

Vasoconstriction is determined by interstitial rather than circulating angiotensin II

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1 We investigated why angiotensin (Ang) I and II induce vasoconstriction with similar potencies, although Ang I-II conversion is limited.

2 Construction of concentration-response curves to Ang I and II in porcine femoral arteries, in the absence or presence of the AT₁ or AT₂ receptor antagonists irbesartan and PD123319, revealed that the ≈ 2 fold difference in potency between Ang I and II was not due to stimulation of different AT receptor populations by exogenous and locally generated Ang II.

3 Measurement of Ang I and II and their metabolites at the time of vasoconstriction confirmed that, at equimolar application of Ang I and II, bath fluid Ang II during Ang I was ≈ 18 times lower than during Ang II and that Ang II was by far the most important metabolite of Ang I. Tissue Ang II was $2.9 \pm 1.5\%$ and $12.2 \pm 2.4\%$ of the corresponding Ang I and II bath fluid levels, and was not affected by irbesartan or PD123319, suggesting that it was located extracellularly.

4 Since $\approx 15\%$ of tissue weight consists of interstitial fluid, it can be calculated that interstitial Ang II levels during Ang II resemble bath fluid Ang II levels, whereas during Ang I they are $8.8–27$ fold higher. Consequently at equimolar application of Ang I and II, the interstitial Ang II levels differ only 2–4 fold.

5 Interstitial, rather than circulating Ang II determines vasoconstriction. Arterial Ang I, resulting in high interstitial Ang II levels *via* its local conversion by ACE, may be of greater physiological importance than arterial Ang II.

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Abbreviations: ACE, angiotensin-converting enzyme; Ang, angiotensin; AT₁, angiotensin II type 1; AT₂, angiotensin II type 2; CRC, concentration response curve; PGF_{2 α} , prostaglandin F_{2 α} ; U46619, 9,11-dideoxy-11 α ,9 α -epoxymethano-prostaglandin F_{2 α}

Introduction

Angiotensin (Ang) I and II both induce vasoconstriction *via* stimulation of AT₁ receptors, the former following its conversion to Ang II by ACE and/or chymase (Urata *et al.*, 1990; Wei *et al.*, 1999). In healthy volunteers, during intrabrachial Ang I infusion, one third of arterially delivered Ang I is converted to Ang II in the forearm vascular bed (Saris *et al.*, 2000), whereas in isolated human coronary arteries mounted in organ baths, the Ang II levels reached in the organ bath at the time of contraction are <1% of the levels of Ang I (MaassenVanDenBrink *et al.*, 1999). Yet, despite the relatively low Ang II levels during Ang I administration, the potencies of Ang I and II *in vivo* as well as *in vitro* are similar (Borland *et al.*, 1998; MaassenVanDenBrink *et al.*, 1999; Saris *et al.*, 2000; Voors *et al.*, 1998). Likewise, in isolated perfused rat hindquarters, renin infusion causes equal increases in perfusion pressure as Ang II infusion, although the Ang II levels during renin perfusion are five times lower than during Ang II perfusion (Hilgers *et al.*, 1998).

Several explanations may solve these discrepancies. Firstly, in view of the vasodilator effects of AT₂ receptors (Muller *et al.*, 1998; Zwart *et al.*, 1998), it is possible that locally generated Ang II reaches different AT receptors than exogenously applied Ang II. For instance, exogenous Ang II may stimulate both endothelial (vasodilatory) AT₂ receptors and AT₁ receptors on smooth muscle cells, whereas locally generated Ang II might stimulate the latter predominantly. Secondly, Ang I and II are degraded to vasoconstrictor (Ang-(2–8) and Ang-(3–8), also known as Ang III and IV) and vasodilator (Ang-(1–7)) metabolites (Li *et al.*, 1995; Ferrario *et al.*, 1997; Tom *et al.*, 2001), and this may affect their vasoconstrictor potency. Thirdly, the Ang II levels in the circulation and organ bath may not be representative for the tissue (interstitial fluid) levels in the immediate vicinity of the AT receptors that actually cause vasoconstriction. This theory has also been put forward to explain the beneficial effects of ACE inhibitors on blood pressure and cardiac remodelling in the absence of clear reductions in circulating Ang II (Nussberger *et al.*, 1986; van Kats *et al.*, 2000). Fourthly, results obtained with Ang I and II in organ baths, as well as during systemic infusion *in vivo*, may be of little physiological relevance, since normally vascular Ang

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II (i.e., the Ang II that is responsible for vasoconstriction) is largely derived from local synthesis by renin and converting enzymes from angiotensinogen (Campbell, 1987; Danser *et al.*, 1992b; Admiraal *et al.*, 1993). In this respect, it is of interest to note that the majority of vascular Ang I-II conversion, at least *in vitro*, is mediated by chymase, a converting enzyme that is located in the adventitia (Urata *et al.*, 1993; Borland *et al.*, 1998; MaassenVanDenBrink *et al.*, 1999).

To investigate these issues, we constructed Ang I and II concentration-response curves (CRCs) in porcine femoral and carotid arteries mounted in organ baths. Ang I and Ang II-mediated effects were also studied following preconstriction, to facilitate the detection of vasodilator effects, and in the presence of AT₁ and AT₂ receptor antagonists. Additional studies with Ang I were performed in the presence of the ACE inhibitor quinaprilat, to investigate the importance of ACE versus chymase. Subsequently, we compared the tissue and bath fluid levels of Ang I and II and their metabolites at the time of maximal vasoconstriction. The amount of vascular Ang II presented in interstitial fluid was estimated after correction of the tissue levels for Ang II bound to AT receptors (van Kats *et al.*, 1997). Finally, we studied the vasoconstrictor effects of Ang I and II applied to the adventitial side of perfused carotid arteries, thereby mimicking the vascular origin of Ang II mediating vasoconstriction.

