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# Norepinephrine increases calcium sensitivity of mouse afferent arteriole, thereby enhancing angiotensin II–mediated vasoconstriction

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**Many agents constrict isolated afferent arterioles only at concentrations higher than their physiological levels. Here we determined if norepinephrine, as released by sympathetic nerve activity, could influence the angiotensin II responsiveness of isolated mouse afferent arterioles. Pretreatment of the arterioles for short periods with norepinephrine significantly increased the ability of 10 picomolar angiotensin II to constrict the vessels, an effect inhibited by the alpha receptor blockers prazosin (α-1) or yohimbine (α-2). Although the intracellular calcium transients induced by angiotensin were not different, phosphorylation of the 20 kDa myosin light chain was significantly increased in the presence of norepinephrine. Phosphorylation of the p38 mitogen-activated protein kinase was not changed. Phosphorylation of the myosin phosphatase targeting subunit at Thr696, but not at Thr850, was significantly enhanced by, norepinephrine pretreatment, thus increasing the calcium sensitivity of the arteriolar smooth muscle. Our results show that norepinephrine increases afferent arteriolar sensitivity to angiotensin II by means of alpha receptor activation, causing increased calcium sensitivity through phosphorylation of the myosin phosphatase targeting subunit.**

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Afferent arterioles are important in the regulation of renal blood flow as they are the point of preglomerular resistance that produces the largest change in resistance, for example, with angiotensin II (Ang II) or norepinephrine (NE) stimulation.[1] The tone of the afferent arteriole is determined by endocrine and paracrine factors, as well as by sympathetic nerve activity[2] and autoregulation.[3] The aim of this study was to investigate whether NE, as released by sympathetic nerves, can augment vasoconstriction caused by Ang II in isolated renal afferent arterioles from mice. This may be a mechanism by which sympathetic activation may enable afferent arteriolar control by Ang II, even at physiological Ang II concentrations. We further identify the main signaling pathways responsible for the effect of NE on Ang II-induced contraction.

On rabbit and mouse afferent arterioles, NE causes a constriction, primarily by activating α-1 adrenoceptors.[4,5] α-2 receptors contribute to a lesser extent to NE-induced constriction.[5] Ang II causes the afferent arteriole to constrict in a concentration-dependent manner,[4] which is mediated primarily by the AT1a receptor in the rat.[6]

The interaction between Ang II and NE has been studied *in vivo*[7–9] and long-term Ang II infusion has been shown to affect afferent arteriolar sensitivity to NE.[10,11] Such an interaction may have a role in different physiological and pathophysiological situations, in which the sympathetic and/or the renin–angiotensin system is activated. However, the NE–Ang II interactions on the afferent arteriole remain to be determined. In this context, the role of intracellular calcium signaling is yet unknown, and our understanding of the activation pathways for contractile proteins in renal resistance vessels remains scant with regard to Ang II and NE and their potential interaction.

The contraction of smooth muscle is regulated by the phosphorylation and dephosphorylation of the 20 kDa myosin light chain (MLC(20)).[12] MLC(20) is phosphorylated by MLC kinase, which is activated through increased cytosolic calcium concentrations. Dephosphorylation of MLC(20) is mediated by MLC phosphatase (MLCP).[12] MLCP activity is modulated by phosphorylation of the

myosin phosphatase-targeting subunit at Thr696 or Thr850, which inhibits MLCP binding to MLC(20).[13] Interestingly, NE can cause phosphorylation of both the Thr696 and Thr850 sites, which may increase the calcium sensitivity of the smooth muscle.[14–16] Another possible pathway for increasing calcium sensitivity is the p38-mitogen-activated kinase (p38-MAPK), which has been shown to induce MLC(20) phosphorylation and constriction at low calcium concentrations,[17,18] potentially independent of calcium transients.[19] Both Ang II and NE can induce p38-MAPK phosphorylation,[20,21] which may thereby contribute to an interaction between NE and Ang II.

The aim of this study was to investigate whether NE stimulation affects the reaction of isolated mouse afferent arterioles to Ang II through its α-receptors. Furthermore, we wanted to show whether this is mediated through increased calcium signaling or increased calcium sensitivity as discussed above.

