Renal microvascular actions of angiotensin II fragments

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During the last decade, evidence has accumulated that in addition to ANG II, ANG II fragments such as the heptapeptide ANG-(1–7) and hexapeptide ANG IV (i.e., ANG II3–8) possess vasoactive properties. ANG-(1–7) is formed from ANG II by hydrolysis of the COOH-terminal Pro7–Phe8 bond (1). In addition, ANG IV, on the other hand, is generated predominantly from ANG II by hydrolysis of two amino acids from the NH2-terminal end (1). The enzymes involved in the formation of both ANG-(1–7) and ANG IV are abundantly present in the kidney (10, 37), resulting in higher intrarenal than plasma levels of these fragments (3). Thus, besides ANG II, ANG-(1–7) and ANG IV may exert vasoactive actions in the kidney and contribute to the control of glomerular hemodynamics by affecting pre- and/or postglomerular resistance. These actions could especially become relevant when fragment levels get elevated, for instance, during antihypertensive treatment with ANG-converting enzyme inhibitors (19, 21, 27).

At present, the renal microvascular actions of ANG-(1–7) are still largely unclear. In normal isolated rat kidneys, ANG-(1–7) slightly increased the glomerular filtration rate, although no effect on the total vascular resistance was found (9, 18). In vivo, intrarenal infusion of ANG-(1–7) reduced the local blood flow in rats and dogs (15, 17, 33), indicating that this fragment acts as a constrictor of at least some renal resistance vessels. In several other vascular beds, however, ANG-(1–7) was found to induce vasodilation (2, 22, 30, 31).

In addition, in humans, ANG-(1–7) attenuated vascular responses to ANG II (32, 36), suggesting that this fragment may also possess renal vasodilator properties and counteract ANG II-related actions (11).

Intrarenal infusion of ANG IV in rats has been reported to induce both a decrease (12, 13, 38) and an increase (7) in renal blood flow. Similarly, ANG IV-induced constriction was found in pulmonary (6, 28), mesenteric (5, 13, 23), and hindlimb vascular beds (4, 14), whereas it induced vasodilation in cerebral (20) and cochlear circulation (8). In general, a reduction in local flow by either ANG-(1–7) or ANG IV is sensitive to ANG II type 1 (AT1) receptor blockade (12, 13, 15, 17, 38), indicating that this receptor subtype mediates vasoconstrictor responses to these ANG II fragments. By contrast, little is known about the receptors mediating vasodilator responses to ANG-(1–7) and ANG IV.

In the present study, we used the in vitro perfused hydronephrotic rat kidney model to investigate the effects of ANG-(1–7) and ANG IV on distal interlobular arteries (ILAs), afferent arterioles (AAs), and efferent arterioles (EAs). We used irbesartan and PD-123319 to assess involvement of the AT1 and the ANG II type 2 (AT2) receptors. In addition, we determined whether ANG-(1–7) and ANG IV were able to counteract renal microvascular actions of ANG II and whether subnanomolar concentrations of ANG-(1–7) attenuated pressure-induced constriction of preglomerular vessels.

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Our findings indicate that relatively high concentrations of ANG-(1–7) and ANG IV elicit constriction of both pre- and postglomerular vessels by activation of the AT1 receptor. ANG-(1–7) and ANG IV failed to alter renal microvascular constriction to ANG II. Moreover, subnanomolar concentrations of ANG-(1–7) had no effect on pressure-induced tone, indicating that ANG II fragments do not exert ANG II antagonistic and/or vasodilator actions in the kidney.

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats (225–250 g) were obtained from Harlan (Zeist, The Netherlands). They were housed individually in macrolon cages and had ad libitum access to pelleted food and water. The Institutional Animal Care and Use Committee approved all procedures as described below.

**Chemicals.** The perfusion medium consisted of DMEM supplemented with (in mmol/l) 23.8 bicarbonate, 5.5 d-glucose, 1 sodium pyruvate, and 5.6 HEPES. The medium was equilibrated with 95% air-5% CO2 at 37°C, resulting in a PO2 and PCO2 of ~150 and 35 mmHg, respectively. The pH of the medium was maintained at 7.4. DMEM and d-glucose were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), bicarbonate was from Merck (Darmstadt, Germany), and pyruvate and HEPES were purchased from GIBCO BRL (Breda, The Netherlands).