Methods

Chemicals

Angiotensin I and II, prostaglandin F_{2 α} (PGF_{2 α}), 9,11-dideoxy-11 α ,9 α -epoxymethano-prostaglandin F_{2 α} (U46619) and substance P (acetate salt) were purchased from Sigma (Zwijndrecht, The Netherlands). Irbesartan was a kind gift of Bristol-Myers Squibb (Princeton, NJ, U.S.A.). PD123319 and quinaprilat were a kind gift of Parke Davis (Hoofddorp, The Netherlands). Irbesartan was dissolved in ethanol and quinaprilat in dimethylsulphoxide. All other chemicals were dissolved in distilled water.

Tissue collection

Porcine femoral and carotid arteries were obtained from 33 2–3 month-old pigs (Yorkshire x Landrace, weight 10–15 kg). The pigs had been used in *in-vivo* experiments studying the effects of α -adrenoceptor and serotonin receptor (ant)agonists under pentobarbital (600 mg, i.v.) anaesthesia (Willems *et al.*, 2001). The Ethics Committee of the Erasmus University Rotterdam dealing with the use of animals for scientific experiments approved the protocol for this investigation. After sacrificing the animal with an overdose of pentobarbital, the right femoral artery and both carotid arteries were removed and stored overnight in a cold, oxygenated Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25 and glucose 8.3; pH 7.4.

Organ bath studies with femoral arteries

To study Ang I- and II-induced vasoconstriction and/or vasodilatation, femoral arteries were cut into segments of

approximately 3–4 mm length, suspended on stainless steel hooks in 15-ml organ baths containing Krebs bicarbonate solution, aerated with 95% O₂/5% CO₂, and maintained at 37°C. All vessel rings were allowed to equilibrate for at least 30 min and the organ bath fluid was refreshed every 15 min during this period. Changes in tissue contractile force were recorded with a Harvard isometric transducer (South Natick, MA, U.S.A.). The vessel rings, stretched to a stable force of about 50 mN, were exposed to 30 mM K⁺ twice. Subsequently, the tissue was exposed to 100 mM K⁺ to determine the maximal contractile response to K⁺. The rings were then allowed to equilibrate in fresh organ bath fluid for 30 min. Next, the vessel rings were pre-incubated for 30 min with or without the AT₁ antagonist irbesartan (1 μ M), the AT₂ antagonist PD123319 (1 μ M) or the ACE inhibitor quinaprilat (10 μ M). Thereafter, Ang I or Ang II (0.3 nM–10 μ M) CRCs were constructed. To facilitate detection of vasodilatation due to AT₂ receptor stimulation, Ang I or II CRCs were also constructed following preconstriction with 1 μ M PGF_{2 α} or 10 nM U46619. Preconstrictions amounted to approximately 40–50% of the maximal contraction induced by 100 mM K⁺. In the presence of irbesartan, the amount of PGF_{2 α} or U46619 required to obtain such preconstrictions was approximately 5–6 times higher, because, at a concentration of 1 μ M, irbesartan acts as an antagonist of the thromboxane A₂/prostaglandin endoperoxide receptor (Li *et al.*, 2000). After the addition of Ang I or II, it took 10–15 min to reach a stable contraction. Subsequent angiotensin concentrations were applied as soon as a stable contraction had been reached. Construction of the CRC was discontinued when desensitization occurred, i.e. when subsequent angiotensin doses elicited no response or decreased contraction. At the end of the experiment the functional integrity of the endothelium was verified by observing relaxation to 1 nM substance P or 100 nM bradykinin after pre-constriction with PGF_{2 α} or U46619. To measure the release of newly formed Ang II into the organ bath fluid during Ang I CRCs, bath fluid samples (150 μ l) for Ang II measurements were taken as soon as a stable contraction had been reached. All samples were collected in chilled tubes containing 15 μ l inhibitor solution (125 mM disodium EDTA and 25 mM 1,10-orthophenanthroline) and 15 μ l 0.1% bovine serum albumin (BSA) in distilled water. The samples were stored at –80°C until analysis.

Metabolism studies with femoral arteries

To study vascular Ang I-II conversion and Ang I and II metabolism in further detail, femoral arteries were cut into segments of approximately 3–4 mm length (weight 10–45 mg; mean 22 mg), put into test tubes containing 1.0 ml Krebs bicarbonate solution ('incubation fluid'), aerated with 95% O₂/5% CO₂, and maintained at 37°C. After a 10-min equilibration period, vessel rings were pre-incubated for 30 min with or without irbesartan (1 μ M), PD123319 (1 μ M) or quinaprilat (10 μ M). Next, Ang I (10 or 100 pmol) or Ang II (10 pmol), randomly combined with ¹²⁵I-Ang I (100,000 c.p.m.) or ¹²⁵I-Ang II (100,000 c.p.m.), were added to the incubation fluid. The use of radiolabelled angiotensins facilitates the detection of the various angiotensin metabolites, by gamma-counting after their high-performance liquid chromatographic (HPLC) separation (see below). After either

15 or 60 min the vessel segments were removed, washed in fresh Krebs solution and dried on tissue paper. The remaining incubation fluid (≈ 1 ml) as well as the dried segment were rapidly frozen in liquid nitrogen and stored at -80°C until analysis.

