

# Adenosine Restores Angiotensin II–Induced Contractions by Receptor-Independent Enhancement of Calcium Sensitivity in Renal Arterioles

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**Abstract**—Adenosine is coupled to energy metabolism and regulates tissue blood flow by modulating vascular resistance. In this study, we investigated isolated, perfused afferent arterioles of mice, which were subjected to desensitization during repeated applications of angiotensin II. Exogenously applied adenosine restores angiotensin II–induced contractions by increasing calcium sensitivity of the arterioles, along with augmented phosphorylation of the regulatory unit of the myosin light chain. Adenosine restores angiotensin II–induced contractions via intracellular action, because inhibition of adenosine receptors do not prevent restoration, but inhibition of NBTI sensitive adenosine transporters does. Restoration was prevented by inhibition of Rho-kinase, protein kinase C, and the p38 mitogen-activated protein kinase, which modulate myosin light chain phosphorylation and thus calcium sensitivity in the smooth muscle. Furthermore, adenosine application increased the intracellular ATP concentration in LuciHEK cells. The results of the study suggest that restoration of the angiotensin II–induced contraction by adenosine is attributable to the increase of the calcium sensitivity by phosphorylation of the myosin light chain. This can be an important component of vascular control during ischemic and hypoxic conditions. Additionally, this mechanism may contribute to the mediation of the tubuloglomerular feedback by adenosine in the juxtaglomerular apparatus of the kidney. (*Circ Res.* 2006;99:1117-1124.)

**Key Words:** adenosine ■ angiotensin II ■ desensitization ■ calcium sensitivity ■ myosin light chain  
■ afferent arterioles ■ kidney

Several *in vivo* studies as well as investigations in isolated afferent arterioles suggest an interplay of adenosine (Ado) and angiotensin II (Ang II) in the control of renal vascular resistance.<sup>1–5</sup> Ang II induces constriction in renal arteries and microvasculature via angiotensin type 1 receptors (AT<sub>1</sub>ARs),<sup>6</sup> whereas Ado exerts a vasoconstrictive effect via Ado type 1 receptors (A<sub>1</sub>ARs) and dilation via A<sub>2</sub>ARs.<sup>7</sup> Ado enhancement of Ang II–induced vasoconstriction is commonly observed. However, a recent study has shown augmentation as well as blunting of the Ang II response of afferent arterioles by Ado acting on both A<sub>1</sub>ARs and A<sub>2</sub>ARs.<sup>5</sup> The amplification of Ang II responses by Ado is probably mediated by a common action of second messenger pathways of both AT<sub>1</sub>ARs and A<sub>1</sub>ARs on phospholipase C.<sup>7</sup> This enhancement of the contractile response on Ang II may play a significant role in the control of perfusion and filtration rate of the kidney, including the tubuloglomerular feedback (TGF).

AT<sub>1</sub>ARs belong to the G protein–coupled receptor family. They are frequently subjected to desensitization,<sup>8</sup> ie, repeated application of the agonist diminishes the response of the smooth muscle cells.<sup>9</sup> One common mechanism of desensitization of G protein receptors is the phosphorylation resulting in uncoupling from the G protein, internalization, and recycling of the protein.<sup>8</sup> Downregulation of the receptor seems to be another, long-term mechanism for desensitization.<sup>10,11</sup>

Recent investigations suggest an intracellular, vascular tone–enhancing action of Ado in smooth muscle cells.<sup>12</sup> In the present study, we therefore investigated a possible receptor independent effect of Ado on the desensitization of Ang II–induced contractions in the renal microvasculature. We show that Ado completely restores the response of isolated, perfused afferent arterioles on repeated applications of Ang II in mice. The restoration of the contractile response is not mediated by Ado-sensitive P1 receptors. In fact, Ado enters the cell and enhances the Ca<sup>2+</sup> sensitivity of the

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contractile machinery. The p38 mitogen-activated protein kinase (MAPK) and possibly Rho-kinase and protein kinase C, perhaps in conjunction with an increased intracellular ATP, participate in this process.

## Materials and Methods

### Animals

Mice of the C57BL6 strain (male; body mass, 22 to 28 g; Scanbur BK AB, Sollentuna, Sweden) were used. 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. 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.<sup>13</sup> Criteria for using an arteriole consisted of a good remaining basal tone and no pronounced 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 (100 mmol/L) solution.

### Measurement of Isotonic Contraction

The experiments were recorded by a video system, off-line digitized, and analyzed as described previously.<sup>13</sup> Each arteriole was only used in 1 protocol, ie, no successive protocols were performed. This design is based on our observation of sustained effects of agents even when the control diameter was reestablished after the washout. Solutions used for dissection and perfusion and bath were the same as described before.<sup>13</sup>

### Calcium Imaging in Perfused Arterioles

Afferent arterioles were isolated and perfused as described above. Vessels were loaded with Fura-2 AM (solved in DMSO,  $10^{-5}$  mol/L) in the bath solution for 45 minutes. Loading was facilitated with Pluronic F-127 (end concentrations of DMSO and Pluronic F-127 were <0.1%). The perfusion system and the experimental chamber were both mounted on the stage of an inverted microscope (Nikon Eclipse). 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 nm/380 nm emission ratio was used to determine the intracellular 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.<sup>14</sup> Modifications were (1) the method to perfuse the kidneys, which was performed via cannulation of the aorta; and (2) the use of smaller sizes for the needles needed for separation of the tissue; as well as (3) use of sieves with smaller pores (100 and 80  $\mu$ m). Isolated nonperfused preglomerular vessels were treated 5 times with Ang II ( $10^{-8}$  mol/L; 2 minutes) separated by 10-minute washout periods with and without Ado treatment. Vessels were shock frozen exactly 2 minutes after the last Ang II application in 10% trichloroacetic acid/acetone and kept at  $-80^{\circ}\text{C}$ .