Stock solutions of ANG-(1–7), ANG IV, and ANG II (all Sigma-Aldrich) and PD-123319 (Bachem, Budendorf, Switzerland) were dissolved in distilled water and stored at −20°C until use. Irbesartan was kindly provided by Sanoﬁ (Montpellier, France) and dissolved in 50% dimethylformamide (Sigma-Aldrich) on the day of an experiment.

**Induction of unilateral hydronephrosis.** Rats were anesthetized with hypnorm (4 mg/kg fluanison and 0.126 mg/kg fentanyl im; Janssen Pharmaceutica, Beerse, Belgium) and diazepam (2 mg/kg ip; Centrafarm, Etten-Leur, The Netherlands). To induce hydronephrosis, the left ureter was exposed through a small midabdominal incision and subsequently tied off with a suture. Six to eight weeks after surgery, renal tubular tissue had undergone almost complete atrophy, allowing direct microscopic visualization of the renal microvasculature (25, 35). At that stage, the hydronephrotic kidney was excised for in vitro perfusion.

**In vitro perfusion of hydronephrotic kidneys.** The technique for isolation and in vitro perfusion of hydronephrotic rat kidneys has been described in detail by Loutzenhiser (24). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg ip; Sanoﬁ Sante, Maassluis, The Netherlands) and ketamine (25 mg/kg im; Kombivet, Etten-Leur, The Netherlands). The hydronephrotic kidney was exposed through a wide abdominal incision, and its renal artery was cannulated via the abdominal aorta. Under continuous perfusion with DMEM, the kidney was excised and connective tissue, fat, and the renal capsule were removed. Subsequently, the kidney was moved to the stage of an inverted microscope (Axiophot vert 100; Zeiss, Weesp, The Netherlands) that was equipped with a thin glass viewing port on the bottom surface (25). Preheated and oxygenated DMEM that was supplied to the renal artery was pumped on demand from a large reservoir through a heat exchanger to a small reservoir. Inflow of gas (95% air-5% CO2) into this small reservoir provided the force needed for perfusion of the hydronephrotic kidney. The perfusion pressure was monitored at the level of the renal artery and kept constant at 80 mmHg by adjusting the pressure within the small reservoir with a backpressure regulator (Fairchild Industrial Products, Winston-Salem, NC). A roller pump (Masterflex, Chicago, IL) was used to pump the venous effluent away from the microscope chamber.

**Determination of renal microvascular diameters.** A small region of the transparent renal cortex was transilluminated and immobilized with a light rod connected by means of an optic fiber to a halogen lamp (model KL1500 electronic; Schott, Wiesbaden, Germany). Video images of ILAs, AAs, and EAs were recorded by means of a camera (model HV7355; Hitachi) and a time-date generator on a videocassette recorder (model RTV-925 HIFI; Blauptuk, Hildesheim, Germany) for off-line analysis. An ×40 objective lens (LD achroplan, 0.6 numerical aperture; Zeiss) yielded a final magnification at the video monitor (model LDH 2135/10; Philips, Eindhoven, The Netherlands) of ×1,370. Video images were digitalized by a computer acquisition board (model IVG-128; Datacube, Peabody, MA), and luminal vessel diameters were assessed by using an automated custom-designed wall tracking system that measured the distance between dark parallel edges on either side of the vessel wall. A vessel segment of ~5 μm in length was scanned at 1- to 2-s intervals for a period of 30 s. Mean vessel diameters were determined by averaging all measurements: ILAs just before branching into AAs, AAs just after branching from ILAs, and EAs within 50 μm of the point where the vessel emerged from the glomerulus.

**Perfusion experiment protocols.** Kidneys were allowed to equilibrate for at least 30 min before experiments began. Investigated drugs were added to the large reservoir in a cumulative way, and luminal diameters were assessed 10 min later. Four series of experiments were performed.