## RESULTS

### NE treatment enhances the effect of Ang II

Before NE treatment, the vessel diameter was 8.65 ± 0.72 μm. NE treatment caused a sustained constriction to 0.08 ± 0.06 μm and the vessels returned to the basal diameter after washout (8.55 ± 0.46 μm). Ang II does not constrict the afferent arteriole at $10^{-10}$ mol/l or at lower concentrations. The maximal constriction caused by Ang II was by 36.6 ± 5.8%, which was reached at $10^{-8}$ mol/l (Figure 1a and b). Vessels treated with NE showed a constriction already at $10^{-11}$ mol/l (46.3 ± 15.8% reduction of the diameter compared with 4.4 ± 4.3% in nontreated vessels, $P<0.05$). The constriction was also stronger than that seen in control vessels at $10^{-10}$ and $10^{-9}$ mol/l Ang II (Figure 1a and b).

### Calcium transients were not increased by treatment with both NE and Ang II

Cytosolic calcium transients were measured after an application of $10^{-11}$ mol/l Ang II in control vessels ($n=4$) and in vessels treated with $10^{-4}$ mol/l NE ($n=5$, Figure 2). NE treatment by itself caused a 135.6 ± 15 nM peak calcium increase. The baseline was 177.5 ± 13.9 nM $Ca^{2+}$ before and 190.3 ± 24.2 nM $Ca^{2+}$ after washout of NE. The baseline was similar in control vessels (181.7 ± 23.6 nM $Ca^{2+}$). Remarkably, there was no difference between the maximal calcium response to Ang II in control vessels (29.4 ± 7.1 nM $Ca^{2+}$) and that in NE-treated vessels (31.5 ± 13.3 nM $Ca^{2+}$).

### Both α-1 and α-2 receptors are necessary for the effect of NE on Ang II

Both prazosin and yohimbine inhibit the effect of NE on the dose–response of afferent arteriole to Ang II (Figure 3a and b). The curves are similar to the normal Ang II curve while being different from the Ang II dose–response curve in NE-treated vessels ($P<0.05$ by two-way analysis of variance).

### Myosin phosphatase-targeting subunit phosphorylation was enhanced by NE and Ang II together

Norepinephrine alone caused an increase in the phosphorylation of myosin phosphatase-targeting subunit at thr696, which remained after washout (0.85 ± 0.14 to 1.4 ± 0.5, $P<0.05$). Ang II $10^{-11}$ mol/l did not enhance myosin phosphatase-targeting subunit phosphorylation, but the combination of $10^{-11}$ mol/l Ang II with NE treatment

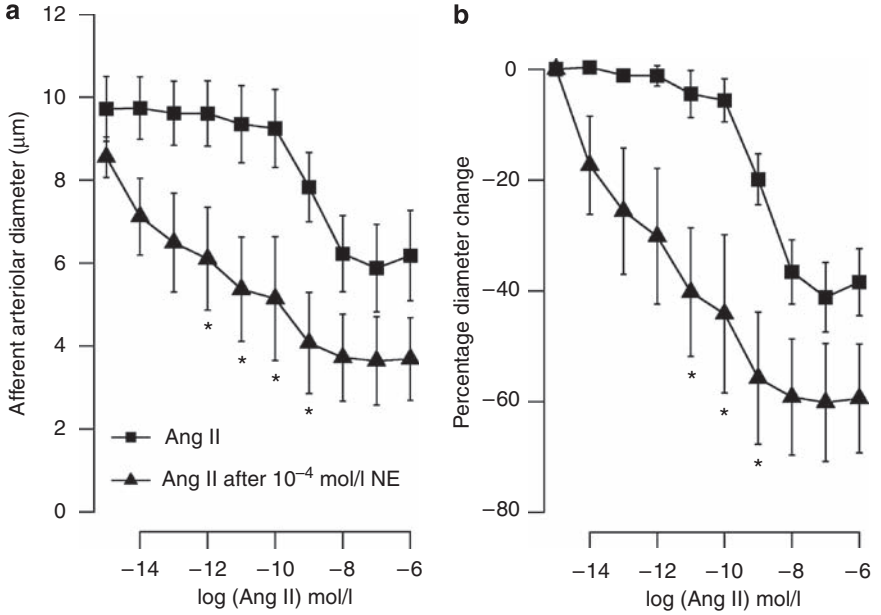


**Figure 1 | Effect of norepinephrine on angiotensin II–mediated constriction.** (**a**) A strong leftward and downward shift of the dose–response curve of the afferent arteriolar diameter to Ang II after treatment with norepinephrine (NE). The normal Ang II dose–response is shown as filled squares ($N=6$), NE-treated vessels are shown as filled triangles ($n=5$). (**b**) The same data as percentages. *Denotes a significant difference between nontreated and treated vessels by Tukey-HSD test ($P<0.05$).

showed a phosphorylation similar to that of NE alone ($1.24 \pm 0.38$, $P < 0.05$ compared with control Figure 4a). The combination of NE treatment with $10^{-11}$ mol/l of Ang II did not change myosin phosphatase-targeting subunit thr850 phosphorylation. No other treatment caused any significant effect on the thr850 phosphorylation of myosin phosphatase-targeting subunit as seen in Figure 4b.