Perfusion and organ bath studies with carotid arteries

To study whether adventitial application of Ang I and II elicits a response that is different from the combined luminal and adventitial application obtained after addition of Ang I or II to the organ bath in the above set-up, carotid arteries (diameter 2–3 mm) were divided in 3–4 mm rings as well as 1–1.5 cm sections. The rings were mounted in organ baths as described above. The vessel sections, which did not contain side branches, were mounted horizontally in a double-jacketed 4-ml bath containing a carbogenated Krebs bicarbonate solution and maintained at 37°C , as described by Hulsmann *et al.* (1992). Krebs solution was perfused through the vessels using a roller pump (Ismatec IPS, Zürich, Switzerland). This approach allows the adventitial application (i.e., into the bath) of drugs. Fluids were refreshed every 15 min. Changes in pressure were recorded with a Viggo-spectramed disposable pressure transducer (Bilthoven, The Netherlands). The vessel sections and rings, at a stable force of 44 mmHg and 20 mN, respectively, were exposed to 30 mM K^{+} twice. Subsequently, the tissues were exposed to 100 mM K^{+} to determine the maximal contractile response to K^{+} . The vessels were then allowed to equilibrate in fresh organ bath fluid for 30 min. Thereafter, Ang I or II (1 nM–1 μM) CRCs were constructed as described above. At the end of the experiment the functional integrity of the endothelium of the vessel rings was verified by observing relaxation to 100 nM bradykinin after pre-constriction with 10 nM U46619.

Measurements of angiotensins

In the metabolism studies, Ang I and II and radiolabelled Ang I and II in vascular tissue and incubation fluid were measured as described previously (Danser *et al.*, 1992a; van Kats *et al.*, 1997), using SepPak extraction and HPLC separation. In short, frozen vessel segments were homogenized in 4 ml ice-cold 0.1 M HCl/80% ethanol. The homogenate was centrifuged at $20,000 \times g$ for 10 min at 4°C . Ethanol in the supernatant was evaporated under constant air flow. The remainder of the supernatant was diluted in 8 ml 1% ortho-phosphoric acid and concentrated on SepPak cartridges. SepPak extracts were dissolved in 100 μl HPLC elution buffer and injected into the HPLC column. Incubation fluid was directly applied to the column without prior SepPak extraction. The concentrations of Ang I and II and of radiolabelled Ang I and II in the HPLC eluate fractions were measured by radioimmunoassays and gamma counting, respectively. Measurements were not corrected for losses occurring during extraction. These losses were maximally 20–30% (van Kats *et al.*, 1997). The lowest Ang I levels that could be measured were 50 fmol g^{-1} tissue and 10 fmol ml^{-1} incubation fluid. The lowest Ang II levels that could be measured were 25 fmol g^{-1} tissue and 5 fmol ml^{-1} incubation fluid. The lowest ^{125}I -Ang I and ^{125}I -Ang II levels that could be measured were 500 c.p.m. g^{-1} tissue and 100 c.p.m. ml^{-1} incubation fluid.

In the organ bath studies, Ang I and II in organ bath fluid were measured by radioimmunoassay without prior SepPak extraction or HPLC separation (MaassenVanDenBrink *et al.*, 1999). The lowest Ang I and II levels that could be measured in these experiments were 20 and 10 fmol ml^{-1} bath fluid, respectively.

Data presentation and statistical analysis

Data are given as mean \pm s.e.mean. Data of the functional studies are expressed as a percentage of the maximal contraction to 100 mM K^{+} (88 ± 5 mN in the organ bath studies with femoral arteries ($n=15$); 220 ± 20 mmHg in the perfusion studies with carotid arteries ($n=7$) and 111 ± 8 mN in the organ bath studies with the carotid arteries ($n=7$)). Data of metabolism experiments are expressed as percentage of the angiotensin concentration in the bath fluid at the start ($t=0$) of the experiment, assuming that 1 g of tissue equals 1 ml of fluid (de Lannoy *et al.*, 1998). To allow statistical evaluation, angiotensin levels that were below the detection limit were taken to be equal to this limit.

CRCs were analysed using the logistic function described by de Lean *et al.* (1978) to obtain pEC_{50} ($-10 \log \text{EC}_{50}$) values. Statistical analysis was by ANOVA, followed by *post hoc* evaluation according to Dunnett. P values <0.05 were considered significant. Statistics were performed using the software package SigmaStat.

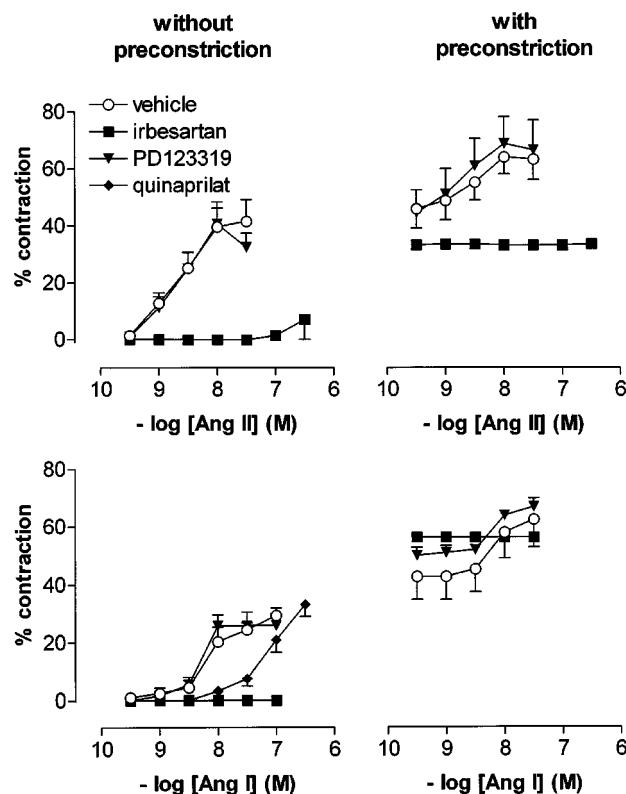


Figure 1 Contractions of femoral arteries, at baseline and after preconstriction with 1 μM PGF_{2 α} or 10 nM U46619, to Ang II (upper panels; $n=8$) or Ang I (lower panels; $n=5$) in the absence or presence of 1 μM irbesartan, 1 μM PD123319 or 10 μM quinaprilat. Data are expressed as a percentage (mean \pm s.e.mean) of the response to 100 mM K^{+} .

