusion pressure and assessing the change in the luminal diameter, which corresponds to transient constriction. A further criterion was a fast and complete constriction in response to KCl (100mmol/l) solution.

Measurement of isotonic contraction

The experiments were recorded by a video system, off-line digitized, and analyzed as described before (24). Luminal diameters of the arteriole were measured to estimate the effect of vasoactive substances. Since we did not see systematic differences in arteriolar responses to agonists, except for the part connected to the holding and perfusion pipette, the site with the strongest contraction was chosen for the measurement. In all series, the last 10 sec of a control or treatment period were used for statistical analysis of steady state responses.

Calcium imaging

Afferent arterioles were isolated, perfused, and loaded with Fura-2 AM (solved in DMSO, 10^{-5} mol/l) in the bath solution for 45 min. Loading was facilitated with Pluronic® F-127 (end concentrations of DMSO and Pluronic® F-127 were <0.1%). Fluorescence was measured using the digital imaging system QC-900 (Applied Imaging, Sunderland, UK). The arterioles were excited alternately at 340 nm and 380 nm, and the emission was measured at 510 nm. The 340/380 emission ratio was used to determine the cytosolic calcium concentration after calibration using Fura-2 Calcium Imaging Calibration Kit (F-6774) in vitro according to the protocol of Molecular Probes Inc.
Isolation of preglomerular vessels and phosphorylation studies

Preglomerular vessels (including mainly interlobular arteries and afferent arterioles) of mice were isolated using a modified iron oxide-sieving technique according to Chaudhari and Kirschenbaum (6). Modifications were (i) the method to perfuse the kidneys, which was performed via cannulation of the aorta, and (ii) the use of smaller sizes for the needles needed for separation of the tissue, as well as (iii) use of sieves with smaller pores (100 and 80 µm). Isolated, non-perfused preglomerular vessels were treated with physiological salt solution, to simulate Ado application (handling control, n=5), or with Ado (10⁻¹¹ to 10⁻⁴ mol/l, cumulatively, 2 min each concentration, n=5), or with Ado followed by 10 min washout and application of Ang II (10⁻⁸ mol/l, 2 min, n=10), norepinephrine (10⁻⁵ mol/l, 2 min, n=10) or ET-1 (10⁻⁸ mol/l, 2 min, n=10). Vessels were shock frozen exactly 2 min after the Ang II application in 10% TCA/ aceton and kept at -80°C.

Quantification of Myosine LC20- and p38 MAPK phosphorylation

Cellular extracts from preglomerular vessels were prepared as described in (17). Protein extracts were separated by SDS-PAGE and transferred to Hybond™-P membranes. Myosine regulatory light chain phosphorylation was detected using a pS19/pS20 specific anti-MLC antibody (Acris antibodies GmbH, R1535P). Membranes were stripped for 5 min with distilled water, 5-15 min 0.2 M NaOH, 5 min distilled water, and reprobed using an anti-phosphorylated p38 MAPK antibody (Calbiochem, #506119). Detection of relative smooth muscle specific alpha-actin levels using an anti-alpha-actin antibody (Acris antibodies GmbH, AB5694) served as loading control.
Solutions
DMEM/F12 with 10mmol/l HEPES (Invitrogen AB, Lidingö, Sweden) was used for dissection, bath and perfusion. The pH was adjusted to 7.4 after addition of bovine serum albumin (BSA). The concentration of BSA was 0.1% in dissection and bath solutions, and 1% in the perfusion solution. BSA was obtained from SERVA Electrophoresis (Heidelberg, Germany) and DMEM from Sigma-Aldrich (Munich, Germany). The K⁺-solution had the following composition: NaCl 20, KCl 95, NaHCO₃ 25, K₂HPO₄ 2.5, CaCl₂ 1.3, MgSO₄ 1.2, glucose 5.5 (mmol/l), and was equilibrated with 5% CO₂ in air.