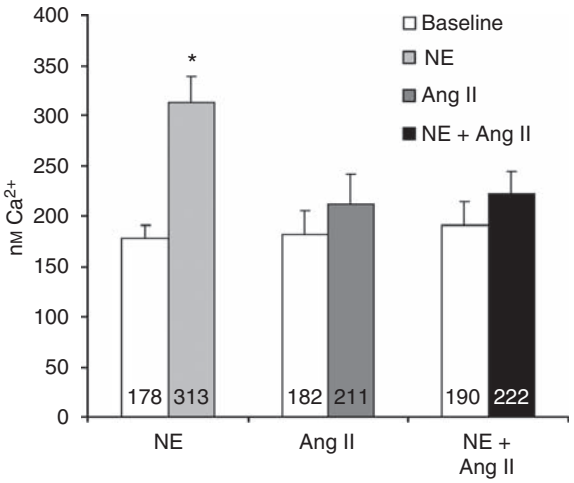


**Figure 2 | Intracellular calcium measured as Fura-2 340/380 ratio in perfused afferent arterioles.** White bars show baseline before norepinephrine (NE) treatment and the maximum calcium concentration measured for 2 min after application of either NE (light gray), Ang II (gray), or NE and Ang II (black). *Denotes a significant difference, $P < 0.05$, compared with that of the corresponding baseline.

### p38-MAPK phosphorylation was reduced by the combination of NE and Ang II

Norepinephrine by itself increased p38-MAPK to $0.75 \pm 0.23$ compared with the $0.29 \pm 0.12$ of baseline. Ang II did not affect p38-MAPK phosphorylation by itself ($0.55 \pm 0.29$ for $10^{-11}$ mol/l Ang II), but in combination with NE, a concentration of $10^{-11}$ mol/l Ang II inhibited the increase in phosphorylation caused by NE ($0.27 \pm 0.09$, Figure 4c).

### Myosin light chain phosphorylation was increased by NE and Ang II together

Norepinephrine strongly increased MLC(20) phosphorylation ($0.96 \pm 0.5$–$2.9 \pm 0.67$, $P < 0.05$), whereas Ang II did not affect MLC(20) phosphorylation significantly ($1.3 \pm 0.57$ at $10^{-11}$ mol/l and $1.22 \pm 0.36$ at $10^{-8}$ mol/l Ang II, respectively) with the protocols used in this study. The combination of NE pretreatment with low-dose Ang II did not increase MLC(20) phosphorylation above that of NE treatment alone, but it was significantly higher than the baseline phosphorylation ($1.81 \pm 0.18$, $P < 0.05$, Figure 4d).

## DISCUSSION

The main finding presented in this paper is the greatly enhanced response to Ang II in the afferent arteriole after transient treatment with NE (2 min, followed by 10 min washout). This effect of NE seems to be dependent on both