Results

Organ bath studies with femoral arteries

In non-preconstricted femoral arteries (Figure 1), Ang I and II displayed similar maximal effects (E_{max} $31 \pm 2\%$ and $37 \pm 7\%$, respectively, $n=5$). Ang I was 2 fold less potent than Ang II (pEC_{50} 8.21 ± 0.13 and 8.59 ± 0.09 , respectively; $P < 0.05$). In femoral arteries that had been preconstricted with PGF_{2 α} or U46619 to 34 ± 4 mN (or $\approx 45\%$ of the maximal contraction induced by 100 mM K^+ ; $n=13$), Ang I and II increased contraction further to 63 ± 9 and $62 \pm 6\%$ (Figure 1). The potencies of Ang I and II in the preconstricted vessels (pEC_{50} 8.08 ± 0.11 and 8.51 ± 0.05 , respectively) were not different from those in the non-preconstricted vessels.

The AT₁ receptor antagonist irbesartan abolished all contractile responses to both Ang I and II, whereas the AT₂ receptor antagonist PD123319 was ineffective under all circumstances. In non-preconstricted vessels, the ACE inhibitor quinaprilat shifted the Ang I CRC approximately 10 fold to the right (pEC_{50} 7.12 ± 0.10 , $n=5$; $P < 0.001$ vs control).

Despite the small difference in potency between Ang I and II, the Ang II levels measured in the organ bath fluid during the Ang I CRC, at the time a stable contraction had been reached, were $<3\%$ of the levels of Ang I. Figure 2 (left panel) relates the organ bath fluid levels of Ang II measured during the Ang I CRC ('endogenous Ang II') to the contractile response. For comparison the CRC obtained with exogenous Ang II is also shown in this Figure. The pEC_{50} value of endogenous Ang II (9.84 ± 0.14) illustrates that, for a given contractile response, the organ bath fluid levels of endogenous Ang II are ≈ 18 fold lower than for exogenous Ang II.

Metabolism studies with femoral arteries

Incubation fluid Femoral artery segments slowly metabolized ^{125}I -Ang I and Ang I (Figure 3). After 1 h of incubation at $37^\circ C$, $56 \pm 6\%$ ($n=4$) and $64 \pm 9\%$ ($n=6$) of the incubation fluid levels at $t=0$ was still present as intact ^{125}I -Ang I and Ang I, respectively. Metabolism was partly due to Ang I-II conversion, as evidenced by the rise in the ^{125}I -Ang II and Ang II levels over time (Figure 3). After 1 h, the incubation fluid levels of ^{125}I -Ang II and Ang II were 4.5 ± 2.6 and $5.4 \pm 2.0\%$ of the respective ^{125}I -Ang I and Ang I levels at $t=0$. HPLC separation of the incubation fluid samples revealed that ^{125}I -Ang II was by far the most important metabolite of ^{125}I -Ang I (Figure 4). The metabolism of ^{125}I -Ang II and Ang II occurred at a similarly slow rate as the metabolism of ^{125}I -Ang I and Ang I, resulting in incubation fluid ^{125}I -Ang II and Ang II levels after 1 h that were $>60\%$ of the levels at $t=0$ (data not shown). Metabolites that could be detected in the incubation fluid during incubation with ^{125}I -Ang II were ^{125}I -Ang-(4-8) and ^{125}I -tyrosine. Irbesartan and PD123319 did not affect the metabolism of ^{125}I -Ang I, ^{125}I -Ang II, Ang I or II (Figure 3). Quinaprilat virtually completely prevented the appearance of ^{125}I -Ang II and Ang II in the incubation fluid during incubation of the vessel segments with ^{125}I -Ang I and Ang I, but did not significantly affect the decrease in ^{125}I -Ang I or Ang I over time (Figure 3), thereby indicating that Ang I-II conversion is not the only metabolic pathway which results in destruction of Ang I.

Vessel segment Under all conditions that were investigated, the angiotensin levels measured in the vessel segment at $t=15$ min were not different from those at $t=60$ min. Therefore, the data in Figure 5 represent the average of the measurements at these two time points ('steady-state' levels). The ^{125}I -Ang I and Ang I levels in the vessel segments

