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# Activation of $A_2$ adenosine receptors dilates cortical efferent arterioles in mouse

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**Adenosine can induce vasodilatation and vasoconstriction of the renal afferent arteriole of the mouse. We determined here its direct effect on efferent arterioles of mouse kidneys. Using isolated-perfused cortical efferent arterioles, we measured changes in luminal diameter in response to adenosine. Extraluminal application of adenosine and cyclohexyladenosine had no effect on the luminal diameter. When the vessels were constricted by the thromboxane mimetic U46619, application of adenosine and 5′-*N*-ethylcarboxamido-adenosine dilated the efferent arterioles in a dose-dependent manner. We also found that the adenosine-induced vasodilatation was inhibited by the $A_2$-specific receptor blocker 3,7-dimethyl-1-propargylxanthine. In the presence of this inhibitor, adenosine failed to alter the basal vessel diameter of quiescent efferent arterioles. Using primer-specific polymerase chain reaction we found that the adenosine $A_1$, $A_{2a}$, $A_{2b}$, and $A_3$ receptors were expressed in microdissected mouse efferent arterioles. We conclude that adenosine dilates the efferent arteriole using the $A_2$ receptor subtype at concentrations compatible with activation of the $A_{2b}$ receptor.**

*Kidney International* (2009) **75,** 793–799; doi:10.1038/ki.2008.684; published online 21 January 2009

KEYWORDS: glomerular arterioles; renal hemodynamics; vascular purin

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**Received 13 June 2008; revised 28 October 2008; accepted 18 November 2008; published online 21 January 2009**

The resistance vessels in the kidney include afferent and efferent glomerular arterioles. Changes in their resistances regulate renal blood flow and are important determinants of glomerular ultrafiltration pressure and glomerular filtration rate. The afferent arteriolar resistance is modulated by changes in adenosine concentration. Thus, the nucleoside has been held responsible for the afferent arteriolar constriction in response to an increased NaCl concentration at the macula densa that underlies the tubuloglomerular feedback (TGF) mechanism.[1–4] An increased NaCl concentration at the macula densa leads to release of adenosinetriphosphate[5] followed by interstitial conversion to adenosine through 5′-ectonuclotidase.[6] The ability of adenosine to constrict the renal afferent arteriole through activation of adenosine $A_1$ receptors ($A_1$AR) is well established.[7–12] By contrast, the effects of adenosine on the efferent arteriole are controversial.[13]

Adenosine in the interstitium initiates its effects by activating members of the P1 class of purinergic receptors. P1 receptors are G-protein-coupled receptors with seven membrane-spanning domains.[14] Four members of the P1 receptor family have been identified, designated $A_1$, $A_{2a}$, $A_{2b}$, and $A_3$ adenosine receptors, and all four receptor subtypes are expressed in the rat kidney.[15,16] In the renal vasculature, the presence of $A_1$AR has been demonstrated in preglomerular resistance vessels both at the mRNA and protein levels.[17–19] Furthermore, adenosine $A_2$ receptors ($A_2$AR) are expressed in preglomerular vessels although it is unclear whether both $A_{2a}$AR and $A_{2b}$AR are present.[19,20] The response to adenosine in the afferent arteriole therefore reflects a net effect of competing influences resulting from the concomitant activation of $A_1$AR and $A_2$AR. The precise identity of adenosine receptors in efferent arterioles is controversial.

Adenosine elicits marked dilatations in most vascular beds that are mediated by $A_{2a}$ (high-affinity) and $A_{2b}$ (low-affinity) receptors coupled to Gs proteins.[14] This is in contrast to the adenosine-mediated constriction at low concentrations observed in the afferent arteriole, which is elicited by $A_1$AR activation coupled to Gi proteins. The increase in adenosine concentration in the interstitium of the juxtaglomerular apparatus during TGF adjustments could