In the first series, we determined the vasoactive actions of ANG-(1–7) and ANG IV per se (i.e., without inducing vascular tone) and investigated the role of the AT1 receptor. Vessel diameters were measured after exposure to increasing concentrations of either ANG-(1–7) (10 nmol/l–3 μmol/l, n = 6) or ANG IV (1 nmol/l–0.1 μmol/l, n = 7). Subsequently, kidneys were perfused for 1 h with drug-free medium, after which they were reexposed to ANG-(1–7) (1 μmol/l, n = 5) or ANG IV (30 nmol/l, n = 5). In the presence of these fragments, increasing concentrations of the AT1 receptor blocker irbesartan (0.1–10 nmol/l) were administered. For comparison of the potency by which ANG-(1–7) and ANG IV exerted their renal microvascular effects with ANG II, a separate group of kidneys (n = 5) was treated with increasing concentrations of ANG II.

In the second series, the involvement of the AT2 receptor in the renal microvascular actions of ANG-(1–7) and ANG IV was assessed. Kidneys were treated with either 1 μmol/l ANG-(1–7) (n = 4) or 30 nmol/l ANG IV (n = 3). Subsequently, in the presence of ANG-(1–7) or ANG IV, increasing concentrations of the AT2 receptor blocker PD-123319 (0.1–100 nmol/l) were administered.

In the third series, we investigated whether ANG-(1–7) and ANG IV were able to attenuate renal vascular responses to ANG II. Renal microvessels were preconstricted with 0.3 nmol/l ANG II. Subsequently, in the continuous presence of ANG II, increasing concentrations of either ANG-(1–7) (1 nmol/l–1 μmol/l, n = 4) or ANG IV (0.1 nmol/l–1 μmol/l, n = 4) were administered.

Some studies have reported vasodilator responses to ANG-(1–7) with the use of lower concentrations than mentioned in the series above (29). To exclude the possibility that ANG-(1–7) induces renal vasoconstriction only at low concentrations, we administered subnanomolar concentrations of this fragment in a fourth series of experiments. Vascular tone was...
induced by either ANG II (0.3 nmol/l, n = 4) or pressure elevation (to 160 Torr; n = 3). Subsequently, acetylcholine (0.3 μmol/l) was added to test endothelial integrity. After acetylcholine had been washed out and vascular tone had recovered, increasing concentrations of ANG-(1–7) were administered (1–100 pmol/l in the case of ANG II and 1 pmol/l–1 nmol/l in the case of pressure-induced constriction). Because EAs do not respond to pressure elevation, the effect of subnanomolar concentrations of ANG-(1–7) during pressure-induced tone was determined in ILAs and AAs only.

Analysis of data. All data are presented as means ± SE; n refers to the number of kidneys examined. When multiple vessels were studied in a kidney, the mean value obtained for each vessel type was used. For each angiotensin, the concentration at which half-maximal constriction was observed (EC₅₀) was calculated by using Prism 3 (Graphpad Software, San Diego, CA). This computer program was also used for statistical analyses. Data were evaluated by using ANOVA for repeated measurements followed by the Newman-Keuls post hoc test. A value of P < 0.05 was considered statistically significant.

RESULTS

Renal microvascular responses to ANG-(1–7) or ANG IV. As shown in Fig. 1A, ANG-(1–7) elicited a concentration-dependent constriction of ILAs, AAs, and EAs. At the highest concentration, i.e., 3 μmol/l, diameters of ILAs, AAs, and EAs were reduced by 33.5 ± 11.4% (from 32.1 ± 0.4 to 21.4 ± 3.8 μm, n = 6), 35.1 ± 8.6% (from 19.3 ± 0.3 to 12.5 ± 1.6 μm, n = 6), and 34.3 ± 8.3% (from 18.0 ± 0.7 to 11.9 ± 1.6 μm, n = 5), respectively (P < 0.05 vs. basal diameter). Similarly, ANG IV constricted renal microvessels in a concentration-dependent manner (Fig. 1B); at 0.1 μmol/l, diameters of ILAs were reduced by 47.1 ± 3.4% (from 29.1 ± 0.8 to 15.4 ± 1.1 μm, n = 7), AAs by 44.0 ± 3.9% (from 19.0 ± 0.3 to 10.7 ± 0.8 μm, n = 7), and EAs by 39.0 ± 3.3% (from 19.5 ± 0.7 to 12.0 ± 1.0 μm, n = 7) (P < 0.05 vs. basal diameter).