### Quantification of Myosin LC<sub>20</sub> Phosphorylation

The frozen tissue samples (preglomerular vessels) were thawed and washed 3 times with acetone at room temperature. The tissue was air dried and proteins were extracted at  $95^{\circ}\text{C}$  in buffer (25 mmol/L Tris, 200 mmol/L glycine, 0.1% SDS) for 30 minutes under vigorous

shaking. Protein extracts were separated by SDS-PAGE and transferred to Hybond-P membranes. Myosin regulatory light chain phosphorylation was detected using a pS19/pS20-specific anti-myosin light chain (anti-MLC) antibody (R1535P; Acris Antibodies GmbH). Detection of relative smooth muscle specific  $\alpha$ -actin levels using an anti- $\alpha$ -actin antibody (AB5694; Acris Antibodies GmbH) served as loading control.

### Measurement of Intracellular ATP

#### Molecular Cloning

A plasmid pINpip2 containing the luciferase gene and an IRES element and puromycin resistance gene was constructed. The *NotI/XhoI* cassette from pIRESpuo2 (catalog no. 6937-1; Clontech) was inserted in a pGL3-derived (Promega) plasmid at the *SalI/FseI* using an *FseI/NotI* adapter. The adapter was generated by hybridizing the 2 oligos: ada1, 5'-CAGCCTGCAGCGC-3'; and ada2, 5'-GGCCGCGCTGCAGGCTGCCGG-3'.

#### Generation of LuciHEK Cells

The luciferase-containing plasmid pINpip2 was linearized by restriction digestion with *FspI* and cotransfected with pHyEGFP (catalog no. 6014-1; Clontech) at a ratio of 20:1 in HEK 293 cells (CRL-1573; American Type Culture Collection) using FuGENE 6 transfection agent (catalog no. 1815075; Roche). Cell culture was maintained in medium (DMEM high glucose, FCS 10%, HEPES, P/S/G), and the cells were preselected with hygromycin (1 g/L) over 2 weeks. Further selection was performed with puromycin (2 g/L) over 3 weeks. Stable luciferase gene expression was achieved.

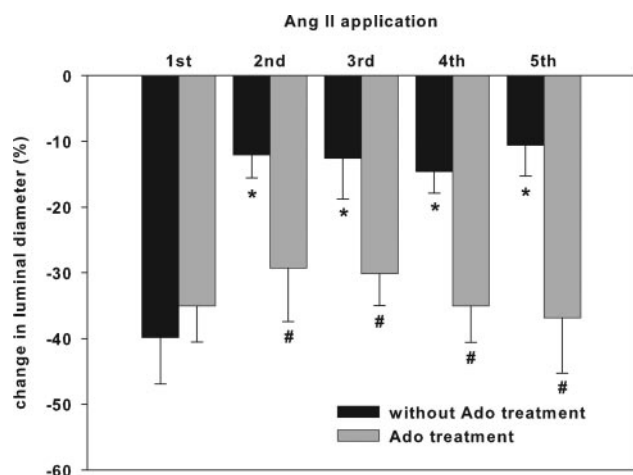
#### Protocol of ATP Measurement

The reaction of D-luciferin to oxyluciferin is ATP dependent. D-Luciferin (catalog no. S039; Synchem, Felsberg/Altenburg, Germany) solubilized in PBS (without calcium and magnesium; PAA, Linz, Austria) 60 mg/100 mL was added to the medium of LuciHEK cells at a ratio of 1:1.

Luminescence was measured 10 minutes after adding D-luciferin (C2400-40; Hamamatsu) and integrated for 5 minutes. Then Ado (final concentration of  $2.5 \times 10^{-5}$  mol/L in PBS) with and without NBTI (final concentration  $10^{-7}$  mol/L in PBS) was added to the test wells. The control wells did not receive a treatment, only the PBS carrier. Luminescence was again integrated for 5 minutes. Integrated light density (ILD) was quantified with a software (Aida-Quantification Software; Raytest, Straubenhardt, Germany). The ILD ratio was defined as the ratio of ILD of treatment period and ILD of pretreatment period.

### Pharmacological Agents

The following drugs were used: Ang II; Ado; 8-(*p*-sulfophenyl)theophylline (8-SPT); *S*-(4-nitrobenzyl)-6-thioinosine (NBTI); ethyleneglycol-bis( $\beta$ -aminoethyl)-*N,N,N',N'*-tetraacetic acid (EGTA); 1,2-dimethoxy-12-[1,3]benzodioxolo[5,6-*c*]phenanthridinium chloride (Chelerythrine); (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632); (4-(4-fluorophenyl)-2-(4-methylsulfinyl)-5-(4-pyridyl)1*H*-imidazole) (SB-203580); and 8,8'-[carbonylbis(imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)carbonylimino)]bis-1,3,5-naphthalenetrisulfonic acid hexasodium (suramin, P2 receptor antagonist) from Sigma-Aldrich (Stockholm, Sweden). 4-(2-[7-Amino-2-(2-furyl) [1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385, selective A<sub>2A</sub>AR antagonist) and *N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS1706, selective A<sub>2B</sub>AR antagonist) were from Tocris Cookson Ltd (Bristol, UK). 1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy) ethane-*N,N,N',N'*-tetraacetic acid (Fura-2 AM) and Fura-2 Calcium Imaging Calibration Kit (F-6774) were from Calbiochem (VWR International AB, Stockholm, Sweden); Pluronic F-127 was from Molecular Probes (AB Göteborgs Termometerfabrik, Sweden).



