Interaction of Angioteins II and Nitric Oxide in Isolated Perfused Afferent Arterioles of Mice

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Abstract. The present study was performed to evaluate angioteins II (Ang II)–nitric oxide (NO) interaction in afferent arterioles (Af) of wild-type mice and mice that are homozygous (−/−) for disruption of the endothelial NO synthase (eNOS) gene. Af were microperfused, and the dose responses were assessed for the NO precursor L-arginine (n = 4), NO inhibitor NG-nitro-L-arginine methyl ester (L-NAME, n = 5), L-NAME after pretreatment with L-arginine (n = 5), Ang II (n = 8), and Ang II after pretreatment with L-NAME (n = 7). Acute administration of L-arginine and L-NAME (both in doses from 10⁻⁶ to 10⁻⁷ mol/L) did not change arteriolar diameter. Moreover, pretreatment with L-arginine did not change the response to L-NAME. However, Ang II, applied in doses of 10⁻¹², 10⁻¹⁰, 10⁻⁸, and 10⁻⁶ mol/L, significantly reduced the lumen to 66.5 ± 7.0% and 62.2 ± 8.0% at 10⁻⁸ and 10⁻⁶ mol/L Ang II, respectively. The contraction was augmented after L-NAME pretreatment (19.5 ± 13.6% and 25.5 ± 10.2% at 10⁻⁸ and 10⁻⁶ mol/L Ang II, respectively). In eNOS (−/−) mice (n = 8), the response to Ang II also was enhanced (9.1 ± 6.0% and 11.2 ± 8.2% at 10⁻⁸ and 10⁻⁶ mol/L Ang II, respectively). Female mice did not differ from male mice in their reactivity to Ang II (n = 9) and Ang II + L-NAME pretreatment (n = 11). The study shows that (1) it is feasible to microperfuse mouse Af, (2) the basal production of endothelial NO is very low and not inducible by L-arginine in Af of mice, and (3) a counteracting effect of NO is initiated by Ang II. High Ang II sensitivity in eNOS (−/−) mice underscores the considerable role of endothelial-derived NO to balance Ang II vasoconstriction in Af.

Nitric oxide (NO) released from endothelial cells and the macula densa is involved in the control of glomerular hemodynamics by acting as a potent vasodilator. Unspecific blockade of the NO synthase (NOS) isoforms leads to increases in mean arterial BP and decreases in renal blood flow and GFR (1,2). Moreover, in vitro studies that used the juxtamedullary nephron preparation showed significant reductions in diameters of afferent arterioles (Af) during NOS inhibition (3). This suggests a tonic NO production. Enhanced Af vasoconstrictor responses to NOS blockade were observed in angioiteins II (Ang II)–induced hypertensive rats in comparison with responses in normotensive controls (3). These results suggest an Ang II–induced NO production that is mediated by either subtype 1 (AT₁) or subtype 2 (AT₂) angioitein receptors (4,5). Experiments in isolated perfused rabbit arterioles underscore an important interaction between Ang II and NO: Ang II–induced arteriolar constriction in this preparation is transient only because of activation of local NO (6).

It remains unclear whether this effect is due to endothelial-derived NO or whether the neuronal NOS isoform located in the macula densa cells is responsible for counteracting Ang II vasoconstriction. The availability of mice that are homozygous (−/−) for disruption of the endothelial NOS (eNOS) gene allows the study of selectively inhibited NO production by eNOS. However, to perfuse isolated mouse Af, a perfusion setup must be miniaturized. In the present study, this was put into effect.

Recent studies demonstrated significantly higher BP in eNOS knockout mice (7,8). Furthermore, overexpression of eNOS causes hypotension and reduces NO-elicited vasorelaxation in mice (9). Thus, in this animal model, an important contribution of endothelial-derived NO on vascular resistance was apparent. However, the role of NO in adjustment of renal arterioles in the mice still is not understood fully. Therefore, in the present study, we examined to what extent NO modulates the constriction of Af because of Ang II. Reduction of NO production was achieved by application of the unspecific NO inhibitor NG-nitro-L-arginine methyl ester (L-NAME). In addition, eNOS knockout mice were studied. Experiments were carried out on isolated perfused Af, which are not under control of the sympathetic nervous system and circulating vasoactive substances.