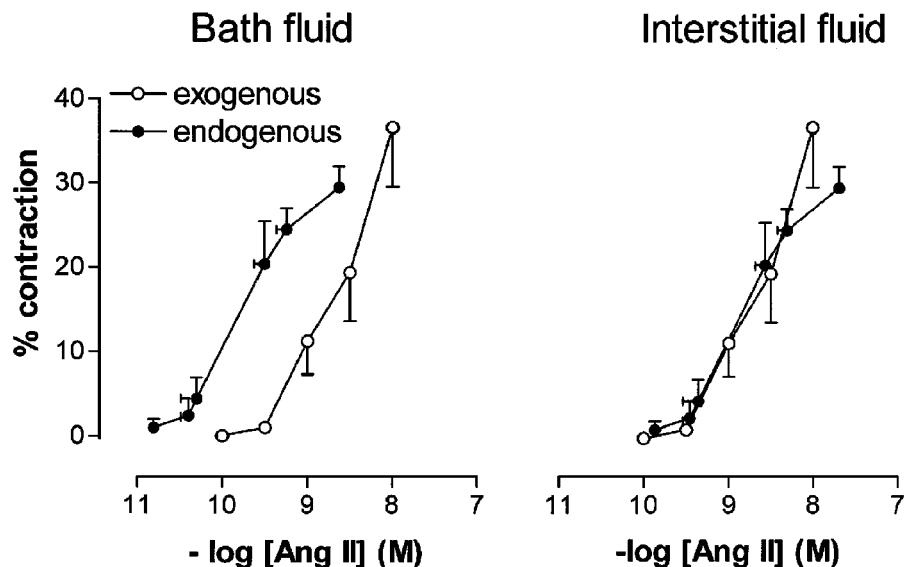


Figure 2 Contractile responses of femoral arteries versus organ bath fluid Ang II levels (left panel) or interstitial fluid Ang II levels (right panel), during Ang I ('endogenous') or Ang II ('exogenous') application. Contractions are expressed as a percentage (mean \pm s.e.mean; $n=5$) of the response to 100 mM K^+ . Interstitial fluid levels of endogenous and exogenous Ang II were calculated by multiplying the Ang II levels in the organ bath with the interstitial fluid/incubation fluid Ang II concentration ratios obtained 15 min following Ang I (8.5 ± 4.4) or Ang II (1.1 ± 0.2) administration in the metabolism studies.

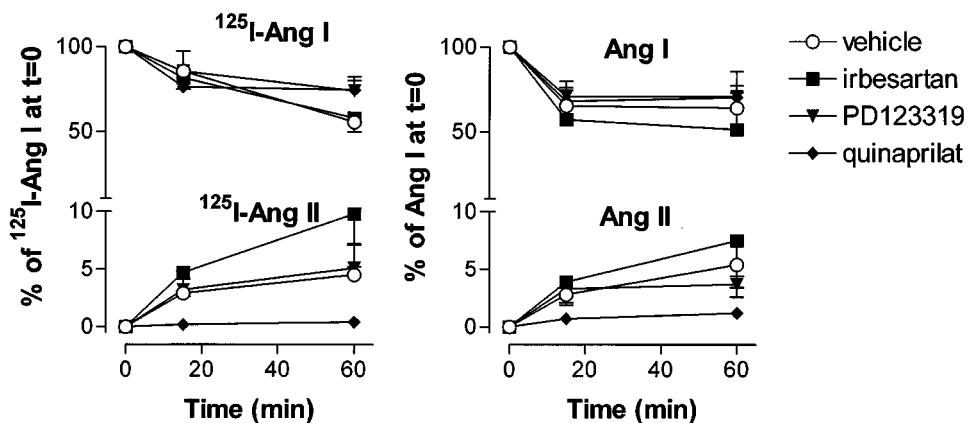


Figure 3 Metabolism of ^{125}I -Ang I and generation of ^{125}I -Ang II (left panel) and metabolism of Ang I and generation of Ang II (right panel) by femoral artery rings incubated in 1 ml incubation fluid containing ^{125}I -Ang I (100,000 c.p.m.) or Ang I (10 or 100 pmol) in the absence or presence of 1 μM irbesartan, 1 μM PD123319 or 10 μM quinaprilat. Incubation fluid levels are expressed as a percentage (mean \pm s.e.mean, $n=4$ –6) of the ^{125}I -Ang I and Ang I levels at $t=0$.

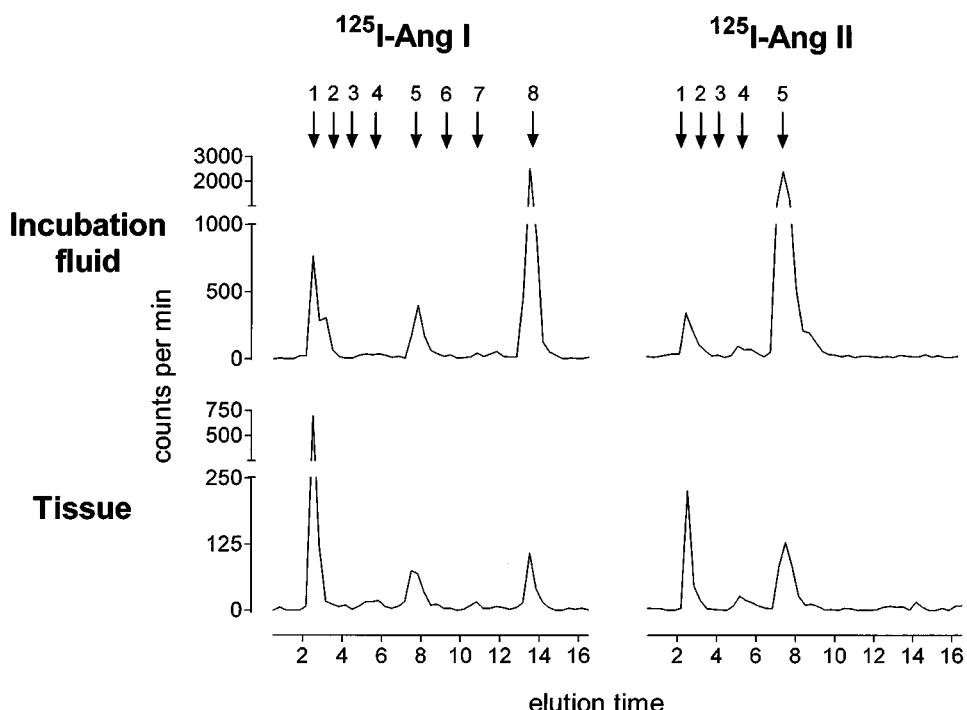


Figure 4 HPLC elution profile of ^{125}I -labelled antiotensins in femoral artery tissue (22 mg) and incubation fluid (100 μl) obtained after incubation of femoral artery rings at 37°C for 60 min in 1 ml incubation medium containing 100,000 c.p.m. ^{125}I -Ang I or ^{125}I -Ang II. The retention times of ^{125}I -labelled standards are indicated by arrows at the top (1, ^{125}I -tyrosine; 2, ^{125}I -Ang-(1–7); 3, ^{125}I -Ang III; 4, ^{125}I -Ang-(4–8); 5, ^{125}I -Ang II; 6, ^{125}I -Ang IV; 7, ^{125}I -Ang-(2–10); 8, ^{125}I -Ang I).