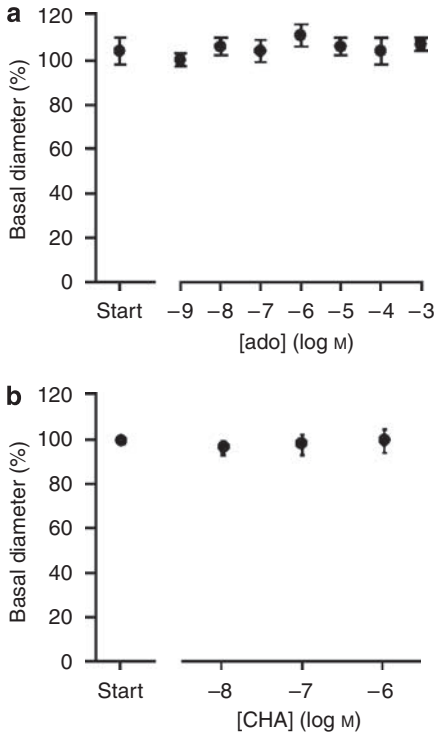


**Figure 1 | Concentration–response relationship for adenosine (ado) and CHA in mouse superficial efferent arterioles.** (**a**) Adenosine was administered to the bath solution every third minute in cumulatively increasing concentrations. Data are means ± s.e.m. ($n = 5$). (**b**) Increasing concentrations of CHA applied to the bath solution. Data are means ± s.e.m. ($n = 5$).

potentially affect the efferent arteriole.[6,21] Therefore, the present experiments were designed to determine the direct functional response to adenosine of isolated perfused efferent arterioles from mice and the nature of the adenosine receptors involved.

## RESULTS

### Cumulative concentration–response curves to adenosine and cyclohexyladenosine in quiescent preparations

No significant change in blood vessel diameter could be observed after application of increasing concentrations of adenosine (Figure 1a). A second sequence of adenosine application also failed to cause significant changes in diameter (data not shown). The luminal diameter was not affected significantly by the application of the $A_1$AR agonist cyclohexyladenosine (CHA) (Figure 1b).

### Constrictions to U46619

U46619 constricted the efferent arterioles from 3.5 ± 0.2 to 3.0 ± 0.3 µm ($2 \times 10^{-8}$ mol/l) to 0.1 ± 0.1 µm ($10^{-7}$ mol/l) (Figure 2a). Repeated application of U46619 ($10^{-7}$ mol/l) caused maximal and reproducible (data not shown) tonic constrictions of the efferent arterioles for 21 min (the time frame required for six consecutive applications of adenosine) (Figure 2b). Photographs of a perfused efferent arteriole with and without U46619 are shown in Figure 2c and d.

### Cumulative concentration–response curve to adenosine and the $A_2$AR agonist 5′-(*N*-ethylcarboxamido)-adenosine in constricted preparations

In efferent arterioles constricted with U46619, $10^{-9}$–$10^{-8}$ mol/l adenosine did not have a significant effect, whereas higher concentrations dilated the vessels in a concentration-dependent manner; a statistically significant and reproducible dilatation was reached at $10^{-5}$ mol/l adenosine (Figure 3a). In the first application sequence basal diameter was 5.9 ± 0.7 µm, and $10^{-7}$ mol/l U46619 reduced vessel diameter to 0.0 ± 0.0 µm. During the first application sequence, addition of $10^{-4}$ mol/l adenosine increased the diameter to 5.0 ± 0.3 µm, which corresponds to 87 ± 7% of the basal diameter. After washout, the basal diameter averaged 6.3 ± 0.8 µm. In the second application series, $10^{-7}$ mol/l U46619 reduced vessel diameter to 0.2 ± 0.2 µm, and addition of $10^{-4}$ mol/l adenosine reversed the constriction to 5.7 ± 0.7 µm, which is 92 ± 7% of second application basal diameter. The effect of adenosine was reproducible (Figure 3a). The EC50 for adenosine averaged $7.0 \times 10^{-6}$ and $1.3 \times 10^{-6}$ mol/l for the first and second application sequences, respectively. These values were not significantly different. Vessels preconstricted with U46619 dilated dose dependently after application of 5′-(*N*-ethylcarboxamido)-adenosine (NECA) (Figure 3b, $P < 0.05$). The EC50 for NECA was $5.2 \times 10^{-8}$ mol/l.

### Inhibition by the $A_2$AR antagonist DMPX

Under control conditions, adenosine caused a concentration-dependent increase in luminal diameter in U46619-constricted vessels, with an EC50 of $1.4 \times 10^{-6}$ mol/l. 3,7-Dimethyl-1-propargylxanthine (DMPX) ($10^{-4}$ mol/l) did not significantly change the resting luminal diameter (from 5.1 ± 0.6 to 5.1 ± 0.7 µm). After incubation with DMPX ($10^{-4}$ mol/l), the addition of $10^{-7}$ mol/l U46619 still constricted the vessels to 0.7 ± 0.7 µm but the addition of increasing concentrations of adenosine did not significantly alter the arteriolar diameter (Figure 4).