We tested further whether the Ang II–NO interaction is gender dependent. There are some indications that sex hormones interact with the renin-Ang and NO system. For exam-
ple, it has been found that estrogen reduces Ang II plasma levels (10) and downregulates the AT₁ receptor expression in ovarioctomized female rats (11). Moreover, a significant association between the number of estrogen receptors and basal release of NO in mouse aorta has been described elsewhere (12).

Materials and Methods

Solutions

Physiologic salt solution (PSS) was used with the following composition: 115 mmol/L NaCl, 25 mmol/L NaHCO₃, 2.5 mmol/L K₂HPO₄, 1.3 mmol/L CaCl₂, 1.2 mmol/L MgSO₄, and 5.5 mmol/L glucose. The K⁺ solution consisted of 100 mmol/L KCl, whereby 95 mmol/L NaCl was substituted by KCl. Dulbecco’s modified Eagle’s medium (DMEM) was supplied with 100 mg of streptomycin and 100,000 U of penicillin. The bicarbonate-buffered solution was equilibrated with 5% CO₂ and 95% O₂. The pH was adjusted to 7.4 after addition of bovine serum albumin (BSA). The concentration of BSA in DMEM (preparation solution) and in PSS was 0.1% (bath solution). The perfusion of bovine serum albumin (BSA) was obtained from SERVA Electrophoresis (Heidelberg, Germany), and L-arginine and L-NAME were obtained from Alexis (Lausanne, Switzerland). Dulbecco’s modified Eagle’s medium (DMEM) was supplied with 100 mg of streptomycin and 100,000 U of penicillin. The bicarbonate-buffered solution was equilibrated with 5% CO₂ and 95% O₂. The pH was adjusted to 7.4 after addition of bovine serum albumin (BSA). The concentration of BSA in DMEM (preparation solution) and in PSS was 0.1% (bath solution). The perfusate consisted of PSS with a BSA concentration of 1%. BSA was obtained from SERVA Electrophoresis (Heidelberg, Germany), and L-NAME and L-arginine were obtained from Alexis (Lausanne, Switzerland).

Animals and Microperfusion Procedure

A total of 57 mice (20 to 30 g) of both genders were used in this study. The mice were wild types from the C57BL/6 strain and eNOS mutants (8) (The Jackson Laboratory, Bar Harbor, ME). Animals were fed with standard mouse chow and allowed free access to tap water. All animal procedures conformed with the guidelines for care and handling of animals established by the U.S. Department of Health and Public Services and published by the National Institutes of Health.

After each animal was killed, the kidneys were removed immediately and sliced along the corticomedullary axis. The AF were prepared in DMEM at 4°C. The dissection of individual arterioles was performed under a stereoscopic microscope at magnifications up to 250X and with the help of sharpened forceps (No. 5; Dumont, La Chaux-de-Fonds, Switzerland). The position of AF in the slice was indicated by the glomerulus that belonged to it. Tubuli were removed, and the arterioles were identified by preparation of the arterial tree, including the interlobular artery. Normally, AF were used at the end of the arterial tree. The arteriole with its glomerulus intact was cut with minibilades and transferred into a thermoregulated chamber (vol 1.5 ml; VETEC, Rostock-Warnemünde, Germany) on a stage of an inverted microscope. The perfusion system allowed movement and adjustment of concentric, holding, and perfusion pipettes (Luigs & Neumann, Ratingen, Germany). Pipettes were produced with the help of a self-manufactured apparatus and with the use of custom glass tubes (Drummond Scientific Company, Broomall, PA). The holding pipette (outer diameter, 0.084; inner diameter, 0.064) into which the proximal end of the arteriole was aspirated had an aperture of approximately 26 μm at the tip and a constriction of approximately 20 μm after customization. The inner perfusion pipette (outer diameter, 0.047; inner diameter, 0.040) with an aperture of 5 μm was advanced into the lumen of the arteriole. It was connected to a reservoir that contained the perfusion solution and to a manometer. AF of wild-type and eNOS knockout mice were perfused with similar pressures between 60 and 80 mmHg at 37°C. Although the arterial BP in eNOS knockout mice is approximately 20 mmHg higher than that of wild types (8), pressure was unchanged in all series to exclude the influence of this factor. If perfusion was not achieved within 120 min after the mouse was killed, then the experiment was stopped. The time of adaptation after the start of perfusion and heating of the bath chamber was 20 min. Only arterioles with remaining basal tone were used. Hypoxic or otherwise injured vessels were identified readily by pronounced vasodilatation and failed constriction to K⁺-rich solution, which was used to test the viability of the arterioles at the beginning of the experiment. Thereafter, a recovery of 10 min was allowed. In all series, the last 10 s of the treatment period were used for statistical analysis of steady-state responses.