Pharmacological agents
The following drugs were used: angiotensin II (Ang II), adenosine (Ado), norepinephrine (NE), endothelin 1, N⁶-cyclopentyladenosine (CPA), 8-(p-sulfophenyl)theophylline (8-SPT), 8-cyclopentyltheophylline (CPT), 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385), nitrobenzylthioinosine (NBTI), and SB203580 from Sigma-Aldrich (Stockholm, Sweden).

Statistics
Analysis of variance (ANOVA) for repeated measurements (non-parametric Brunner test) was used to test time-dependent changes in the arteriolar diameter and to check for differences between the groups (SAS system®). Post hoc comparisons were performed using Tuckey’s test. Wilcoxon tests were applied for treatment effects on basal diameter, for comparison of control diameters between groups and luminescence signals of Western blots. Data are presented as mean±SEM. The confidence level p was set to 0.05.
**Experimental protocols for contraction measurement and imaging**

**Effect of Ado on Ang II induced constriction:** Ado was applied in cumulative concentrations, each for 2 min, from $10^{-11}$ to $10^{-4}$ mol/l. After washout for 10 (n=5) or 30 min (n=5) Ang II was applied cumulatively from $10^{-12}$ to $10^{-6}$ mol/l (each dose 2 min). For manipulation control, physiological solution (DMEM + albumin) was applied instead of Ado followed by 10 min washout and measurement of an Ang II concentration response curve in another series of experiments (n=6). The control curve for the Ang II concentration response was measured by application of Ang II in cumulative concentrations from $10^{-12}$ to $10^{-6}$ mol/l in separate vessels (n=10).

**Specificity of the Ado effect:** Ado was applied in cumulative doses from $10^{-11}$ to $10^{-4}$ mol/l. After washout for 10 min, the norepinephrine (NE) concentration response ($10^{-9}$ to $10^{-5}$ mol/l, n=5) or the endothelin 1 (ET-1) concentration response ($10^{-12}$ to $10^{-7}$, n=6) were measured. For control, the concentration response curves were measured for both substances, but without Ado pre-treatment in separate arterioles (NE: n=5, ET-1: n=8).

**Role of Ado receptors:** The selective A1AR inhibitor CPT alone ($10^{-5}$ mol/l, n=5), CPT together with the A2AR inhibitor ZM241385 ($10^{-7}$ mol/l, n=6), or the non-selective Ado receptor inhibitor 8-SPT ($5*10^{-5}$ mol/l, n=6) were applied simultaneously with Ado. Inhibitors were also present during the washout period of 10 min. In another series, inosine ($10^{-11}$ to $10^{-4}$ mol/l, n=6), which acts on A3AR was applied instead of Ado followed by washout of 10 min. In all these series, Ang II was administered cumulatively from $10^{-12}$ to $10^{-6}$ mol/l. Further, the effect of A1AR stimulation was tested by application of the selective A1AR agonist CPA ($10^{-11}$ to $10^{-6}$ mol/l (n=6)), followed by 10 min washout. The efficacy of the receptor agonist and inhibitors has been shown in mouse arterioles. CPA induced a constrictor response, and CPT inhibited the constrictor effect of Ado effectively, while inhibition of A2AR receptor with ZM241385 (all drugs with same concentration as in the present study) induced
concentration dependent constrictor responses to Ado (18). The ability of 8-SPT to block adenosine receptors in the concentration used here was also shown in mouse arterioles (18).

**Contribution of cytosolic calcium:** To test whether the enhancement of the Ang II response of the arterioles is calcium dependent, cytosolic calcium transients were measured using load with Fura-2. Transients were obtained for Ang II concentrations of 10^{-10} and 10^{-8} mol/l with (n=9 and n=6, respectively) and without Ado pre-treatment (n=7 and n=8, respectively).