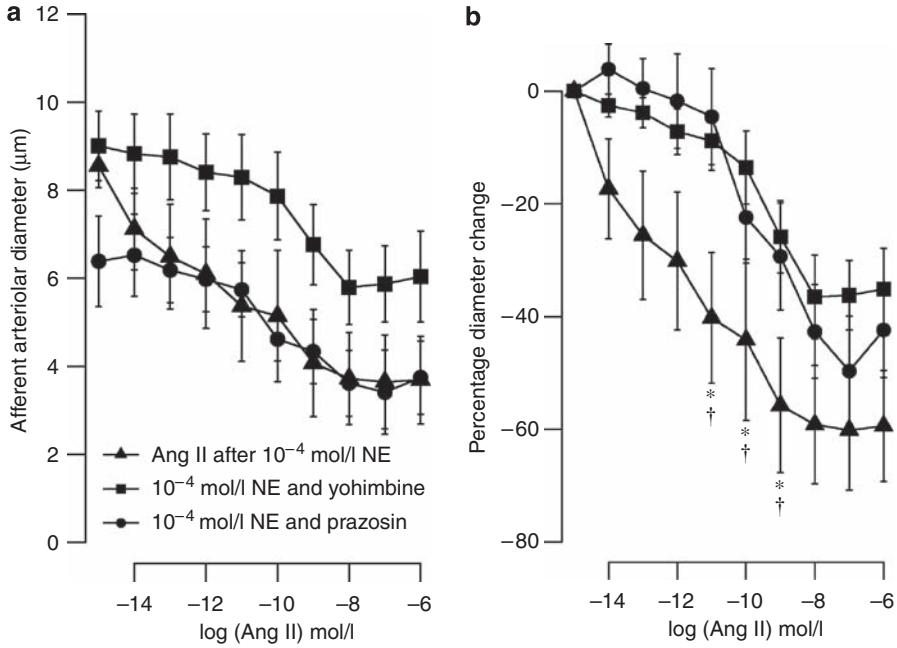


**Figure 3 | The α-1 antagonist prazosin and α-2 antagonist yohimbine inhibition of the $10^{-4}$ mol/l norepinephrine (NE) effect on Ang II-elicited constriction (shown in Figure 1 as well).** (**a**) Prazosin is depicted as solid circles ($n = 6$), yohimbine as solid squares ($n = 7$). (**b**) The same data shown as percentages. *Denotes a significant difference between yohimbine and NE-treated vessels and NE-treated vessels (solid circles in Figure 1b). †Denotes a difference between prazosin and NE-treated vessels and NE-treated vessels by Tukey-HSD test ($P < 0.05$) (solid triangles).