Experimental Protocols

Series 1. The effect of the NO precursor L-arginine on arteriolar diameter was determined. L-arginine was added to the bath in concentrations of 10⁻⁶, 10⁻⁵, 10⁻⁴, and 10⁻³ mol/L. A 3-min treatment period was used. AF diameters were measured under control conditions and during exposure of L-arginine.

Series 2. To determine whether an endogenous, tonic NO release plays a role for the arteriolar diameter in this preparation, we added the NO synthase inhibitor L-NAME to the bath solution. The L-NAME concentration was increased from 10⁻⁶ to 10⁻³ mol/L with treatment periods of 3 min. The diameter was determined before and during the exposure of L-NAME.

Series 3. To exclude that the lack of the NO precursor L-arginine prevents a tonic production and release of NO as tested in series 2, all solutions were enriched with L-arginine (10⁻³ mol/L). The protocol corresponds to that in series 2.

Series 4. In this series, the dose response to Ang II was determined at concentrations of 10⁻¹², 10⁻¹⁰, 10⁻⁸, and 10⁻⁶ mol/L. The treatment period was 2 min. AF diameters were measured before and during the addition of Ang II. Female as well as male mice were used, to assess any gender-specific effects of Ang II on arteriolar diameter.

Series 5. Experiments were performed to determine whether NO modulates the arteriolar reactivity to Ang II. The bath solution was enriched with L-NAME at a concentration of 10⁻⁴ mol/L. The doses of Ang II were the same as those in series 3. Female and male mice were included, to test for gender-specific effects.

Series 6. Mice that were homozygous (−/−) for disruption of the eNOS gene were used to test the effect of total blockade of endothelial NO production by eNOS. The reactivity of the vessel to Ang II was determined by addition of Ang II in concentrations of 10⁻¹², 10⁻¹⁰, 10⁻⁸, and 10⁻⁶ mol/L.

Data Analyses

Experiments were recorded on SVHS videotapes (video recorder AG-MD 830; Panasonic, Yokohama, Japan). The magnification results from an objective (×40; Zeiss, Oberkochen, Germany) and projection (×1) on a 0.3" chip digital camera (CB-3803S; GKB, Tai Chung, Taiwan). Video sequences were digitized by use of a frame grabber card (UDT 55-LC-EZ-50; Data Translation, Marlboro, MA). The vessel diameter was determined by use of customized software (Dr. H. Siegmund, Institute of Physiology, Humboldt-University of Berlin, Berlin, Germany). The equipment allowed a resolution of 0.2 μm of the vessel structures.

Statistical Analyses

The luminal diameter of the arterioles was determined. In all series, the last 10 s of the treatment and control period were used for statistical analysis of steady-state responses. For each 10-s period, five measurements were averaged. To test diameter dose responses, we
performed a nonparametric Friedman two-way ANOVA. If significance was found, then the Mann-Whitney U test was applied. The same test was used for the comparison of independent measures. The confidence level, α, was set to 0.05. Data are presented as mean ± SEM.

Results
Pharmacologic Stimulation and Inhibition of NOS

Acute addition of L-arginine to the bath solution in increasing doses did not change the diameter of the arterioles (Figure 1, n = 4, male mice). However, the highest dose led to a transient contraction of the vessels, which, however was completely normalized at the time of measurement. L-NAME also had no effect on the vessel diameter (Figure 2, n = 5, male mice). Pretreatment with L-arginine, which was added to all solutions, did not change the response of the vessel to L-NAME (Figure 2, n = 5, male mice).