**Role of Ado transport through the membrane:** Since Ado receptors participated only to a small amount in mediation of the Ado effect, it was tested whether transport of Ado through the cell membrane by an equilibrative transporter plays a role. Arterioles were treated with nitrobenzylthioinosine (NBTI, 3x10^{-7} mol/l) during extracellular Ado application. After washout, the Ang II concentration response curve was measured (n=5). To exclude a non-specific effect of NBTI on arteriolar constriction, arterioles were treated with NBTI alone and the Ang II concentration response curve was measured after 10 min washout (n=5).

**Intracellular action of Ado:** To test a contribution of p38 MAPK to the Ado effect, the selective inhibitor of this enzyme SB203580 (10^{-5} mol/l) was applied 10 min before and during the Ado treatment, then SB203580 and Ado were washed out (10 min), and the Ang II concentration response was measured (n=5).

All drugs were added to the bath solution.

**Results**

**Long-term effect of Ado on Ang II induced contractions**

Ado induced modest changes in arteriolar diameter during cumulative application, which agrees with results of a recent study (18). There was no significant change in the diameter at the end of the cumulative application, namely at Ado 10^{-4} mol/l, except for a slight tendency to greater diameters. Washout of Ado re-established control diameters. The Ang II response of afferent arterioles was significantly stronger after Ado pre-treatment compared with the
Ang II concentration curve without Ado pre-treatment, especially at low Ang II concentrations (ANOVA, p<0.05, Fig. 1). The enhancement of the Ang II response was similar for washout times of 10 and 30 min (Fig. 1). Time control experiments revealed no effect of the procedure on the Ang II concentration response of arterioles (Fig. 1).

Specificity of the Ado effect on the contractility

The concentration responses to NE and ET-1 were not influenced by an Ado treatment (Fig. 2 and 3).

Role of Ado receptors for the enhancement effect

Using selective and non-selective inhibitors of Ado receptors, their possible contribution to the Ado effect was tested. CPT, a selective A₁AR inhibitor, did not influence luminal diameters in the control situation. It did not prevent the augmented Ang II response when applied simultaneously with Ado (ANOVA, p<0.05, Fig. 4). Combination of CPT and ZM241385, which is a selective A₂AAR inhibitor, also had no influence of arteriolar diameters, and did not prevent an increase of the arteriolar constriction upon lower Ang II concentrations, i.e. the Ang II response was enhanced compared to the control Ang II response in the low concentration range. For higher Ang II concentrations the constriction corresponded to that of the control Ang II response (Fig. 4). Inosine, an A₃AR agonist did not influence the Ang II response (Fig. 4). The non-selective inhibition of Ado receptors with 8-SPT did not affect the Ado induced enhancement of the Ang II response (Fig. 4). This substance had no effect on arteriolar diameters in the control situation. To further investigate the role of A₁AR arterioles were pre-treated with CPA. This A₁AR agonist constricted the arteriole concentration dependent. The diameter was reduced to 89.9±3.9% at 10⁻⁶ mol/l CPA (ANOVA, p<0.05). Diameters returned to control values during the washout period. The Ang II concentration response was significantly attenuated at Ang II concentrations of 10⁻⁸ and 10⁻⁷ mol/l compared to the control Ang II response (ANOVA, p<0.05, Fig. 5).
In A1(-/-) mice as well as in their control wild types (A1(+/+)), Ado treatment induced a significant enhancement of the Ang II response (ANOVA, p<0.05, Fig. 6), similar to that observed in the C57BL6 mice (cf. Fig. 1).

**Contribution of cytosolic calcium**

Ang II application of 10^{-10} and 10^{-8} mol/l increased cytosolic calcium concentrations by 21.4±4.9% and 90.7±22.3%, respectively. Calcium transients did not significantly change after Ado pre-treatment (33.6±11.3% and 86.4±15.2% for 10^{-10} and 10^{-8} mol/l Ang II, respectively, Fig. 7 and Fig. 8).

**Role of Ado transport through the membrane**

Blockade of the Ado transport into the cell using NBTI during extracellular Ado application inhibited the Ado effect on the Ang II response significantly (Fig 9, ANOVA, p<0.05). NBTI itself did not change arteriolar diameters in the control situation (not shown), and did not change the Ang II response significantly (Fig. 9).