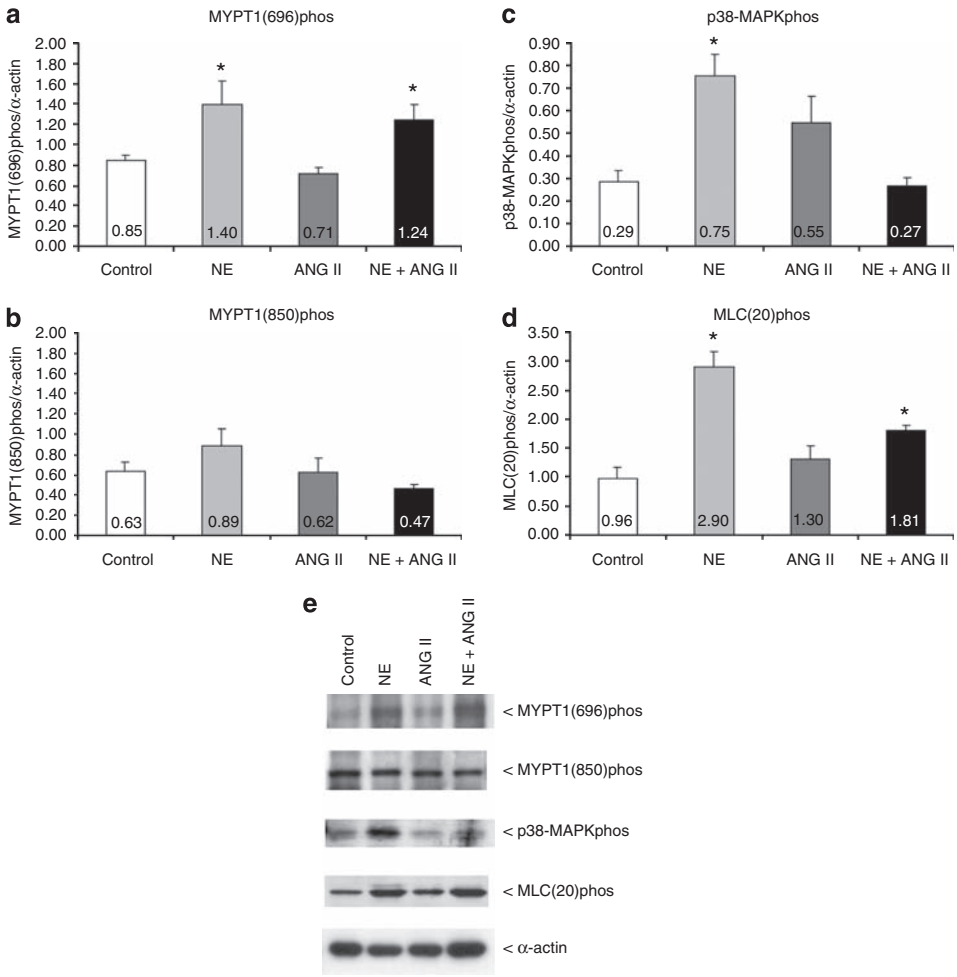


**Figure 4 | Western blot results for phosphorylated proteins.** (**a**) Myosin phosphatase-targeting subunit phosphorylation at thr-696. (**b**) Myosin phosphatase-targeting subunit phosphorylation at thr-850. (**c**) p38-MAPK phosphorylation. (**d**) MLC(20) phosphorylation at ser-20. Relative α-actin levels served as a loading control. *Denotes a significant difference compared with control, $n = 10$ isolated afferent arterioles for each group. (**e**) Representative western blots were created by pooling the protein extracts from 10 vessels per group, indicating the phosphorylation status of myosin phosphatase-targeting subunit (MYPT-1) at thr-696 or thr-850, p38-MAPK, and MLC(20) after NE ($10^{-4}$ mol/l), Ang II ($10^{-11}$) or the combined treatment of both substances. Relative α-actin levels were used to normalize results.

α-1 and α-2 receptors, but does not involve calcium signaling. Rather it involves a phosphorylation-dependent inactivation of the myosin phosphatase-targeting subunit of MLC phosphatase. This goes along with a significant increase in the phosphorylation of the 20 kDa MLC.

Interaction of Ang II and NE, that is, of the renin–angiotensin system and the sympathetic nervous system, has been shown in *in vivo* studies in the rat.[7–9] Interrupting the sympathetic nervous system decreased the effect of Ang II on renal blood flow, whereas inhibition of Ang II blunted the effect of renal sympathetic nerve stimulation.[22] It has been indicated that α-1 receptors contribute to the vasoconstrictive effect of infused Ang II,[23] and a dual knockout of the AT1a and AT1b Ang II receptors has been shown to decrease the effects of NE.[24] In this study, α-1 as well as α-2 blockade can inhibit the effect of transient NE application on Ang II response in mice. Such a cooperative action of both α-receptor types was also shown for the enhancing effect of adenosine triphosphate on NE-induced constrictions,[5] and may indicate that α-receptors are to some degree physically associated in the plasma membrane.

Cytosolic calcium changes did not differ between treated and nontreated arterioles. The fact that we show relatively small calcium transients at concentrations in which we have considerable constriction (NE followed by Ang II $10^{-11}$ mol/l) indicates that calcium sensitization may be an important mechanism for the effect of NE.

The myosin phosphatase-targeting subunit showed differential phosphorylation states at the known sites, Thr696 and Thr850, in response to NE, Ang II, and their combination. This study demonstrates an increase in the phosphorylation of Thr696 in response to NE and Ang II. It seems that this NE effect contributes to the enhanced phosphorylation after combined NE and Ang II treatment than the Ang II application alone. A known pathway for changing calcium sensitivity is through the MLCP that dephosphorylates

MLC(20) and reduces the contractile force. The myosin phosphatase-targeting subunit is necessary for MLCP binding to MLC(20) and phosphorylation of Thr696, as shown here, inhibits MLC(20) binding and reduces dephosphorylation, causing a net increase in the phosphorylation rate of MLC(20).[25] Thus, the enhanced phosphorylation of myosin phosphatase-targeting subunit at Thr696 may contribute to an increased MLC(20) phosphorylation by inhibition of MLCP.[14]

The fact that activation of both receptors is necessary and that the α-1, G(q)-associated,[26] signaling pathway and the α-2, G(i)[27] pathway converge on phospholipase C activation may indicate that the difference lies upstream of the activation of phospholipase C, that is, on the level of G-proteins or direct receptor interactions. However, the convergent phospholipase C activation has been shown to be important by itself in the interaction between Ang II-induced AT1 activation and NE-induced α-2 activation in the rat during simultaneous stimulation.[28] On the other hand, repeated treatment with Ang II does not lead to sensitization, but rather to a decreased reactivity,[29] which is also observed in our results.[17] Repeated applications of NE do not change the reaction from one application to the next,[29] which is consistent with our observations in the mouse (Hultström M., unpublished observation). Taken together, this indicates that there is some effect beyond calcium sensitization that makes the mechanism specific to the interaction between NE and Ang II.