(expressed per g tissue) were 3–6 times lower than the concomitant levels in the incubation fluid (expressed per ml fluid). In contrast, the tissue ^{125}I -Ang II and Ang II levels, measured after the addition of ^{125}I -Ang I and Ang I to the incubation fluid, were as high as (or slightly higher than) the incubation fluid ^{125}I -Ang II and Ang II levels. Quinaprilat reduced these levels by >75% (data not shown).

The tissue ^{125}I -Ang II and Ang II levels, measured after the addition of ^{125}I -Ang II and Ang II to the incubation fluid were 5–6 times lower than the concomitant levels in the incubation fluid. Other metabolites that could be detected in

the vessel segments during ^{125}I -Ang I or ^{125}I -Ang II application were ^{125}I -Ang-(2–10), ^{125}I -Ang-(4–8) and ^{125}I -tyrosine (Figure 4).

Neither irbesartan nor PD123319 affected the tissue ^{125}I -Ang II and Ang II levels under any of the conditions studied, thereby indicating that the majority of tissue ^{125}I -Ang II and Ang II was not bound to cell surface – or internalized AT receptors. Assuming therefore that all tissue ^{125}I -Ang II and Ang II is located extracellularly (i.e., present in vascular interstitial fluid, which accounts for \approx 15% of tissue weight (Linde, 1975; Plank *et al.*, 1976;

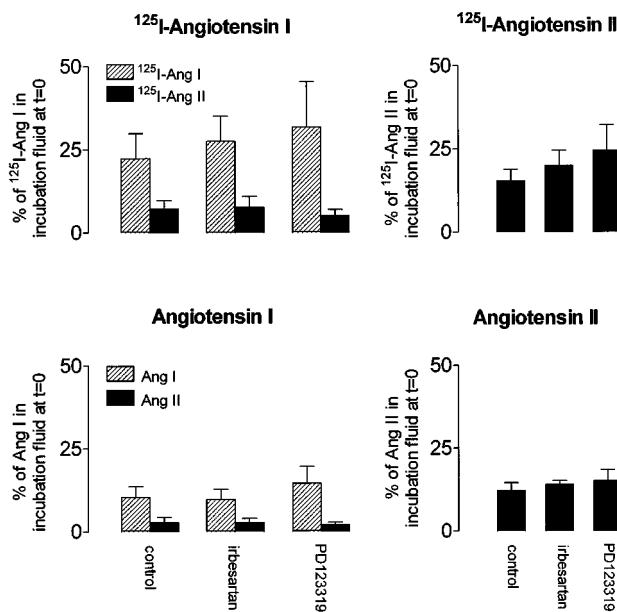


Figure 5 Steady-state tissue ^{125}I -Ang I, ^{125}I -Ang II, Ang I and II levels in femoral artery rings incubated with ^{125}I -Ang I (100,000 c.p.m.) or Ang I (10 or 100 pmol) (left panels) or with ^{125}I -Ang II (100,000 c.p.m.) or Ang II (10 pmol) (right panels) in the absence or presence of 1 μM irbesartan or 1 μM PD123319. Data are expressed as a percentage of the radiolabelled and endogenous Ang I or II levels in incubation fluid at $t=0$ (mean \pm s.e.mean, $n=4-6$).

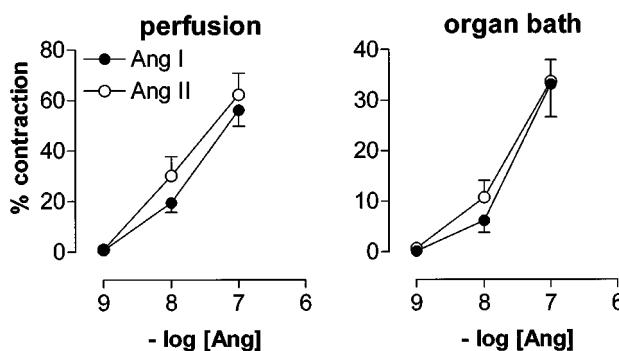


Figure 6 Contractions of carotid arteries to Ang I or II added adventitiously to perfused vessel sections (left panel) or into the bath fluid of vessel rings mounted in organ baths (right panel). Data are expressed as a percentage (mean \pm s.e.mean; $n=7$) of the response to 100 mM K^+ .

Reed & Wiig, 1984; Katz *et al.*, 1997), it can be calculated that during ^{125}I -Ang I and Ang I application, the interstitial ^{125}I -Ang II and Ang II levels are 49 ± 17 and $19 \pm 10\%$, respectively, of the initial ^{125}I -Ang I and Ang I levels in the incubation fluid. Moreover, during ^{125}I -Ang II and Ang II application, the interstitial ^{125}I -Ang II and Ang II levels are 103 ± 23 and $81 \pm 16\%$, respectively, of the initial ^{125}I -Ang II and Ang II levels in the incubation fluid. In other words, 15 min after the application of ^{125}I -Ang I and Ang I, the interstitial fluid ^{125}I -Ang II and Ang II levels are, respectively, 27 ± 13 and 8.8 ± 4.4 times higher than the ^{125}I -Ang II and Ang II levels in the incubation fluid (the latter

levels are shown in Figure 3), whereas 15 min after the application of ^{125}I -Ang II and Ang II, the interstitial fluid ^{125}I -Ang II and Ang II levels equal the levels in the incubation fluid.