A single injection of $10^{-7}$ mol/l of adenosine did not change the diameter of quiescent efferent arterioles (Figure 5). After incubation with DMPX, adenosine did not induce significant changes in diameter (Figure 5).

### RT–PCR

The reverse transcriptase–PCR (RT–PCR) analysis showed bands of the expected size for $A_1$AR, $A_{2a}$AR, $A_{2b}$AR, and $A_3$AR in superficial efferent arterioles and in the positive control using whole kidney (Figure 6a). The negative controls, water and –RT, showed no bands. The presence of $A_1$AR, $A_{2a}$AR, $A_{2b}$AR, and $A_3$AR was also detected in microdissected afferent arterioles (Figure 6b).

## DISCUSSION

This study shows for the first time the direct effect of adenosine on cortical efferent arterioles of the mouse perfused *in vitro*. The findings demonstrate that adenosine

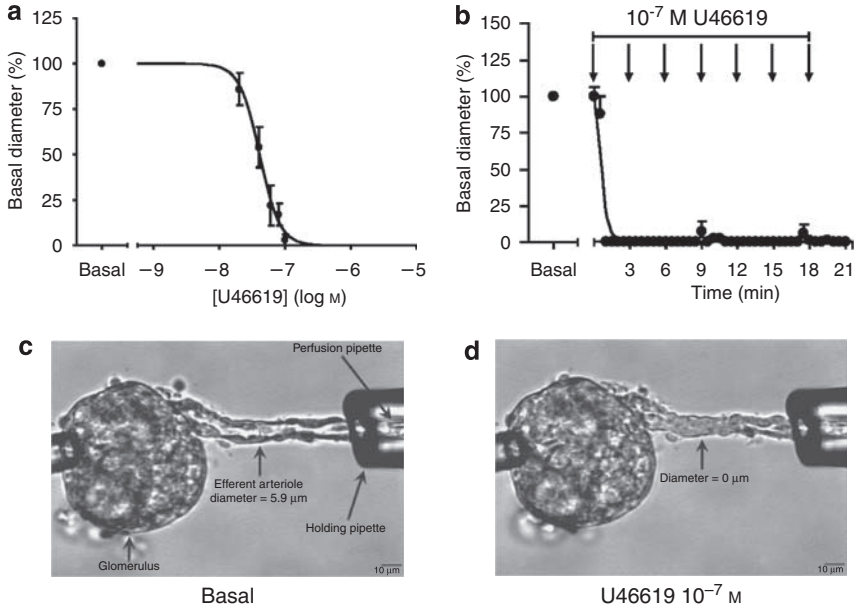


**Figure 2 | Effect of the thromboxane receptor agonist U46619 in mouse superficial efferent arterioles.** (**a**) Cumulatively increasing concentrations of U46619 were added to the bath every third minute. Full constriction was seen with $10^{-7}$ mol/l. Basal diameter (Basal) before U46619 administration is set to 100%. Data are means ± s.e.m. ($n = 5$). (**b**) Application of $10^{-7}$ mol/l U46619 for 21 min. Each arrow represents bath substitution with new medium containing $10^{-7}$ mol/l U46619. Data are means ± s.e.m. ($n = 3$). (**c**) Photograph of an isolated perfused mouse efferent arteriole under basal conditions. (**d**) Efferent arteriole constricted with U46619 ($10^{-7}$ mol/l).

and the $A_2$AR agonist NECA elicit a concentration-dependent dilatation in constricted preparations whereas the nucleoside and the $A_2$AR agonist CHA did not change the luminal diameter of quiescent blood vessels. The ability of $A_2$AR antagonist DMPX to inhibit the dilatation suggests that the relaxation is mediated by activation of $A_2$ adenosine receptors.[22] All four known subtypes of adenosine receptors were expressed in both pre- and postglomerular arterioles. In resemblance to the afferent arteriole, the net effect of adenosine on the efferent arteriole could reflect competing activation of $A_1$AR and $A_2$AR. However, in contrast to the afferent arteriole, the main effect of adenosine on the efferent arteriole is vasodilatation. This direct evidence for dilatation of cortical efferent arterioles by adenosine is in agreement with the overall suggested vascular effect for the nucleoside. Indeed, adenosine elicits a decrease in filtration fraction[23,24] that is consistent with a dilatation in the postglomerular arterioles. Furthermore, an adenosine-induced dilatation has been observed in efferent arterioles of hydronephrotic kidney of the rat.[8,25]

The thromboxane analogue U446619 caused a concentration-dependent constriction of the efferent arterioles and led to sustained constriction over time, making it well suitable for the study of dilator responses in this preparation. In this study, the EC100 concentration of the thromboxane-prostanoid agonist was used, as lower levels of constriction would lead to a magnitude of dilatation that would be difficult to measure accurately in view of the small size of the preparation.