Although ANG-(1–7) and ANG IV constricted renal microvessels in a concentration-dependent way, both fragments were much less potent in eliciting renal microvascular constriction than ANG II. As depicted in Fig. 2, concentration-response curves for ANG IV and ANG-(1–7) were ∼3 and 4 orders of magnitude to the right of that for ANG II. Concomitantly, the −log of the derived EC₅₀ for each fragment was significantly lower than that of ANG II itself (Table 1).

Involvement of AT₁ or AT₂ receptors. Figure 3 summarizes the effect of the AT₁ receptor blocker irbesartan on the renal microvascular constriction to either ANG-(1–7) or ANG IV. ANG-(1–7) (1 μmol/l) decreased diameters of ILAs by 37.7 ± 4.8% (from 32.3 ± 0.5 to 20.2 ± 1.8 μm, n = 5), AAs by 36.9 ± 2.7% (from 19.1 ± 0.3 to 12.0 ± 0.7 μm, n = 5), and EAs by 40.0 ± 9.6% (from 17.6 ± 1.1 to 10.5 ± 1.7 μm, n = 4) (P < 0.05 vs. basal diameter). This ANG-(1–7)-induced constriction was completely reversed after addition of 3 nmol/l or more of irbesartan (not significant vs. basal diameter).

Similarly, irbesartan completely reversed the renal microvascular constriction to ANG IV. ANG IV (30 nmol/l) decreased diameters of ILAs by 44.7 ± 8.8% (from 30.3 ± 1.2 to 16.9 ± 3.1 μm, n = 4), AAs by 43.8 ± 3.7% (from 19.1 ± 0.5 to 10.7 ± 0.7 μm, n = 5), and EAs by 39.3 ± 5.6% (from 19.5 ± 1.3 to 11.9 ± 2.0 μm, n = 5) (P < 0.05 vs. basal diameter). Diameters of ILAs, AAs, and EAs had returned to their basal values after addition of 3 nmol/l irbesartan (not significant vs. basal diameter). In contrast, the AT₂ receptor blocker PD-123319 failed to reverse the renal microvascular constriction to both ANG-(1–7) (n = 4) and ANG IV (n = 3) up to a concentration of 0.1 μmol/l (Fig. 4).

Table 1. Sensitivity of renal microvessels to different angiotensins

<table>
<thead>
<tr>
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<th>ANG II</th>
<th>ANG IV</th>
<th>ANG-(1–7)</th>
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<tbody>
<tr>
<td>ILAs</td>
<td>10.5 ± 0.2</td>
<td>7.7 ± 0.1*</td>
<td>7.0 ± 0.4*</td>
</tr>
<tr>
<td>AAs</td>
<td>10.4 ± 0.3</td>
<td>7.7 ± 0.1*</td>
<td>6.2 ± 0.2†</td>
</tr>
<tr>
<td>EAs</td>
<td>10.9 ± 0.2</td>
<td>7.6 ± 0.2*</td>
<td>6.3 ± 0.2†</td>
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Values are means ± SE; n = 5–7 kidneys, expressed as −log of the EC₅₀ value. ILAs, interlobular arteries; AAs, afferent arterioles; EAs, efferent arterioles. *P < 0.05 vs. ANG II; †P < 0.05 vs. ANG IV.

Fig. 2. Comparison of the vasoconstrictor effects of ANG-(1–7) and ANG IV with that of ANG II. Each substance was tested for its effect on the diameter of ILAs, AAs, and EAs. Values are means ± SE; n = 5–7 kidneys. See Table 1 for the −log of the corresponding EC₅₀ values.