**Figure 1.** Desensitization of Ang II-induced constrictions (relative changes in the luminal diameter from the first to the fifth application) in afferent arterioles (black bars). Ado treatment in between the successive applications restored the response almost completely (gray bars). \*Significant differences compared with the first application; #significant differences between Ado-treated and untreated arterioles.

### Statistics

ANOVA for repeated measurements (nonparametric Brunner test) was used to test time-dependent changes in the arteriolar diameter and to determine differences between the groups (R-program, <http://www.ams.med.uni-goettingen.de/de/sof/ld/makros.html>). Post hoc comparisons were performed using Tukey's test. Wilcoxon tests were applied for the comparison of treatment effects on basal diameter and for comparison of control diameters between groups. Data are presented as mean  $\pm$  SEM. The confidence level  $\alpha$  was set to 0.05.

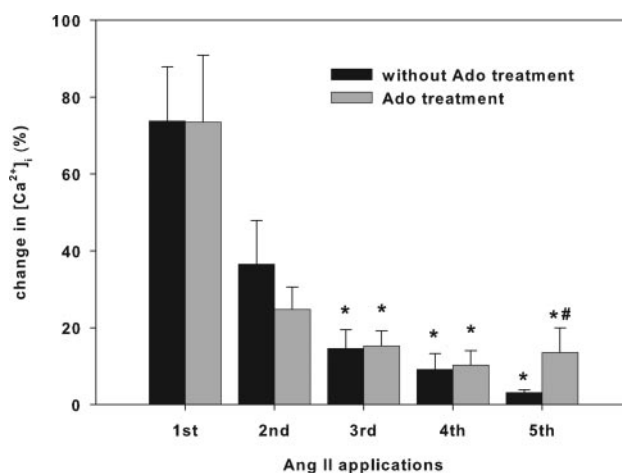
## Results

### Desensitization of Ang II Responses and $\text{Ca}^{2+}$ Transients and Restoration of Contraction by Ado

Successive applications of Ang II ( $10^{-8}$  mol/L, for 2 minutes, 10 minutes washout between applications) from bath side resulted in a clear desensitization of the Ang II-induced constriction beginning with the second application in the isolated perfused arterioles ( $n=5$ ; ANOVA,  $P<0.05$ ; Figure 1). In a next series, Ado ( $10^{-5}$  mol/L) was applied during the Ang II washout period (10 minutes) and then removed from the bath immediately before the following Ang II application. Ado did not significantly change arteriolar diameters during the 10 minutes treatment. However, Ado treatment restored the contractile Ang II response ( $n=5$ ; ANOVA,  $P<0.05$ ; Figure 1). Ado ( $10^{-8}$  mol/L) had no significant effect on the contractile response of afferent arterioles on repeated application of Ang II ( $n=5$ , data not shown). The  $\text{Ca}^{2+}$  imaging experiments showed increased intracellular  $\text{Ca}^{2+}$  concentration in the wall of afferent arterioles on the Ang II application (Figure 2). These  $\text{Ca}^{2+}$  transients diminished during subsequent Ang II applications ( $n=15$ ; ANOVA,  $P<0.05$ ). Ado treatment did not change  $\text{Ca}^{2+}$  transients significantly, except for the fifth Ang II applications, in which Ado resulted in a small but significant increase ( $n=11$ ; Figure 2).

### Role of Ado Receptors and P2 Receptors

To test the role of Ado receptors for the resensitization, the nonselective inhibitor 8-SPT was used. Figure 3A shows the



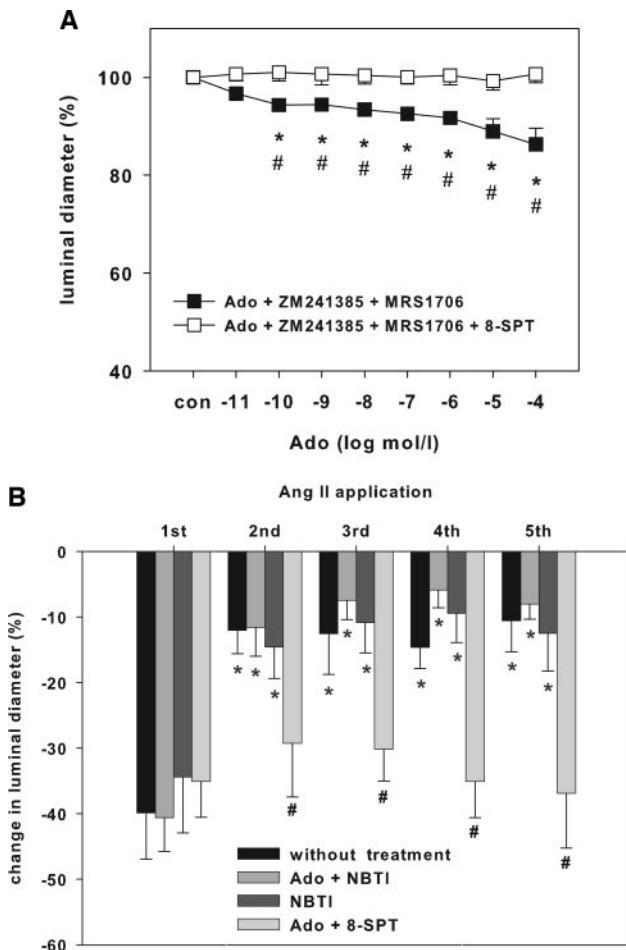
**Figure 2.** Relative changes in the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) in the afferent arteriolar wall (measured using the fluorescent  $\text{Ca}^{2+}$  indicator Fura-2).  $[\text{Ca}^{2+}]_i$  decreases on successive Ang II applications (black bars). There was no effect of Ado treatment in between successive Ang II applications on  $[\text{Ca}^{2+}]_i$  transients, except for the fifth application (gray bars). \*Significant differences compared with the first Ang II application; #significant differences between Ado-treated and untreated arterioles.