Both adenosine and the $A_2$AR agonist NECA dilated the efferent arterioles by 80% after complete closure with U46619. This effect is in agreement with several studies showing the ability of adenosine and adenosine receptor agonists to dilate postglomerular vessels.[8,25] Thus, Holz and Steinhausen[25] reported that in the hydronephrotic kidney of the rat the adenosine receptor agonist NECA induces a small efferent dilatation and an increase in glomerular blood flow. In the same preparation, adenosine increased the diameter of the efferent arteriole by 14%, and the dilatation was abolished by the $A_2$AR blocker DMPX.[8]

The vasodilatation observed in this study can be attributed to activation by adenosine of $A_2$AR. This conclusion is based on the observation that DMPX abolished the response and that NECA elicited a vasodilatation similar to the adenosine-induced effect. DMPX is a nonselective $A_2$AR antagonist and thus the present observations do not provide further evidence for the involvement of $A_2$AR subtypes. Because the $A_{2b}$AR subtype is a low-affinity receptor, it is likely responsible for the observed efferent dilatation caused by adenosine, as high concentrations of adenosine were necessary to elicit the response. In addition to a receptor-mediated effect, an intracellular action of adenosine may also affect afferent arteriolar tone.[26] However, in whole kidney experiments, inhibition of nucleoside transport reduces glomerular filtration rate with an insignificant decrease in renal vascular resistance, which is consistent with an increase in efferent arteriolar diameter.[27] Because the effect after nucleoside transport inhibition was blocked completely by DMPX,

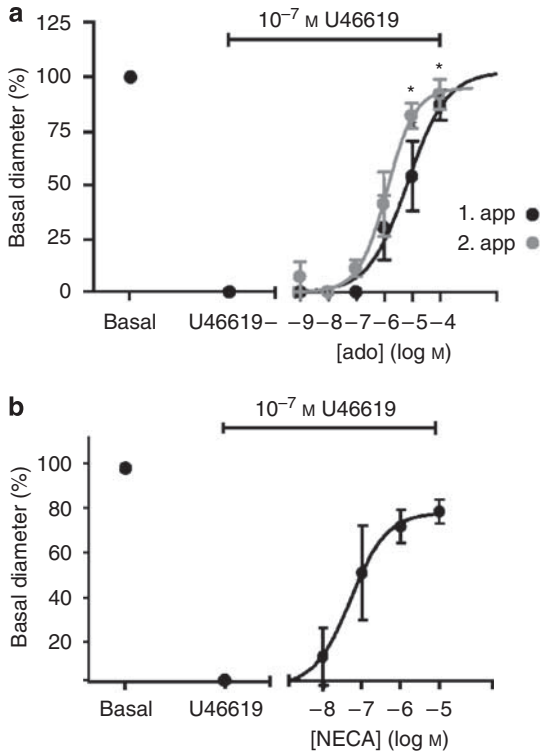


**Figure 3 | Reproducible concentration–dilatation curves to adenosine (ado) and NECA in arterioles constricted with U46619.** 'Basal' represents basal vessel diameters before application of adenosine or NECA. (**a**) Adenosine was added to the bath solution every third minute in cumulatively increasing concentrations. Data are means ± s.e.m. ($n = 4$; *$P < 0.05$ vs U46619). (**b**) Increasing concentrations of NECA applied to the bath every third minute. Data are means ± s.e.m. ($n = 5$).