Effect of Ang II

Ang II reduced the diameter significantly, from 8.1 ± 0.5 μm (control) to 5.6 ± 0.8 μm (66.5 ± 7.0%) and to 5.2 ± 0.8 μm (62.2 ± 8.0%) at doses of 10⁻⁸ and 10⁻⁶ mol/L, respectively. There were no differences between female (n = 9) and male (n = 8) mice, either for the absolute diameter or for percentage changes (Figure 3).

Effect of L-NAME on Responses to Ang II

Ang II significantly reduced arteriolar diameter in vessels that were pretreated with L-NAME (male mice, n = 7). The diameter averaged 8.9 ± 1.0 μm during control, 1.8 ± 1.3 μm (19.5 ± 12.6%; P < 0.05) at 10⁻⁸ mol/L Ang II, and 2.4 ± 1.1 μm (25.5 ± 10.2%; P < 0.05) at 10⁻⁶ mol/L Ang II. As shown in Figure 3, Ang II + L-NAME led to further constriction, in comparison with Ang II without L-NAME pretreatment (1.8 ± 1.3 versus 5.6 ± 0.8 μm [P < 0.05] and 19.5 ± 12.6% versus 66.5 ± 7.0% [P < 0.05] at 10⁻⁸ mol/L Ang II; 2.3 ± 1.1 μm versus 5.2 ± 0.8 μm [P = 0.054], 25.5 ± 10.2% versus 62.2 ± 8.0% [P < 0.05] at 10⁻⁶ mol/L Ang II, male mice). Female (n = 11) and male (n = 7) mice did not differ in regard to their response to Ang II during the exposure to L-NAME (Figure 3).

Ang II Effect in eNOS Knockout Mice

In mice that were homozygous (−/−) for disruption of the eNOS gene (female mice, n = 8), Ang II reduced diameters from 8.2 ± 0.9 μm (control) to 0.7 ± 0.5 μm (P < 0.05) and 9.1 ± 6.0% (P < 0.05), respectively, at 10⁻⁸ mol/L Ang II and to 0.8 ± 0.6 μm (P < 0.05) and 11.2 ± 8.2% (P < 0.05), respectively, at 10⁻⁶ mol/L Ang II (Figure 4). The values at 10⁻⁸ and 10⁻⁶ mol/L were significantly less in comparison with arteriolar diameters of wild-type mice that were treated with the same Ang II dose (female mice, n = 9). No significant differences in Ang II reactivity were found between eNOS knockout and wild-type mice that were pretreated with L-NAME (10⁻⁶ mol/L Ang II, n = 5).

Figure 1. Effect of L-arginine administration to the bath solution in increasing doses from 10⁻⁶ to 10⁻³ mol/L on the luminal diameter (male mice, n = 4).

Figure 2. Effect of N⁵-nitro-L-arginine-methyl ester (L-NAME; 10⁻⁶ to 10⁻³ mol/L, male, n = 5) on luminal diameter without (■) and after pretreatment with (□) L-arginine (10⁻⁴ mol/L, male mice, n = 5).

Figure 3. Effect of angiotensin II (Ang II) and Ang II after pretreatment with L-NAME (10⁻⁴ mol/L L-NAME) on the luminal diameter of afferent arterioles (Af) of female and male mice. The doses of Ang II were 10⁻¹², 10⁻¹⁰, 10⁻⁸, and 10⁻⁶ mol/L. * and # indicate significant changes in the diameter, compared with the control value for female and male mice, respectively. Significant differences between the effect of Ang II and Ang II + L-NAME are indicated by ▲ and ■ for female and male mice, respectively.
NAME (female mice, n = 11; Figure 4). In both groups, complete constriction of the vessels usually occurred at $10^{-8}$ and $10^{-6}$ mol/L Ang II (for example, see Figure 5).

**Discussion**

This study shows that endothelial NO is important in countering Ang II vasoconstriction of isolated mouse Af. Remarkably, however, administration of the precursor of the NO production L-arginine or blockage of the NOS activity by L-NAME without Ang II does not influence the diameter of Af (Figures 1 and 2). Thus, the basal activity of NO is negligible in this preparation.