ability of 8-SPT ( $n=6$ ) to completely block  $\text{A}_1\text{AR}$ -mediated vasoconstriction ( $n=7$ ) in afferent arterioles. Intriguingly, restoration of repeated, Ang II-induced contractions was not affected by the inhibition of Ado receptors with 8-SPT ( $5 \times 10^{-5}$  mol/L)<sup>15</sup> during Ado treatment ( $n=5$ ; Figure 3B). Conversely, prevention of the Ado-induced restoration by the NBTI ( $3 \times 10^{-7}$  mol/L), an inhibitor of Ado transporter, suggests an importance for Ado intake into the cell for the restoration ( $n=6$ ; ANOVA,  $P<0.05$ ; Figure 3B). NBTI itself did not influence the Ang II-induced contractions (Figure 3B).

The nonspecific P2 receptor antagonist suramin ( $10^{-4}$  mol/L) was used to exclude a contribution of P2 receptors in the restoration. Suramin ( $n=7$ ) had no effect on the restoration of a second application of Ang II by Ado in afferent arterioles. Arteriolar diameter was reduced by  $61.1 \pm 10.5\%$  at Ang II (first application) and by  $64.7 \pm 7.3\%$  at Ang II (second application after Ado treatment+suramin).

### $\text{Ca}^{2+}$ Sensitivity of the Contractile Apparatus

KCl (100 mmol/L) induced strong and transient constrictions of perfused afferent arterioles, which did not desensitize (data not shown). KCl-induced constrictions were very weak at very low  $\text{Ca}^{2+}$  concentrations, as produced by addition of EGTA to the bath solution ( $5 \times 10^{-3}$  mol/L in bath solution; estimation of free extracellular  $\text{Ca}^{2+}$  concentration  $[\{\text{Ca}^{2+}\}_e]$ :  $1.9 \times 10^{-8}$  mol/L;  $n=15$ ; Figure 4A) and became stronger after addition of  $\text{CaCl}_2$  ( $5 \times 10^{-4}$  mol/L in the bath solution; free  $[\text{Ca}^{2+}]_e$ ,  $\approx 3.3 \times 10^{-8}$  mol/L;  $n=17$ ; ANOVA,  $P<0.05$ ; Figure 4A). Ado aggravated the KCl-induced constriction after treatment with EGTA ( $n=13$ ; ANOVA,  $P<0.05$ ; Figure 4A) and after addition of  $\text{CaCl}_2$  ( $n=13$ ; ANOVA,  $P<0.05$ ; Figure 4A). Ado increased the  $\text{Ca}^{2+}$  sensitivity of afferent arterioles significantly (Wilcoxon,  $P<0.05$ ; Figure 4B).



**Figure 3.** A, Complete inhibition of Ado-induced constrictions of afferent arterioles (during treatment with the selective  $A_{2A}$ AR and  $A_{2B}$ AR inhibitors ZM241385 and MRS1706, respectively) by the nonselective Ado receptor inhibitor 8-SPT. \*Significant differences compared with control; #differences between groups. B, The nonselective Ado receptor antagonist 8-SPT did not influence the restoration of Ang II-induced constriction of afferent arterioles by Ado (same data as in Figure 1). Inhibition of NBTI-sensitive Ado transporter by NBTI prevented the restoration of the Ang II response by Ado. Application of NBTI alone did not influence the Ang II response. \*Significant differences compared with the first application of Ang II; #differences to arterioles without treatment.

The analysis of the phosphorylation of the MLC using a pS19/pS20 specific anti-MLC antibody demonstrates higher degree of phosphorylation in Ado-treated preglomerular arterioles ( $n=8$ ) compared with untreated arterioles ( $n=8$ ; Wilcoxon,  $P<0.05$ ; Figure 5).

Inhibitors of Rho-kinase (Y-27632;  $10^{-6}$  mol/L;  $n=8$ ) and PKC (chelerythrine;  $10^{-5}$  mol/L;  $n=7$ ), which were simultaneously applied with Ado, prevented the restorative effect of Ado (ANOVA,  $P<0.05$ ; Figure 6). Constrictions were reduced to levels seen without Ado treatment (Figure 1). Both inhibitors had no influence on arteriolar tone. In the control situation, Y-27632 changed afferent arterioles diameter by  $-1.1\pm 4.1\%$  ( $n=6$ ;  $P=NS$ ) and chelerythrine by  $4.3\pm 0.8\%$  ( $n=7$ ;  $P=NS$ ). However, both inhibitors reduced contractions to Ang II ( $10^{-8}$  mol/L) compared with untreated arterioles (Y-27632:  $n=6$ ,  $-9.6\pm 1.7\%$  versus  $-39.8\pm 7.1\%$ ,

$P<0.05$  [Wilcoxon test]; chelerythrine:  $n=7$ ,  $-12.3\pm 5.6\%$  versus  $-39.8\pm 7.1\%$ ,  $P<0.05$ ; Figure 1).