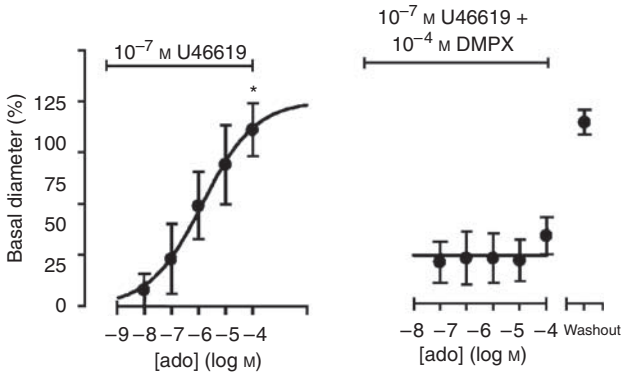


**Figure 4 | Concentration–response relationship for adenosine (ado) in arterioles constricted with U46619 in the absence (left) and presence (right) of DMPX.** Data are means ± s.e.m. ($n = 5$; *$P < 0.001$ vs U46619).

the response was mediated most likely by $A_2$AR activation in response to an increased adenosine concentration in the interstitium. Whether nucleoside transport is involved in the adenosine-elicited response in the efferent arteriole is unknown.

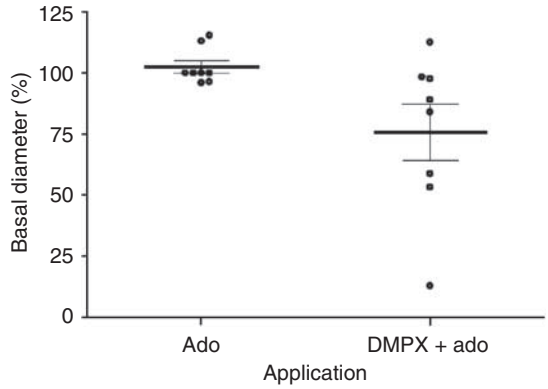


**Figure 5 | Effect of adenosine (ado) in the presence of the $A_{2A}$R antagonist DMPX.** The plot shows diameter of the same blood vessel during adenosine application before and after treatment with DMPX. Diameters for each application are expressed as percent of vessel diameter before treatment. Data are means ± s.e.m. ($n = 8$; $P > 0.05$ for ado vs DMPX + ado).

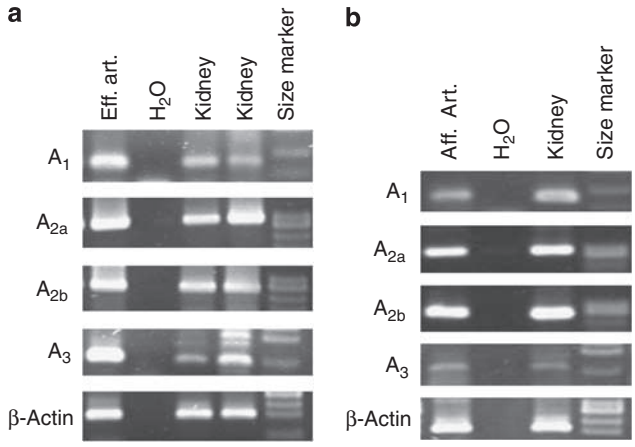


**Figure 6 | Expression of adenosine receptors in mouse superficial arterioles.** Bands of the expected size were obtained for $A_1$AR, $A_{2a}$AR, $A_{2b}$AR, and $A_{3A}$R in efferent arterioles (Eff. art., **a**), afferent arterioles (Aff. art., **b**), and in the positive control using whole kidney. The negative control, water, expressed no bands.

The application of adenosine in quiescent cortical efferent arterioles did not change the luminal diameter at any concentration. By contrast, in the *in vitro* blood-perfused juxtamedullary nephron preparation, adenosine evokes a biphasic response of efferent arterioles with constriction at concentrations in the micromolar range and dilatation at higher doses.[7,9] The vasoconstrictor component of this biphasic response has been attributed to activation of $A_1$AR. Likewise, in afferent arterioles, $A_1$AR are responsible for the constriction elicited by adenosine,[12,28] and in the blood-perfused rat juxtamedullary nephron preparation low concentrations of adenosine decrease afferent arteriolar diameter, an effect that is enhanced by DMPX.[29] In the present experiments, DMPX, at the same concentration which inhibited adenosine-elicited vasodilatation, did not unmask changes in diameter in quiescent efferent arterioles. Because adenosine potentially could be metabolized during

the experiment, we used the nonmetabolized $A_1$AR agonist CHA. This drug also had no effect on luminal diameter. Taken in conjunction, these findings show that activation of $A_1$AR does not contribute to the cortical efferent arteriolar responses to adenosine in the mouse. The discrepancy between the effects of adenosine on cortical efferent arterioles in this study and those observed in juxtamedullary efferent arterioles[7,9] could be due to differences in species and preparation studied. However, it potentially adds further evidence to the view that juxtamedullary efferent arterioles differ from the corresponding cortical preparations.[30,31]