In these studies, vessels are treated with a very high concentration of NE. The results may be directly applicable only to situations with a very strong sympathetic activity, such as hemorrhagic shock, in which it may be important. However, the mechanism described could well have a role in other situations with an increased renal sympathetic nerve activity, such as hypertension and edema in heart failure and nephrotic syndrome.[30] This is an area that warrants further investigation.

In conclusion, after NE treatment, there is a stronger vasoconstrictor response of the afferent arteriole to low concentrations of Ang II. This provides a mechanism by which physiological concentrations of Ang II may affect renal circulation. Second, the apparent dependence on both α-1 and α-2 receptors suggests a closer relationship between these or their respective G-proteins in the interaction with other transmitter systems. Finally, the lasting effect after washout of NE indicates a change in the contractile machinery, which increases the sensitivity of the afferent arteriole for longer times; this seems to be an effect of phosphorylation of the myosin phosphatase-targeting subunit.

## MATERIALS AND METHODS

### Animals

Male mice of the C57 black 6J strain (Scanbur BK AB, Solna, Sweden) were used in these experiments. The mice weighed between 25 and 30 g. The animals were fed standard pelleted food (Scanbur BK AB, Solna, Sweden) and had free access to tap water. The experiments were approved for ethics by the board of animal experiments at the county court of Uppsala.

### Dissection and perfusion

Dissection and perfusion were performed as previously published.[31] In short, the animals were killed by cervical dislocation and kidneys were removed and immediately placed in ice-cold Dulbecco's modified Eagle's medium with 0.1% albumin. The kidneys were sliced into thin transversal sections and afferent arterioles were dissected using sharpened forceps (no. 5, Dumont) under a stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany).

Arterioles from the outer cortex with attached glomeruli were selected and transferred to a thermoregulated chamber on the stage of an inverted microscope (Noran, Middleton, WI, USA). Glass pipettes (Drummond Scientific Company, Broomall, PA, USA) mounted in a perfusion system (Vestavia Scientific, Vestavia Hills, AL, USA) were used to fix and perfuse the arteriole and glomerulus. The holding pipette (outer diameter (OD) 2.13 mm, inner diameter (ID) 1.63 mm) had an aperture of roughly 26 μm at the tip and a constriction of about 20 μm after customizing. The proximal end of the arteriole was aspirated into this pipette. The inner, perfusion pipette (OD 1.19 mm, ID 1.02 mm), with a diameter of the tip of 5 μm, was advanced into the lumen of the arteriole. This pipette was connected to a reservoir containing the perfusion solution and to a manometer. The arterioles were perfused at 37°C with a pressure of 100 mm Hg in the pressure head, which, as previously shown, produces a perfusion pressure within the autoregulatory range and with a physiological perfusion flow rate.[31] Arterioles that exhibited a basal tone and clear lumen were tested for viability by changing the bath solution to 100 mmol/l KCl, after which the arterioles were allowed to recover for 10 min. Only arterioles showing a strong constriction to the KCl challenge were used.

### Measurement of arteriolar diameter

These experiments were recorded on super video home system videotapes (video recorder Panasonic NV-HS830, Matsushita Audio Video GmbH, Lueneburg, Germany). The final magnification results from a Nikon ×60/1.2 water-immersion objective lens and projection (×1) were obtained using a 0.3″ chip digital camera (VCAM110; Phytec Technology Holding AG, Mainz, Germany). Video sequences were digitized using a frame grabber card (pciGrabber-4plus; Phytec Technologie Holding AG). Vessel diameters were determined using customized software (Dr H. Siegmund, Johannes-Müller-Institute of Physiology, Humboldt-University of Berlin, Germany). The equipment allowed a resolution of 0.11 μm of the vessel structure. The luminal diameter of the afferent arteriole was measured at one point in five consecutive pictures with 1-s intervals during the last 10–15 s of each 2-min treatment period. Each vessel was used for one experiment only. Control diameter was obtained after recovery from the KCl test, shortly before starting the experimental protocol.