In contrast, treatment with SB-203580, an inhibitor of p38 MAPK ( $10^{-5}$  mol/L;  $n=5$ ), did not significantly affect contractions to Ang II ( $10^{-8}$  mol/L) (Figure 1;  $-27.0\pm 6.9\%$  versus  $-39.8\pm 7.1\%$ ;  $P=NS$ ). It also did not change arteriolar tone ( $-1.8\pm 3.3\%$ ;  $n=5$ ) during the control situation but completely prevented the Ado-induced restoration of repeated, Ang II-induced contractions (Figure 7;  $n=5$ ; ANOVA,  $P<0.05$ ).

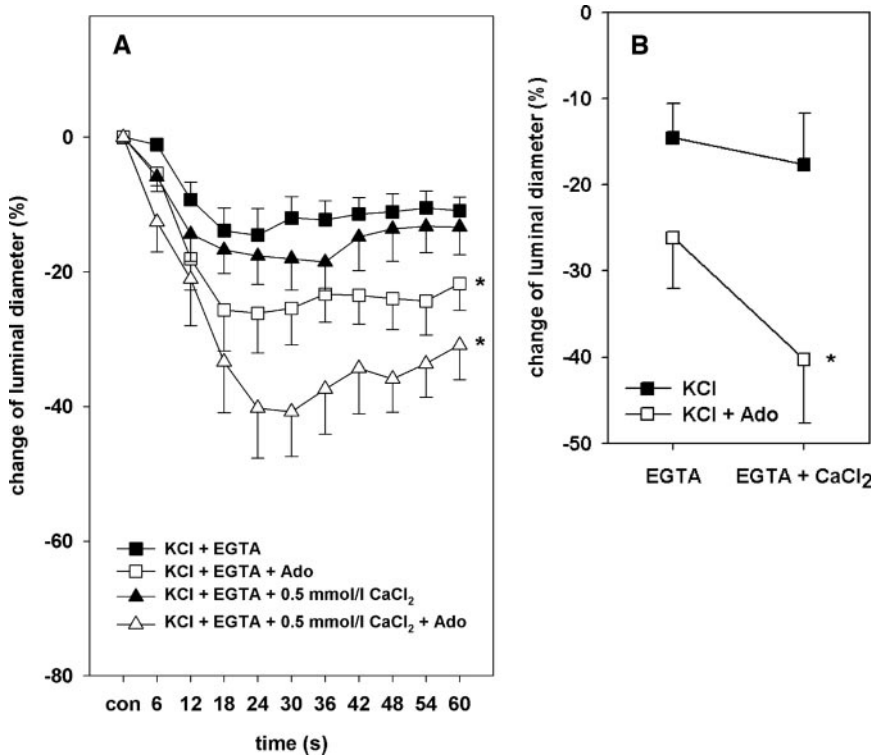
### Intracellular ATP

We further tested the hypothesis that an increased intracellular Ado concentration can also influence the purinergic metabolism, which in turn influences the contractile response. Application of Ado ( $2.5\times 10^{-5}$  mol/L) induced a rise of the bioluminescence in cultured LuciHEK cells. The ILD ratio increased from  $0.302\pm 0.026$  (untreated control;  $n=8$ ) to  $0.348\pm 0.016$  ( $n=8$ ; Wilcoxon,  $P<0.05$ ). Blockade of Ado transporters with NBTI inhibited a significant increase of ATP during Ado application ( $0.328\pm 0.020$  versus  $0.302\pm 0.026$ ;  $P=NS$ ;  $n=8$  [Figure 8]).

### Discussion

The present study shows that Ado completely restores the response on repeated applications of Ang II in afferent arterioles of mice. However, resensitization of the receptor-mediated signal cascade is not involved. In fact, this restoration is caused by an increase of the  $Ca^{2+}$  sensitivity of the contractile apparatus in the vascular smooth muscle. Restored Ang II response of the arterioles may be important in the control of arteriolar tone and consequently for the control of renal perfusion and filtration during ischemic and postischemic periods. This mechanism may potentially play a role for the TGF. Ado had earlier been found to be involved in the TGF mechanism as a mediator of the signal from an increase in distal fluid delivery to a contraction of the afferent arteriole. Possibly an increased delivery enhances the leakage of ATP from the Macula densa cells into the interstitium in which, by the action of enzymes, ATP is degraded to Ado. It then mediates the arteriolar contraction by acting on  $A_1$ ARs and through a release of calcium in the afferent arteriolar smooth muscle cells. As found in the present study, Ado also markedly sensitizes the contractile response of Ang II. Thus, a stronger contraction of the afferent arteriole can be achieved in at least 2 different ways.

Successive applications of Ang II resulted in a decrease of the contractile response and a concomitant attenuation of the cytosolic  $Ca^{2+}$  transients. This agrees with earlier investigations.<sup>9</sup> The fast desensitization is most likely attributable to the phosphorylation and endocytosis of  $AT_1$  receptors with the consequence of a reduction of  $Ca^{2+}$  mobilization.<sup>8</sup> Although treatment with Ado between the successive applications of Ang II completely restored the contractile response, intracellular  $Ca^{2+}$  transients were not reestablished. Therefore, it does not appear that a classical mechanism of resensitization occurring by restoration of the receptors and intracellular  $Ca^{2+}$  transients<sup>8,16</sup> takes place.