**Intracellular action of Ado**

Selective inhibition of the p38 MAPK with SB203580 prevented the enhancing effect of Ado on the Ang II induced constriction in afferent arterioles (Fig. 10). The response to Ang II did not differ from the control curve for Ang II (cf. Fig. 1).

Investigation of p38 MAPK using western blot technique showed increased phosphorylation of p38 MAPK after treatment with Ado. The phosphorylation was enhanced 10 min after finishing the treatment with Ado and following washout compared to the untreated situation (Fig. 11). Ado treatment also enhanced the phosphorylation of p38 MAPK after exposure to Ang II compared to non-treated vessels (Fig. 12 A). However, the phosphorylation of the p38 MAPK was reduced in Ado treated arterioles, which were subjected to ET-1 and NE (Fig 12 A, B).

Ado treated vessels demonstrated a greater phosphorylation of the MLC20 after Ang II application in comparison to non-treated vessels (Fig. 12 A). In contrast, there was no
significant difference in the MLC_{20} phosphorylation in Ado treated vessels after ET-1 and NE application (Fig. 12 B,C).

**Discussion**

The present study shows that a transient Ado application enhances Ang II vasoconstriction of afferent arterioles. The effect sustains for 10 to 30 min in the absence of Ado. Ado receptors are not critically involved in the mediation of this Ado effect. Ado enters the cell and influences intracellular signaling which involves the p38 MAPK pathway. The finding provides a new perspective on the role of Ado in the control of kidney perfusion.

In addition to the well known Ado-Ang II interactions in the renal vasculature mediated by their receptors, it has been shown that Ado can also restore contraction of Ang II desensitized afferent arterioles. This latter effect takes place by a non-receptor mediated increase of the calcium sensitivity (17). Here we show a clear long lasting increase of the Ang II response after Ado treatment, which is equally receptor independent. The enhanced contractile response to Ado is reflected by an increase in the MLC_{20} phosphorylation.

Both, receptor dependent and independent effects of Ado on the Ang II response may contribute to the tubuloglomerular feedback in the kidney. The tubuloglomerular feedback mechanism links distal tubular chloride load and filtration rate of the same glomerulus: Larger sodium chloride load results in an increased release of Ado or ATP (and subsequent increased interstitial generation of Ado from ATP) from macula densa cells (4; 28). Ado then constricts arterioles by acting on A_{1}AR (10). Data of the present study suggest that a transient elevation of Ado concentration sensitizes the response to Ang II dramatically for at least 30 min. This could result in a prolonged change of glomerular hemodynamics and consequently of the filtration rate, since the tone of the afferent arteriole is increased for a given Ang II concentration in the blood and kidney, respectively. However, the effect of transient
elevations of Ado concentrations on efferent arterioles has not been investigated yet, which limits conclusions regarding the renal resistance and filtration rate.

Interactions of Ado and Ang II in the renal vasculature are well known. In general, constrictor effects of Ang II are enhanced by Ado and vice versa (9; 11; 13; 18; 33). A1AR, A2AR, and AT1 are involved in this interaction (11; 18). In contrast to these findings of a receptor mediated, specific interplay of both substances, the enhancement of the Ang II response in the present study did not, or only to a small degree, involves Ado receptors. Neither selective inhibition of A1AR, A1AR and A2AR together, nor general blockade of Ado receptors prevented the Ado effect on the Ang II response of arterioles. Also, genetically induced lack of A1AR did not influence the increased Ang II response after Ado. Further, A3AR are also not involved, since the specific agonist inosine did not affect Ang II responses. CPA pre-treatment did not mimic the Ado effect supporting the conclusion that A1AR receptors are not involved. Rather, the Ang II response was attenuated at 10^-8 and 10^-7 mol/l. This observation suggests that A1AR activation can have a long-term influence on the contractility. This effect might be neutralized by the simultaneous activation of A2AR during Ado treatment.