Fig. 1. Reaction of interlobular arteries (ILAs), afferent arterioles (AAs), and efferent arterioles (EAs) to the addition of ANG-(1–7) (A) or ANG IV (B). Values are means ± SE; n = 5–7 kidneys. *P < 0.05 vs. basal diameter.
versed both ANG-(1–7) and ANG IV-induced constriction of renal microvessels. At 3 nmol/l, irbesartan completely reversed both ANG-(1–7)- and ANG IV-induced vasoconstriction of ILAs, AAs, and EAs. Values are means ± SE; n = 4–5 kidneys. #P < 0.05 vs. fragment-induced constriction.

**DISCUSSION**

In the present study, we provide evidence for vasoactive actions of ANG II fragments in the renal microcirculation. Both ANG-(1–7) and ANG IV induced a concentration-dependent constriction of ILAs, AAs, and EAs. However, both were much less potent in eliciting renal microvascular constriction than ANG II. Vasoconstriction to both ANG-(1–7) and ANG IV was completely abolished by the nonpeptide AT1 receptor blocker irbesartan, indicating the involvement of the AT1 receptor. By contrast, addition of the AT2 receptor antagonist PD-123319 had no effect. Both fragments failed to counteract renal microvascular constriction to ANG II, even at concentrations that induced no vasoconstriction by themselves. In addition, subnanomolar concentrations of ANG-(1–7) failed to induce vasodilation during pressure-induced renal microvascular tone.

Previous studies have investigated the renal actions of ANG-(1–7) by using normal isolated rat kidneys (9, 18). These studies found that at a low dose, i.e., 3 pmol/ml, ANG-(1–7) exhibited tubular, but no renal, vascular effects. In our study, low concentrations of ANG-(1–7) also failed to affect renal microvascular diameters. However, at micromolar concentrations, ANG-(1–7) induced a significant vasoconstriction. This latter result extends the data of Handa et al. (15) and Heller et al. (17), who found in vivo that intrarenal infusion of relatively high doses of ANG-(1–7) reduced local blood flow in rats and dogs. However, these authors were not able to locate the site of action of ANG-(1–7). We demonstrate that ILAs, AAs, and EAs are susceptible to ANG-(1–7). The ANG-(1–7)-induced constriction of these renal microvessels was sensitive to irbesartan, indicating involvement of the classic AT1 receptor. This result is in agreement with the findings of Handa et al. and Heller et al., who also reported that the reduction in renal blood to ANG-(1–7) was sensitive to AT1 receptor blockade.
At present, the renal vascular effects of ANG IV are still unclear. Intrarenal infusion of ANG IV in conscious or anesthetized rats has been reported to cause a reduction in local blood flow (12, 13, 38), suggesting a prominent renal vascular constriction. By contrast, using laser Doppler flowmetry, Coleman et al. (7) found an ANG IV-induced increase in renal cortical blood flow in rats. These previous studies did not discriminate between the effects of ANG IV on different types of renal microvessels. In this study, we demonstrate by means of videomicroscopy that small preglomerular and postglomerular vessels constrict in response to relatively high concentrations of ANG IV. This response was found to be mediated by means of the AT1 receptor. These data are in line with the above-mentioned studies reporting a reduced renal blood flow in response to ANG IV, which was also sensitive to AT1 receptor blockade (12, 13, 38). Also, in the pulmonary, the mesenteric, and hindquarter vascular beds of rats and cats ANG IV has been reported to induce vasoconstriction by means of activation of the AT1 receptor, whereas blockade of the AT2 receptor had no effect (4–6, 14, 28).