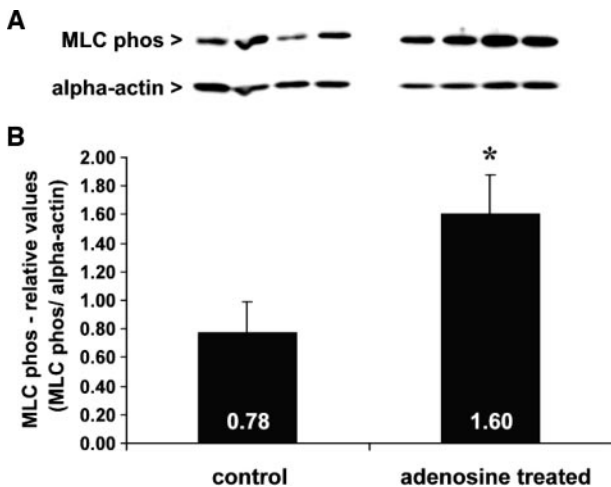


**Figure 4.** A, KCl-induced (100 mmol/L) constrictions of afferent arterioles presented as relative changes of the luminal diameter over time. Measurements were performed in the presence of EGTA ( $5 \times 10^{-3}$  mol/L in bath), which reduces  $[Ca^{2+}]_e$  to a very low level ("EGTA" in B) and after increase of  $[Ca^{2+}]_e$  by addition of  $CaCl_2$  ( $5 \times 10^{-4}$  mol/L in bath) to the bath solution ("EGTA+ $CaCl_2$ " in B). Ado treatment increased the responses significantly, both with and without  $CaCl_2$ . Con is control situation before treatment with KCL. B, Relative changes of the luminal diameter 24 seconds after KCl administration (data from A) demonstrate higher  $Ca^{2+}$  sensitivity of Ado-treated arterioles. \*Differences between Ado-treated and untreated groups.

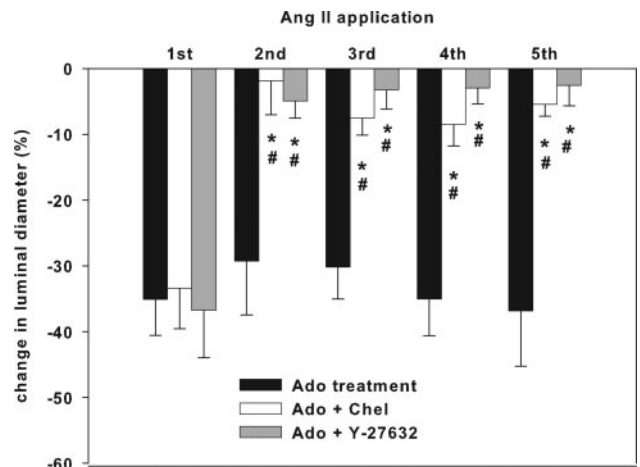
Smooth muscle contraction is initiated by phosphorylation of the regulatory light chain of myosin II (MLC<sub>20</sub>) on serine 19 catalyzed by the MLC kinase (MLCK), which is classically activated by a  $Ca^{2+}$ /calmoduline complex. The MLC phosphatase (MLCP) has an antagonistic effect on the MLC phosphorylation. In addition to the  $Ca^{2+}$ -dependent regulation of MLC, the modulation of the  $Ca^{2+}$  sensitivity by phosphorylation of the MLC by several other kinases plays an important role for smooth muscle contraction.<sup>17,18</sup> We found that Ado treatment increased the  $Ca^{2+}$  sensitivity, as indicated by a significantly greater contraction on KCl when increasing

extracellular  $Ca^{2+}$  concentrations, compared with untreated arterioles. Analysis of the MLC<sub>20</sub> phosphorylation at Ser19 clearly reveals elevated levels and supports the assumption of  $Ca^{2+}$  sensitization of the contractile apparatus by Ado.

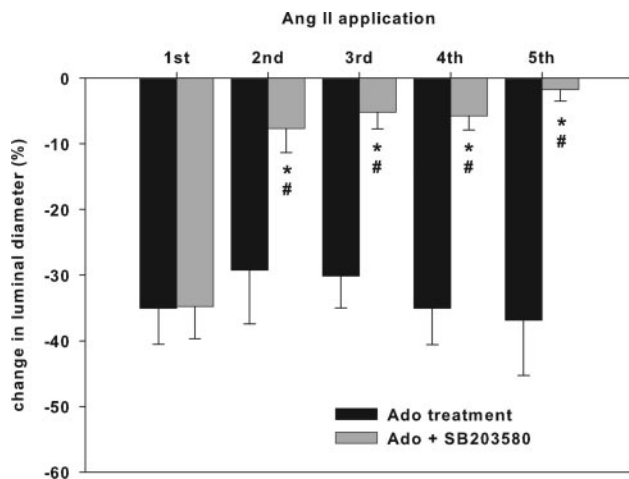
Several proteins are involved in the modulation of MLC phosphorylation and consequently of  $Ca^{2+}$  sensitivity. The RhoA/Rho-kinase pathway inhibit MLCP activity by phosphorylation of a 110- to 130-kDa regulatory subunit (MYPT1) of MLC.<sup>19,20</sup> MLCP can also be inhibited by phosphorylated CPI-17, a substrate of the protein kinase C (PKC),<sup>21,22</sup> and possibly of the Rho-kinase. In the present



**Figure 5.** A, Representative example of Western Blot signals for untreated (control) and Ado-treated arterioles. The figure shows the chemiluminescence signal of the phosphorylated MLC (MLC phos) and the signal for smooth muscle  $\alpha$ -actin (alpha-actin). B, Mean of relative values of phosphorylated MLC protein. \*Significant difference compared with the control value.