Remarkably, the cytosolic calcium transients upon Ang II after Ado treatment do not correspond to the enhanced Ang II response, but behave like control arterioles. This finding suggests a calcium sensitizing mechanism enhancing the Ang II induced constriction. In a previous study, Lai et al. found re-sensitization of Ang II responses of afferent arterioles to repeated Ang II applications induced by Ado treatment between the applications (17). The re-sensitization of the contraction was not due to receptor related mechanisms. Rather, the calcium sensitivity was increased and went along with increased phosphorylation of the regulatory unit of the myosin light chain. Inhibition of p38 MAPK prevented the effect of Ado in their study. We show here that the selective p38 MAPK inhibitor SB203580 also prevents the long term Ado effect on Ang II induced constrictions. It was shown that SB203580 does not influence the Ang II induced constriction of afferent arterioles not treated
with Ado (17). Further, the phosphorylation of p38 MAPK determined by Western blotting was enhanced in Ado treated preglomerular vessels after Ang II application. This observation hints at a contribution of this kinase in mediating Ado effects on Ang II induced contractions. The p38 MAPK is involved in cell responses to several stimuli, which act as stressors (for review see (25)). Ado, which concentration increases in hypoxic and ischemic tissues, activates p38 MAPK as it was shown in the present study in arterioles and in myocardial tissue shown in other studies (1; 14). Apart from a receptor mediated activation (29), the present study suggests that Ado can activate p38 MAPK via intracellular action. The p38 MAPK kinase may in turn activate a signaling pathway resulting in an increased phosphorylation of MLC\textsubscript{20} after Ang II application. Ado and Ang II seem to act collectively in the activation of p38 MAPK, which stimulates MAPK-activated protein (MAPKAP) kinase-2, resulting in the phosphorylation of heat shock protein 27 (20). This induces inhibition of the myosin light chain phosphatase and an increase of MLC\textsubscript{20} phosphorylation.

Remarkably, the phosphorylation of p38 MAPK was reduced in vessels treated with Ado and then subjected to ET-1 or NE. This observation was not expected, since Ado, NE as well as ET-1 increase the phosphorylation of p38 MAPK in vascular smooth muscle cells (19; 22). Whilst the combination of Ado and Ang II, latter also activates p38 MAPK (31), results in increased p38 MAPK phosphorylation, Ado combined with NE and ET-1 decreases it compared to vessels not treated with Ado. This suggests different intracellular pathways of p38 MAPK activation for NE or ET-1 compared to Ang II. It has been shown that Ang II uses redox-sensitive mechanisms (20), while the NE induced activation is dependent on the calcium influx. The response to ET-1 was dependent on a herbacine A sensitive tyrosine kinase and calcium influx in a study in small arteries (22). Ado treatment did not influence the phosphorylation of the MLC\textsubscript{20} in combination with NE or ET-1 in the preglomerular vessels. This agrees with the lack of influence of Ado on NE or
ET-1 induced constrictions of the afferent arterioles. One reason may be the decreased p38 MAPK phosphorylation observed for this combination of drugs.

Data indicate that the long-term Ado effect requires a transportation of Ado through the cell membrane into the cytosol. The transport, and thus the enhancement of the Ang II response are inhibited by nitrobenzylthioinosine (NBTI), which blocks an important part of the Ado transporters. These transporters normally equilibrate the Ado concentrations inside and outside of the cell, i.e. the direction of transport depends on the concentration gradient (32). The efficacy of NBTI to inhibit the adenosine transport has been shown in freshly isolated porcine coronary smooth muscle cells and cultured human coronary smooth muscle cells (7). Concentrations of $3 \times 10^{-7}$ mol/l NBTI as used in the present study reduced the adenosine uptake to 20% of the value without NBTI in the cited study. Further, NBTI significantly reduced the adenosine uptake in the mouse cardiomyocyte cell line HL-1 (5) and in the porcine coronary smooth muscle (26).