Changes in the efferent and afferent arteriole diameters regulate renal blood flow and glomerular filtration rate. Efferent arterioles *in vivo* possess tone. The present *in vitro* observation of a tone-dependent vasodilator effect of adenosine mediated by A2AR may be relevant for the fall in efferent resistance induced by adenosine *in vivo* in situations with decreased glomerular capillary pressure and filtration rate. Furthermore, in addition to the well-documented afferent arteriolar vasoconstrictor response,[32] efferent vasodilatation may be involved in the TGF mechanism.[21,33] Thus, in the isolated perfused juxtaglomerular apparatus, increasing the NaCl concentration at the macula densa dilates the efferent arteriole significantly. This increase in diameter can be blocked by an $A_2$AR antagonist and by preventing extracellular adenosine formation. Therefore, the direct response to high concentrations of adenosine observed in this study implies that the dilatation in response to high NaCl concentrations in the macula densa[21,33] could be due to release of high concentrations of adenosine with a subsequent dilatation. In support of this interpretation, micropuncture studies reveal that activation of the TGF and the resulting decrease in glomerular filtration rate occur with simultaneous decreased or unchanged glomerular capillary pressure.[34] A role for an adenosine-mediated vasodilatation of the efferent arteriole during activation of the TGF could potentially explain in part the decreased glomerular capillary pressure.

Several studies have investigated the distribution of adenosine receptors in the kidney but the precise localization of these receptors in the renal glomerular vessels is still unclear. This study demonstrates the expression of $A_1$AR, $A_{2a}$AR, and $A_{2b}$AR mRNA in microdissected efferent arterioles. The expression of $A_1$AR and $A_{2a}$AR in mouse glomerular arterioles has also been observed by Vitzthum *et al.*[20] although these authors did not detect $A_{2b}$AR in mouse glomerular vessels. Functional data by Lai *et al.*[34] suggest the existence of low-affinity adenosine receptors $A_{2b}$AR in mice afferent arterioles. Existence of $A_{2b}$AR on preglomerular vessels is established in the rat.[10,19,20] For postglomerular vessels only Kreisberg *et al.*[35] have determined the expression of adenosine receptors in the outer medullary vasa recta.

In conclusion, this study provides evidence for the expression of the four known subtypes of adenosine receptors in efferent arterioles of the mouse. In constricted vessels, which are most relevant for the *in vivo* situation, adenosine elicits efferent vasodilatation, through $A_2$AR activation. This vasodilatation elicited by adenosine could be relevant for the regulation of efferent arteriolar diameter during activation of the TGF.

## MATERIALS AND METHODS

### Animals

The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. The C57BL/6 mice used in this study had free access to rodent chow (Altromin, Lage, Germany) and tap water.

### Perfused cortical efferent arterioles

Efferent arterioles with attached glomeruli were microdissected at 4 °C in DMEM/F12 from mice of either sex (17–27 g) and were perfused retrogradely. The method was similar to the perfusion technique described by Hansen *et al.*[12] but adapted to efferent arterioles. Efferent arterioles were identified by the following criteria; they are thinner than the afferent arteriole, they often are curved as opposed to the straight afferent arterioles, and they divide into capillaries. Furthermore, we used the paths of the preglomerular vasculature for comparison. The efferent arteriole with its glomerulus intact (afferent arteriole removed by cutting the vessel close to the glomerulus) was transferred to a thermostated (37 °C) perfusion chamber (Warner), mounted on an inverted microscope (Zeiss Axiovert 10). The segment was perfused by aspiration of the arteriole into a holding pipette (tip diameter 14 μm) followed by cannulation with a perfusion pipette (tip diameter 4–5 μm) and increase in driving pressure until the vessel opened, indicating attained perfusion. The entire experiment was recorded using a digital camera (DAGE MTI). One frame per second was stored on a computer, using a frame grabber card (Pixel Smart).