Perfusion and organ bath studies with carotid arteries

Ang I and II displayed similar maximal effects, both in the organ bath studies and the perfusion experiments (Figure 6). Under both conditions, Ang I tended to be less potent than Ang II ($\text{pEC}_{50} 7.53 \pm 0.11$ vs 7.76 ± 0.08 in the organ bath studies and 7.73 ± 0.07 vs 8.00 ± 0.11 in the perfusion experiments), but the difference did not reach statistical significance. Ang II, but not Ang I, was more potent after adventitial application (perfusion experiment) than after application to the organ bath ($P < 0.05$).

Discussion

In the present *in-vitro* study, in agreement with previous studies measuring Ang II following Ang I administration (Danser *et al.*, 1995; MaassenVanDenBrink *et al.*, 1999; Saris *et al.*, 2000), the levels of Ang II in the organ bath during the construction of the Ang I CRC in femoral artery rings were $<3\%$ of the Ang I levels at the time of maximal vasoconstriction. Yet, in femoral, as well as in carotid arteries, the potency of Ang I was only 2 fold lower than that of Ang II.

The results obtained with the AT_1 receptor antagonist irbesartan and the AT_2 receptor antagonist PD123319 do not support the possibility that exogenous Ang II and locally generated Ang II stimulate different AT receptors. Neither at baseline, nor following preconstriction, we were able to demonstrate Ang I- or II-induced vasodilatation *via* AT_2 receptors in femoral arteries. Thus, either such receptors do not exist in porcine femoral arteries, or AT_2 receptors in these vessels mediate other, non-blood pressure-related effects in this vessel (e.g., effects on vascular growth and remodelling (Stoll *et al.*, 1995; Yamada *et al.*, 1996; van Kesteren *et al.*, 1997). Measurement of radiolabelled angiotensin metabolites during incubation of femoral artery rings with ^{125}I -Ang I and ^{125}I -Ang II, revealed that the metabolism of Ang I and II by these rings is relatively slow (half life >1 h), and that Ang II is by far the most important metabolite of Ang I. The enzyme responsible for Ang I-II conversion is ACE, since the ACE inhibitor quinaprilat shifted the Ang I CRC 10 fold to the right and prevented the appearance of Ang II in the organ bath. The latter contrasts with previous findings in human vessels (Borland *et al.*, 1998; MaassenVanDenBrink *et al.*, 1999), where chymase was by far the most important converting enzyme. Neither the vasoconstrictor metabolites Ang III and IV (Li *et al.*, 1995), nor the vasodilator metabolite Ang-(1-7) (Ferrario *et al.*, 1997; Tom *et al.*, 2001) were present in detectable amounts. Thus, the similar potencies of Ang I and II are not related to the generation of different vasoconstrictor and/or vasodilator metabolites from Ang I and II, nor to differences in the rapidity of their metabolism. Moreover, the rightward shift of Ang I CRC caused by quinaprilat, and the similar potencies of Ang I and II despite the >100 fold lower affinity of Ang I for the AT_1 receptor (Whitebread *et al.*, 1989; Dudley *et al.*, 1990),

exclude the possibility that Ang I mediated its vasoconstrictor effects directly, independent of its conversion to Ang II.

During adventitial application to perfused carotid arteries, Ang I and II were equipotent, thereby showing that their similar potency in organ bath studies (Borland *et al.*, 1998; Voors *et al.*, 1998; MaassenVanDenBrink *et al.*, 1999) or following arterial infusion *in vivo* (Saris *et al.*, 2000) is not a consequence of the experimental set-up. The perfusion studies in the present investigation were performed with carotid arteries, since the many sidebranches of femoral arteries did not allow such studies with these vessels. The slightly higher potency of Ang II following adventitial application as compared to combined adventitial+luminal application (i.e., into the organ bath) is in agreement with earlier studies showing a higher metabolic clearance rate of angiotensins after luminal application than after adventitial application (Danser *et al.*, 1995). Apparently, the majority of the vascular angiotensin metabolizing enzymes is located on endothelial cells.

Based on the above findings, we reasoned that Ang I administration results in high tissue Ang II levels with limited Ang II release into the surrounding bath fluid, and that these high tissue Ang II levels might explain why the potency of Ang I is similar to that of Ang II. We therefore expected the vascular Ang II levels during Ang I administration to be much higher than the organ bath fluid Ang II levels. As shown in Figures 3 and 5, this was not the case. Fifteen minutes after Ang I administration (i.e., at the time maximal vasoconstriction had occurred), the vascular Ang II levels were approximately as high as the bath fluid levels. The tissue concentrations at 15 min represented steady-state levels, since similar concentrations were measured after 60 min. Tissue Ang II, however, is localized in one or more different compartments, and a low tissue level therefore does not argue against the possibility that Ang II is present at high concentrations in a specific tissue compartment. For instance, previous *in-vivo* (Siragy *et al.*, 1995; Dell'Italia *et al.*, 1997) and *in-vitro* (de Lannoy *et al.*, 1997; 1998) studies have shown that interstitial fluid Ang II may be higher than circulating Ang II. In addition, tissue Ang II is, at least partly, localized intracellularly, because of its internalization *via* AT₁ receptors (van Kats *et al.*, 1997; 2001). Such internalization does not appear to occur after binding to AT₂ receptors (Matsubara, 1998; van Kats *et al.*, 1997), nor *via* non-AT₁, non-AT₂ receptor-mediated mechanisms (van Kats *et al.*, 2001). Finally, Ang II may be bound to AT receptors on the cell surface. Receptor binding protects Ang II against rapid metabolism by degrading enzymes (Schuijt *et al.*, 1999). Since in pigs, like in humans, there is currently no evidence for the existence of AT receptors other than the AT₁ and AT₂ receptor, we distinguished cellular (membrane bound or internalized) and extracellular Ang II by measuring tissue Ang II in the presence of irbesartan or PD123319. No significant differences were found in comparison with the control situation. This suggests that only a small fraction of tissue Ang II in femoral artery rings is bound to cell surface – and/or internalized AT receptors, and, thus, that the majority of vascular Ang II is localized in interstitial fluid. Assuming therefore that all vascular Ang II is present in the interstitial space, and taking into account that this compartment represents $\approx 15\%$ of tissue weight (Linde & Chisolm, 1975; Plank *et al.*, 1976; Reed & Wiig, 1984; Katz *et al.*,