In the present study, Ado was applied in concentrations from $10^{-11}$ to $10^{-4}$ mol/l, each for 2 min. To estimate whether there is a netto inward Ado flow, or not, the tissue concentrations of Ado must be known. Measurements of intra- and extracellular Ado concentrations in different models produced variable results. In vitro studies using the microdialysis technique showed values of $2 \times 10^{-7}$ mol/l in the interstitium of the rat renal cortex (2). Tissue concentration of Ado in the rat kidney was measured to be $4 \times 10^{-6}$ mol/kg kidney wet weight in another study (16). Investigations of the NBTI sensitive nucleoside transporter in freshly isolated smooth muscle cells of porcine coronary arteries also provide estimates on Ado concentrations. The $K_m$ of these transporter are about $4-5 \times 10^{-6}$ mol/l (7). The Ado concentrations were comparably higher at the end of the cumulative application of the present study. Therefore, an inward transportation and transient increase of the intracellular Ado concentration must be assumed.

The long-term influence of transient increases of extracellular Ado concentrations on Ang II response in afferent arterioles seems to be specific for Ang II, since the responses upon
norepinephrine and ET-1 were not affected by Ado treatment. This observation suggests specificity of the mechanisms involved in the process of enhancement of the Ang II response. Since receptors do not play an important role, interactions at the receptor level can be excluded. The influence of Ado on the Ang II induced contraction most likely includes Ang II specific intracellular signalling pathways, via protein kinase cascades, enhancing the calcium sensitivity of the contractile machinery in the arterioles.

In summary, the present study suggests a specific, long-term or “memory effect” of transient extracellular Ado on the Ang II mediated constriction in smooth muscle cells of afferent arterioles. Adenosine contributes to the mediation of the tubuloglomerular feedback, i.e. it constricts afferent arterioles and reduces the filtration rate in response to increased sodium chloride load in the distal tubule. An enhanced reactivity of afferent arterioles to Ang II for a longer time after a transient increase of Ado may maintain reduced filtration rate. The Ado effect critically depends on transportation of Ado into the cell, does not require the action on Ado receptors, and includes the activation of p38 MAPK.

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Legends:

Fig. 1: Angiotensin II (Ang II) concentration response curve and long-term effect of Adenosine (Ado, A: absolute diameters, B: relative diameters compared to the control diameter) in afferent arterioles. Ado was washed out for 10 or 30 min after treatment. The Ang II concentration response was significantly enhanced for both situations ‘*’ indicate significant differences of the curve compared to the control Ang II curve. The “procedure” control curve (Ang II after DMEM) suggests no influence of manipulation during Ado treatment (see also text). § and $ indicates significant differences for single Ang II concentrations 10 and 30 min after wash out, respectively, compared to the control Ang II response.

Fig. 2: Norepinephrine (NE) concentration response without Ado treatment as well as after treatment and washout of 10 min in afferent arterioles. The responses do not differ from each other.

Fig. 3: Endothelin-1 (ET-1) concentration response without Ado treatment as well as after treatment and washout of 10 min in afferent arterioles. The responses do not differ from each other.

Fig. 4: The selective A1AR inhibitor CPT alone, CPT together with the A2AAR inhibitor ZM241385, or the non-selective Ado receptor inhibitor 8-SPT did not prevent the adenosine induced enhancement of the Ang II response in afferent arterioles. However, application of antagonists and their combinations resulted in slightly different Ang II response patterns. Inosine, an A3AR agonist has no effect on the Ang II response in afferent arterioles.
“*” indicate a significant difference in the response compared to control Ang II response (ANOVA). “§” indicate differences for CPT treated, “$” differences for CPT + ZM241385 treated, and “&” differences in the constriction compared to control Ang II response (same data as in Fig. 1).

Fig. 5: Effect of application of the selective A1AR agonist CPA followed by 10 min washout on the Ang II response of afferent arterioles. “*” indicate significant differences in the Ang II response compared to control Ang II response (ANOVA), § indicates significant differences for distinct Ang II concentrations compared to that of the control Ang II response (same data as in Fig. 1).