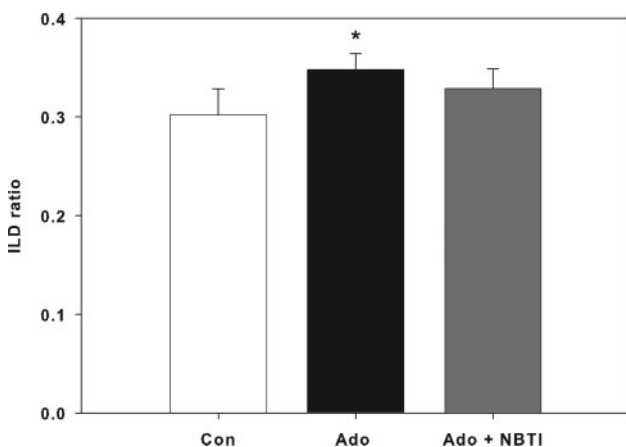
**Figure 6.** The specific inhibitors of Rho-kinase (Y-27632) and PKC (chelerythrine) prevented the restoration of Ang II-induced constrictions of Ado. \*Significant differences compared with the first Ang II application; #significant differences between Y-27632+Ado-treated and chelerythrine+Ado-treated arterioles, respectively, and arterioles treated with only Ado (same data as in Figure 1).



**Figure 7.** The specific inhibitor of p38 MAPK (SB203580) prevented the restoration of Ang II-induced constrictions by Ado. \*Significant differences compared with the first Ang II application; #significant differences between SB203580+Ado-treated arterioles and arterioles treated with only Ado (same data as in Figure 1).

study, the contribution of both Rho-kinase and PKC were investigated using their specific inhibitors Y-27632 and chelerythrine, respectively. Ado-induced restoration was completely blocked by these treatments. This points to a contribution of both pathways in the mechanisms of the restoration effect. However, although Y-27632 and chelerythrine did not influence the tone of nonstimulated arterioles, a single Ang II-induced constriction was clearly diminished. Therefore, it is likely that the inhibition of the Rho-kinase and PKC pathways reduces the constriction independently of the Ado effect.

Recent studies point to the involvement of the p38 MAPK in the Ang II-induced contraction in the rat vascular smooth muscle.<sup>23</sup> It was shown that Ang II-activated p38 MAPK stimulates MAPK-activated protein (MAPKAP) kinase-2 and the phosphorylation of heat shock protein 27 (HSP27). Two



**Figure 8.** Effect of extracellular Ado application on intracellular ATP concentration in LuciHEK cells. The ratio of ILD, a measure of ATP, is presented for cells without treatment (Con), Ado-treated cells (Ado), and cells treated with Ado and the Ado transporter inhibitor NBTI (see text for details). \*Significant increase in ILD compared with control.

other studies showed a contribution of p38 MAPK for the endothelin-1-induced contraction in rat thoracic aorta.<sup>24,25</sup> In the present study, inhibition of p38 MAPK with SB203580 did not change basal tone or a single Ang II-induced contraction. However, SB203580 prevented the Ado-induced restoration, which indicates a contribution of p38 MAPK in the Ado-induced increase of the  $Ca^{2+}$  sensitivity. Activation of p38 MAPK has been observed during stress situations such as hypoxia in several studies in cultured cells.<sup>26,27</sup> Furthermore, inhibition of p38 $\alpha$  MAPK protects from damage of isolated cardiomyocytes during simulated ischemia.<sup>28</sup> It is widely accepted that Ado concentrations increase under hypoxic conditions (hypoxic stress), suggesting that Ado could be a mediator of the stress-induced activation of p38 MAPK. Thus, p38 may form a link between Ado and increase of  $Ca^{2+}$  sensitivity.

Ado acts on  $A_1$ ARs and  $A_2$ ARs, which mediate constriction and dilation in arterial vessels, respectively. The net effect depends on the organ-specific, differential expression of the receptor subtypes.<sup>7</sup> Remarkably, the present study does not support the idea of a receptor-mediated increase of the  $Ca^{2+}$  sensitivity and concomitant resensitization of the response to Ang II. The nonspecific, water soluble Ado receptor antagonist 8-SPT did not prevent restoration, although it was recently shown that 8-SPT completely inhibits the action of Ado on  $A_1$ ARs and  $A_2$ ARs in afferent arterioles.<sup>5</sup> However, inhibition of the NBTI-sensitive Ado transporter, which are equilibrative transporter expressed in vascular smooth muscle cells, did. This strongly suggests that Ado must cross the cell membrane to be effective with regard to the restoration of the contraction. Assuming an intracellular effect of Ado, the question arises regarding the sources and the site of its action. Ado is produced intracellularly by sequential dephosphorylation of ATP to Ado. Also, the extracellular production from cAMP by action of phosphodiesterase and 5'-nucleotidase has a possible physiological role in the kidney.<sup>29</sup> Another important source of Ado results from the hydrolysis of S-adenosylhomocysteine (AdoHcy) by the enzyme AdoHcy hydrolase.<sup>30</sup> Several studies have shown that Ado binds to AdoHcy hydrolase and leads to an inhibition of this enzyme and of the methyltransferases in the cell, which can impair cell energetic and contractility.<sup>31,32</sup> However, there is as yet no experimental evidence for an influence of Ado inhibition of AdoHcy hydrolase for smooth muscle contraction. Other potential intracellular sites of Ado action are the "r" and "p" site of the adenylyl cyclase, which mediate activation and stimulation of the enzyme, respectively.<sup>33</sup> Studies revealed activation of the adenylyl cyclase by Ado in some tissues and inhibition in others, which may result in opposing effects on the intracellular cAMP and contractility.<sup>33,34</sup> Whether this mechanism has an influence on vascular smooth muscle contraction remains to be investigated.