### Measurement of intracellular calcium

In separate experiments, afferent arterioles were dissected and perfused as described above and incubated with Fura-2 AM (VWR International AB, Stockholm, Sweden) at $10^{-5}$ mol/l for 45 min. The arterioles were excited alternately at 340 and 380 nm and emission was measured at 510 nm, with 3-s collection periods for each excitation. The ratio between emissions in successive time periods (340/380 nm) was used to determine intracellular calcium concentration against a calibrated standard (Fura-2 Calcium Imaging

Calibration Kit from Molecular Probes, F-6774, Carlsbad, CA, USA) in the same microscope. Fluorescence was detected using Applied Imaging Quanticell-900 (VisiTech International, Sunderland, UK). Three regions of interest were selected on each vessel, one covering the whole width of the vessel and one from each side of the vessel wall. The tracings from these areas were generally very similar. Results from the three areas were averaged for each vessel. Fura-2 ratio was measured for 2 min after application of Ang II. Calcium increase was calculated as the maximum recorded after application minus the baseline directly before application of Ang II.

### Diameter dose–response curves

A normal dose–response curve for Ang II was obtained by exchanging the bath solution at 2-min intervals in steps of 10 times the dilution from $10^{-14}$ to $10^{-6}$ mol/l. Vessel diameter was measured during the last 10–15 s of each period as a measure of sustained vessel diameter change. Only one experimental protocol for measurement of constriction and calcium transient each (agonists alone, in combination or together with antagonists) was performed in each arteriole.

### NE treatment

To study the interaction of NE and Ang II on the constriction of afferent arterioles, we incubated the isolated perfused arterioles with $10^{-4}$ mol/l NE for 2 min, followed by a 10-min washout period before performing an Ang II dose–response curve as described above.

### Use of inhibitors

All antagonists were purchased from Sigma Chemicals (St Louis, MO, USA). While investigating the effects of specific receptor blockers on the interaction between NE and Ang II, the receptor antagonists were added together with NE. Both NE and the antagonist were washed out before investigating the dose–response for Ang II. The receptor antagonists used were α-1-adrenoceptor blocker prazosin at a concentration of $10^{-7}$ mol/l and α-2-adrenoceptor blocker yohimbine at a concentration of $10^{-7}$ mol/l. We have used these concentrations previously.[5] Prazosin at a concentration of $10^{-7}$ mol/l blunted the constriction caused by $10^{-4}$ mol/l NE to approximately half, whereas yohimbine did not affect constriction, but inhibited the interaction of ATP with NE, although others have shown that $10^{-7}$ mol/l prazosin does not abolish the effect of yohimbine in mice.[32]

### Western blot

Cellular extracts from preglomerular vessels were prepared as described by Lai *et al.*[17] The isolated renal resistance vessels were treated with $10^{-4}$ mol/l NE or with Ang II at $10^{-11}$ mol/l, followed by a washout, or a combination of NE treatment and washout of NE (10 min) and subsequent treatment with Ang II ($10^{-11}$ mol/l), followed by washout. Protein extracts were separated by SDS–polyacrylamide gel electrophoresis and transferred to HybondTM-P membranes (GE Healthcare Europe, GmbH). The following phosphorylation-specific antibodies were used for detection of the protein phosphorylation status: anti-Myosine regulatory light chain (pSer20) antibody (Acris antibodies GmbH, Herford, Germany, R1535P), anti-phos p38-MAPK antibody (Calbiochem, no. 506119), anti-phosphorylated myosin phosphatase-targeting subunit at thr696 (Millipore, Schwalbach/Ts., Germany, no. 07-251), anti-phosphorylated myosin phosphatase-targeting subunit at thr850 (Millipore, no. 36-003). Membranes were stripped for 5 min with distilled water, for 5–15 min with 0.2 M NaOH, and again for 5 min with distilled water, and reprobed using an anti-α-actin antibody (Acris antibodies GmbH, AB5694). Detection of relative smooth muscle-specific α-actin levels served as loading control.

### Statistics

Functions were analyzed with two-way analysis of variance to test for constriction over time and for difference between curves.

Baseline diameter varies between the different groups of vessels used for diameter measurement. In particular, the prazosin-treated group seems to have a lower baseline diameter, and has a borderline significant difference than the others by analysis of variance. Although this is a notable difference, we do not believe that these vessels belong to any special population, but that what we see is an effect of the natural variation in diameters of afferent arterioles in mice. The reactivity of afferent arterioles from mice seems to be very consistent and not related to baseline diameter when studied as relative changes, which therefore forms the basis of our statistics and discussion.

Tukey-HSD test was used as a *post hoc* test between curves and *t*-tests were used to test for significant difference in calcium increase. $P<0.05$ was considered to be significant. Statistics were calculated using R 2.8.0 software (Vienna, Austria).[33]

## DISCLOSURE

All the authors declared no competing interests.

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