Fig. 6: Effect of adenosine treatment and 10 min washout on the Ang II response in mice deficient for the adenosine type 1 receptor (A1(-/-)) and their littermate wild types (A1(+/+)). “*” indicate significant differences in the Ang II response compared to the Ang II response in C57BL6 mice (same data as in Fig. 1). “§” and “$” indicate differences for distinct Ang II concentrations in A1(+/-) and A1(-/-), respectively, compared to control Ang II responses.

Fig. 7: Imaging of intracellular Ca^{2+} in an afferent arteriole loaded with Fura-2. The left panels show the afferent arteriole and parts of the glomerulus (bright mass in lower part of the picture). Intensity of emission is given for excitation wave lengths of 340 and 380 nm (left panel) during control situation and after application of Ang II. Arterioles were pretreated with adenosine (10^{-5} mol/l). Right panel demonstrates Ca^{2+}-transients measured in the wall of the arteriole (area of measurement is indicated by the frame in the upper left figure).

Fig. 8: Changes in cytosolic calcium concentrations upon extracellular application of Ang II in concentrations of 10^{-10} and 10^{-8} mol/l for untreated and adenosine (Ado) pretreated afferent
arterioles. Note significant differences in calcium transients comparing $10^{-8}$ and $10^{-10}$ mol/l Ang II (indicated by *). Ado does not influence calcium transients.

Fig. 9: Transport of Ado through the cell membrane by equilibrative transporters was blocked by NBTI. Transport blockade inhibits the Ado effect on Ang II responses, but does not influence Ang II contractions itself. * indicates a significant difference in the course of the concentration response to Ang II after Ado pre-treatment compared to Ang II without pre-treatment (cf. Fig. 1).

Fig. 10: Selective inhibition of the p38 MAPK with SB203580 prevents the effect of Ado on the Ang II induced contraction in afferent arterioles (cf. Fig. 1). The response does not differ significantly from that of the control group (same data as in Fig. 1).

Fig. 11: Effect of Ado on the phosphorylation of p38 MAPK. “*” indicates a significant difference compared to the value without Ado treatment.

Fig. 12: Representative example of Western Blotting signals for the effect of adenosine (Ado) on phosphorylation of p38 mitogen activated protein kinase (p38 MAPK phos) and myosin light chain (MLC(20) phos) in preglomerular arterioles treated with angiotensin II (ANG II, Fig. 12 A), endothelin 1 (ET1, Fig. 12 B), or norepinephrine (NE, Fig 12 C). The figures show the chemiluminescence-signal of the p38 MAPK phos and MLC(20) phos, the signal for smooth muscle alpha-actin (alpha-actin), and mean of relative values of phosphorylated p38 MAPK and MLC$_{20}$ protein, respectively.

“*” indicates a significant difference compared to the value without Ado treatment, while “n.s.” indicates no significant differences.
Fig 1A

217x337mm (150 x 150 DPI)
Fig 2A
256x337mm (150 x 150 DPI)
Fig 3B
254x337mm (150 x 150 DPI)
Fig 4A
256x337mm (150 x 150 DPI)
Fig 4B
261x337mm (150 x 150 DPI)
Fig 5A

Luminal diameter (µm)

Ang II (log mol/l)

- Ang II
- Ang II after CPA

con -12 -11 -10 -9 -8 -7 -6

Fig 5A
256x337mm (150 x 150 DPI)
Fig 6A
217x337mm (150 x 150 DPI)
Fig 6B

261x337mm (150 x 150 DPI)
Fig 7
190x160mm (300 x 300 DPI)
Fig 8

305x240mm (150 x 150 DPI)
Fig 9B

280x337mm (150 x 150 DPI)
Fig 10A
256x337mm (150 x 150 DPI)
Fig 10B
254x337mm (150 x 150 DPI)
Fig 11
335x270mm (150 x 150 DPI)
Fig 12
194x511mm (150 x 150 DPI)