Increase of Ado concentration in the extracellular space, as it was simulated in our study by Ado application, may lead to an increased intracellular ATP concentration. Transport of Ado along the concentration gradient into the cell increases its intracellular concentration and may stimulate the production of nucleotides upstream from Ado. In fact, using the luciferase approach, we could show that the cytosolic ATP

concentration increased on Ado application in the in vitro cell experiments in the present study. The result agrees with other studies that have also shown higher tissue level of ATP after Ado infusion in vivo.<sup>35</sup> ATP is required for the phosphorylation of MLC by MLCK and is used by the actin-activated myosin ATPase.<sup>18</sup> There is a general consensus that drastic reduction of ATP levels impairs smooth muscle contraction. Some studies have shown a modulation of force and shortening by intracellular changes in ATP and ADP content.<sup>36,37</sup> To our knowledge, investigations focusing on the effect of increased ATP have not been performed. Thus, a causal connection among increased Ado and ATP, Ca<sup>2+</sup> sensitivity, and phosphorylation of the MLC<sub>20</sub> has yet to be explored. Although the extracellular ATP concentration should not change, because of the lack of ATP transporters, we tested a possible influence of ATP via membrane P2 receptors. Inhibition of P2X and P2Y subtypes by suramin did not influence Ado-induced contractile restoration. Therefore, a contribution of the P2 receptor in the increase of the Ca<sup>2+</sup> sensitivity is unlikely.

The increase in the Ca<sup>2+</sup> sensitivity of vascular smooth muscle cells, which was found here, may be important in several pathophysiological and physiological situations. For example, hypoxia induces an enhanced production of Ado in the surrounding tissue of a smaller arteriole. The extracellular Ado concentration increases because of equilibrative transportation of Ado out of the cell and affects the vascular smooth muscle cells, which themselves have a better oxygen supply and possibly lower oxygen demand. In the kidney, a model of metabolic control of the glomerular perfusion and filtration rate by the TGF was introduced by Osswald et al.<sup>38</sup> These authors proposed a release of Ado by Macula densa cells in response to increased sodium load and energetic demands for electrolyte transport. Ado, in turn, constricts afferent arterioles via A<sub>1</sub>ARs, leading to a reduced glomerular filtration rate. Recent studies support the idea of Ado as a mediator of TGF.<sup>39,40</sup> The primary model of Osswald was modified (1) by the finding that ATP can be released by Macula densa cells<sup>41</sup> and (2) by the accumulating evidence for a role of the ecto-5'-nucleotidase in the TGF.<sup>42,43</sup> It is therefore assumed that the signaling of the TGF involves the release of ATP by Macula densa cells and the formation of 5'-AMP and Ado by extracellular enzymes.

Our data show a complete restoration effect of exogenously applied Ado at 10<sup>-5</sup> mol/L, but no changes at a concentration of 10<sup>-8</sup> mol/L. This observation suggests that the lower Ado concentration is close to that of the intracellular Ado. Interstitial Ado concentrations in the rat renal cortex were estimated to ≈2×10<sup>-7</sup> mol/L in vivo using microdialysis. Thereby, the authors assumed similar intra- and extracellular concentration in the steady-state situation.<sup>44</sup> In another study, Ado tissue concentration in the rat kidney was ≈4×10<sup>-6</sup> mol/kg kidney wet weight.<sup>35</sup> These data are only rough estimations, because measurements of Ado are difficult. In freshly isolated smooth muscle cells of porcine coronary, brachial, and femoral arteries, the K<sub>m</sub> of the NBTI-sensitive nucleoside transporter is ≈4 to 5×10<sup>-6</sup> mol/L. Taking into consideration an approximate 4- to 5-fold increase of Ado, for example, during ischemia in the kid-

ney,<sup>35</sup> extracellular Ado concentrations are high enough for effective transportation into vascular smooth muscle cells. Our results indicate that uptake of Ado strongly influences smooth muscle contraction. In a recent study, increased relaxation to exogenously applied Ado was observed during Ado transporter inhibition with NBTI in porcine coronary arteries.<sup>12</sup> This finding supports the idea of an intracellular action of Ado in regulating arterial tone.

The present study suggests that exogenously applied Ado leads to an increased Ca<sup>2+</sup> sensitivity and phosphorylation of the MLC<sub>20</sub> in vascular smooth muscle cells, which has not been demonstrated thus far. This effect restores the Ang II-induced constriction and does not include membrane-located Ado receptors. Rather, enzymes such as p38 MAPK, and possibly the RhoA/Rho-kinase and PKC pathway, are involved in the increase of MLC<sub>20</sub> phosphorylation. This effect could be important in the metabolic control of renal perfusion and filtration, and in the TGF. Therefore, a mechanism of the mediation of the TGF by Ado may be the increase of the Ca<sup>2+</sup> sensitivity of afferent arterioles.

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## Disclosures

None.

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