Besides vasoconstriction, several studies have reported vasodilator responses to both ANG-(1–7) and ANG IV. For example, ANG-(1–7) has been shown to induce vasodilation in the coronary (2, 31), mesenteric (30), and cerebral circulation (26). Similarly, ANG IV-induced dilation was found in cerebral (20) and cochlear circulation (8). In addition, ANG-(1–7) attenuated vascular responses to ANG II in humans (32, 36), suggesting that this fragment may also counteract ANG II-related actions (11). In the present study, ANG-(1–7) and ANG IV failed to alter diameters of ILAs, AAs, and EAs preconstricted with ANG II, even at concentrations that did not induce vasoconstriction by themselves. In addition, subnanomolar concentrations of ANG-(1–7) had no effect on pressure-induced renal microvascular tone. The reason ANG-(1–7) and ANG IV exert different effects on renal microvessels compared with other vascular beds is still unclear. In our preparation, acetylcholine was found to induce renal vasodilation, excluding the possibility of a damaged endothelium. Moreover, ANG-(1–7) failed to alter both ANG II- and pressure-induced constriction, indicating that our failure to observe renal vasodilation of angiotensin fragments is also not determined by the nature of the underlying vascular tone. Osei et al. (30) also found different vascular responses to ANG-(1–7) in the mesenteric and hindlimb vascular bed of the rat, suggesting that the receptor profile or linkage of the receptor to second messenger pathways may differ between vascular beds. In this regard, it is interesting to note that in the kidney, the non-AT1/non-AT2 receptor subtype, designated as AT4, which is thought to mediate vasodilator actions of ANG IV (7), is predominantly located on tubular structures (7, 16).

Although our study demonstrates that both ANG-(1–7) and ANG IV are capable of eliciting vasoconstriction of both pre- and postglomerular resistance vessels, it is uncertain whether these fragments contribute to the control of glomerular hemodynamics. We found that...
ANG-(1–7) and ANG IV were much less potent in eliciting renal microvascular constriction than ANG II. Thus relatively high levels are needed for ANG-(1–7) and ANG IV to influence glomerular hemodynamics. Under normal conditions, the levels of ANG-(1–7) and ANG IV are lower than those measured for ANG II (21, 34). However, these levels may markedly increase during antihypertensive treatment with angiotensin-converting enzyme inhibitors (19, 21, 27) or an AT1 receptor antagonist (34). During AT1 receptor blockade, however, it is unlikely that ANG-(1–7) and ANG IV exert vasoactive actions in the renal microvasculature, because we found that ANG-(1–7) and ANG IV-induced vasoconstriction of ILAs, AAs, and EAs was mediated by means of this receptor subtype.

From Fig. 2, it becomes apparent that besides a higher potency, ANG II also causes a substantially greater constrictor effect on ILAs and AAs than on EAs, compared with ANG-(1–7) and ANG IV. At present, the cause for this difference is still unclear, and one can only speculate about the underlying mechanism. In general, a vasoconstrictor response to ANG II is the result of simultaneous activation of the AT1 and AT2 receptors. Although the precise function of the AT2 receptor is still unknown, it has been suggested that AT2 modulates AT1-mediated actions. Thus differences between renal microvessels in the response to ANG II might be related to a distinct AT1-AT2 receptor ratio at the different renal microvascular sites. Unfortunately, at present, the relative expression ratios of the AT1 receptor vs. the AT2 receptor in different renal microvessels is still unclear. The fact that ANG-(1–7) and ANG IV cause a similar constrictor effect in the three vessel types studied might indicate that these fragments fail to activate the AT2 receptor. In support of this hypothesis is our observation that the AT2 receptor antagonist PD-123319 failed to increase the vasoconstrictor response to both ANG-(1–7) and ANG IV particularly in EAs but also in ILAs and AAs.

In conclusion, the present study demonstrates that in addition to ANG II, ANG-(1–7) and ANG IV are capable of eliciting vasoconstriction of ILAs, AAs, and EAs, albeit at concentrations several magnitudes higher. This vasoconstrictor response is mediated by means of stimulation of the classic AT1 receptor. Blockade of the AT2 receptors had no effect. Both ANG-(1–7) and ANG IV failed to counteract renal microvascular constriction to ANG II. In addition, subnanomolar concentrations of ANG-(1–7) had no effect on the pressure-induced constriction of ILAs and AAs, indicating that ANG-(1–7) and ANG IV do not exert ANG II antagonistic and vasodilator actions in the kidney.

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