In vivo, L-arginine causes an increased plasma flow and GFR in rats (13). Accordingly, pharmacologic blockade of NO production leads to an increase in renal vascular resistance (1,13–15) and a decrease in renal plasma flow in the rat (13,16). A bulk of evidence indicates that the L-arginine–mediated vasodilatation depends on NO (for overview, see reference 17). As was mentioned above, in the present study, L-arginine did not significantly change afferent vessel diameter. This may be explained, in part, by experimental conditions, e.g., the absence of circulating vasoactive substances. Notably, the inhibition with L-NAME did not change the arteriolar diameter either. In isolated perfused vessels, there is no supply of NO precursors during the experiment, which could explain the lacking L-NAME vasoconstriction. However, replenishing L-arginine to the bath solution failed to elicit an L-NAME response. These results differ from studies on isolated Af of the rabbit (6,18). In those studies, a clear reduction of the vessel diameter in response to L-NAME pretreatment was observed. Methods and drug concentration used in these studies match with our experimental protocols. Thus, our results suggest that, in mice, vasoconstriction caused by Ang II (or possibly other vasoconstrictors) is a prerequisite for a functional significance of the NO system in the control of vascular diameter.

In our study, Ang II constricted the Af significantly in a dose-dependent manner. The contraction reached significance at $10^{-8}$ and $10^{-6}$ mol/L Ang II. The results agree well with investigations in vivo and in vitro that demonstrated the vasoconstrictor properties of Ang II in Af in rabbit and rat (6,19–21). In contrast to previous experiments in isolated Af in the rabbit (6), we observed a prolonged contraction. This can be explained by the proposed low eNOS activity in mice. Accordingly, pretreatment with L-NAME augmented the vasoconstrictor action of Ang II at $10^{-8}$ and $10^{-6}$ mol/L.

The advantage of miniaturizing the experimental setup to allow Af perfusion of the mouse is that knockout models can be investigated. In eNOS knockout mice, which have a very low endothelial NO production, the response to Ang II was increased (Figure 4). An antagonistic action of Ang II and NO...
on Af in normotensive rats (19, 21) and in isolated perfused Af in rabbits has been reported elsewhere (6, 18). From these studies, as well as from studies in Ang II–hypertensive rats (22), it is assumed that Ang II induces NO production in renal vessels. Thus, blocking NO withdraws the counterbalance to Ang II vasoconstriction. Remarkably, in the present study, the entire effect of NO relied on Ang II induction. It is not clear whether the activation of AT1 or AT2 receptors stimulates the release of NO from Af. Both AT1 receptors of endothelial cells and AT2 receptors have been discussed elsewhere (4, 18, 23, 24).

In the kidney, all isoforms of NOS have been discovered (25, 26). Neuronal NOS and eNOS are found in the renal vasculature and in the glomerulus (27, 28). The question arises as to which of the isoforms are involved in the Ang II–NO interaction. It could be shown that Ang II acts dose dependently and that the vasoconstrictive effect is enhanced in eNOS knockout mice. Because both the L-NAME treatment and knockout of the eNOS produces comparable increases in Ang II vasoconstriction, it is concluded that the mainly endothelial-derived NO counteracts the Ang II effect in our preparation. The clear enhancement of Ang II reactivity in the eNOS knockout mice is a little surprising, because compensating mechanisms can be assumed in such mice with congenital defect of NO.

Af vasoconstriction in response to Ang II did not differ between male and female mice. Moreover, there were no differences after pretreatment with L-NAME in the Ang II dose response in either of these groups. Our perfusion model is free of circulating hormonal influences and does not contain estrogen. Hormones were not added, and so, in the present model, it is assumed that gender does not change the response to Ang II.

In conclusion, the present study shows that it is feasible to microdissect and microperfuse mouse Af, thereby allowing studies on genetically modified mice. From the data obtained in Af of eNOS knockout mice and Af pretreated with L-NAME, it seems that the Ang II vasoconstriction elicits a counterbalancing NO release. The endothelial NOS isoform is responsible for this action. Furthermore, the NO system of isolated perfused Af in mice differs from other species in regard to its dependence on Ang II. Finally, this study also shows that there are no gender differences in Ang II–NO interaction in Af.

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References