The dose–response curve to adenosine was determined in the concentration range of $10^{-9}$ to $10^{-3}$ mol/l. Each concentration (added extraluminally to the bath) was in contact with the preparations during 3 min each. After a washout period the above was repeated. The same protocol was repeated using the $A_1$AR agonist CHA. Concentration–response curves were obtained also for the thromboxane-prostanoid receptor agonist U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) by adding the compound to the bath for 3 min at increasing concentrations ($2 \times 10^{-8}$–$10^{-7}$ mol/l; 3 min exposure to each concentration). To obtain sustained constrictions of the efferent arterioles, U46619 ($10^{-7}$ mol/l) was added repeatedly to the bath at 3 min intervals for a total of 21 min.

Increasing concentrations of adenosine ($10^{-9}$–$10^{-4}$ mol/l) were administered at 3 min intervals minute during sustained U46619-induced constrictions. This protocol was repeated using the $A_2$AR agonist NECA.

The $A_2$AR antagonist DMPX ($10^{-4}$ mol/l) was added to the bath for a period of 15 min before determining the response to adenosine during sustained U46619-induced constrictions. The effect of DMPX was also tested on the response to adenosine ($10^{-7}$ mol/l) in quiescent preparations.

### Data analysis

Images were transferred to a custom-made imaging software DiaFix (produced by Rozh Husain Al-Mashhadi, University of Southern Denmark). Luminal (interendothelial) blood vessel diameters were measured on the computer screen using images of interest. To

eliminate bias, a randomization algorithm was coded in the software utility, allowing blinded measurements of diameter. Luminal diameter was determined at the most reactive part of the arteriole.

### Statistical analysis

All data are expressed as means ± s.e.m. Blood vessel diameters are expressed in percent of basal. Bartlett's test for equal variances was used to verify that the variances did not differ significantly, thereby allowing for analysis of variance. Further analysis was conducted using one-way analysis of variance followed by Bonferroni's multiple comparison test, two-way analysis of variance or the paired *t*-test. *P*-values less than 0.05 were considered to indicate statistically significant differences.

### Isolation of cortical efferent arterioles for PCR analysis

Mice were killed by cervical dislocation and the kidneys were removed and placed on ice. Kidney slices were digested using enzyme treatment containing collagenase NB8, 0.5 g/l (Serva, Heidelberg, Germany); trypsin inhibitor, 0.1 g/l (Sigma, St. Louis, MO, USA); dispase II, 0.35 g/l (Roche, Basel, Switzerland); and DNAse I, 1.8 ml/l (Roche) for 45 min at 37 °C. Using a sharpened forceps, microdissection of cortical efferent arterioles was performed under a stereomicroscope. Arterioles with glomeruli were moved to a clean Petri dish and the efferent arterioles were cut from the glomeruli. Isolated efferent arterioles were then collected for PCR.

### RT–PCR

RNA was isolated from microdissected efferent arterioles and reverse transcribed using Superscript and oligo as described previously.[36] Double PCR was performed for 30 cycles using template from three efferent arterioles per PCR reaction. One-third of the PCR product was used in the second PCR reaction running for 34 cycles. Specific mouse primers were—$A_1$AR: forward primer, 5′-cag-agc-tcc-atc-ctg-gct-ct-3′, reverse, 5′-cgc-tga-gtc-acc-act-gtc-ttg-3′ covering 100 base pairs (GenBank accession no. NM 001008533); $A_{2a}$AR: forward primer, 5′-ggc-tat-tgc-cat-cga-cag-at-3′, reverse, 5′-agg-tag-atg-gcc-agc-atg-ag-3′ covering 300 base pairs (GenBank accession no. Y13346); $A_{2b}$AR: forward primer, 5′-cgg-gat-cct-ttc-acg-gct-gcc-tct-tc-3′, reverse, 5′-gga-att-cca-tcc-ccc-agt-tct-gtg-c-3′ covering 256 base pairs (GenBank accession no. GI6680655) from Vitzthum *et al.*;[20] and $A_3$AR: forward primer, 5′-acc-gat-acc-tgc-ggg-tca-a-3′, reverse, 5′-tca-gcc-cca-cca-gaa-agg-3′ covering 111 base pairs (GenBank accession no. AF069778). Negative controls included water and RNA where no reverse transcriptase was added to the reaction (−RT). Whole kidney tissue was used as a positive control.

### DISCLOSURE

All the authors declared no competing interests.

### ACKNOWLEDGMENTS

This work was supported by the Danish Medical Research Council, the Carlsberg Foundation, The Danish Heart Foundation, and the Novo Nordic Foundation. We thank Kristoffer Rosenstand for technical assistance.

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