1997), it follows that interstitial Ang II during Ang I administration is 8.8–27 times higher than bath fluid Ang II.

The latter was not the case during Ang II administration. Tissue Ang II following Ang II administration was also restricted to the interstitial fluid compartment (i.e., not affected by irbesartan or PD123319), and the interstitial Ang II fluid levels during Ang II administration were not different from the bath fluid Ang II levels. The latter contrasts with earlier findings in isolated perfused rat Langendorff hearts, where interstitial Ang II during Ang II infusion was <35% of arterial Ang II (de Lannoy *et al.*, 1998), and with *in-vivo* studies in pigs (Schuijt *et al.*, 1999) and dogs (Dell'Italia *et al.*, 1997), where interstitial fluid Ang II during Ang II infusion was <5% of arterial Ang II. These differences are most likely related to the amount of metabolizing enzymes present under the various experimental conditions. In addition, chronic exposure to Ang II is known to affect the level of angiotensin-metabolizing enzymes, including ACE (Schunkert *et al.*, 1993).

Why are the interstitial Ang II levels during Ang I administration higher than the bath fluid Ang II levels? In porcine arteries, Ang I-II conversion occurs both after luminal and after adventitial Ang I administration (Danser *et al.*, 1995), suggesting that ACE is present both in the endothelium and the adventitia. In addition, ACE has been demonstrated on rat and human vascular smooth muscle cells (Coulet *et al.*, 2001). Most likely therefore, Ang I-II conversion occurs by ACE within the interstitial space. This does not exclude Ang I-II conversion on either the endothelial or adventitial surface. In fact, in view of the much larger volume of the organ bath (15 ml) as compared to the interstitial fluid volume of a 22 mg vessel segment ($\approx 3\ \mu\text{l}$), it can be calculated that the majority (>95%) of Ang II is present in the organ bath, and thus is probably generated on the vascular surface. In the artery segment, the interstitial space is continuously supplied, *via* diffusion (de Lannoy *et al.*, 1997), with Ang I from the bath fluid, and this Ang I will be converted by ACE into Ang II in close proximity of the AT₁ receptors. The small volume of the interstitial space allows a rapid rise in the interstitial Ang II levels, resulting in a steady state within 15 min. The rate-limiting factor in this process is most likely the diffusion of Ang I into the interstitial space (de Lannoy *et al.*, 2001). Although the bath fluid Ang II levels continued to rise over time, previous studies (Danser *et al.*, 1995; MaassenVanDenBrink *et al.*, 1999) have shown that it is unlikely that these levels will become as high as the interstitial Ang II levels. This is due to the small volume of the interstitial space, thereby allowing interstitial Ang II to contribute only marginally to the organ bath levels of Ang II. Indeed, in previous perfusion studies with porcine carotid arteries, we found no Ang II in the perfusion fluid upon adventitial Ang I administration, nor was Ang II detectable in bath fluid upon luminal Ang I administration (Danser *et al.*, 1995). Moreover, we were also unable to demonstrate significant release of Ang II from tissue sites into the circulation (Danser *et al.*, 1992b; Admiraal *et al.*, 1993).

Taken together, the similar potencies of Ang I and II in the present and previous studies (Danser *et al.*, 1995; MaassenVanDenBrink *et al.*, 1999; Saris *et al.*, 2000), can be fully explained on the basis of the interstitial Ang II

levels that are reached in the vessel wall during Ang I and II application. During Ang I application, at the time of vasoconstriction, these levels are almost one order of magnitude higher than the levels in the organ bath, whereas during Ang II application the interstitial and bath fluid Ang II levels are virtually equal. The right panel of Figure 2 illustrates the consequences of this concept. Our results not only explain why circulating Ang II does not always

References

ADMIRAAL, P.J.J., DANSER, A.H.J., JONG, M.S., PIETERMAN, H., DERKX, F.H.M. & SCHALEKAMP, M.A.D.H. (1993). Regional angiotensin II production in essential hypertension and renal artery stenosis. *Hypertension*, **21**, 173–184.

BORLAND, J.A.A., CHESTER, A.H., MORRISON, K.A. & YACOUB, M.H. (1998). Alternative pathways of angiotensin II production in the human saphenous vein. *Br. J. Pharmacol.*, **125**, 423–428.

CAMPBELL, D.J. (1987). Circulating and tissue angiotensin systems. *J. Clin. Invest.*, **79**, 1–6.

COULET, F., GONZALEZ, W., BOIXEL, C., MEILHAC, O., PUEYO, M.E. & MICHEL, J.B. (2001). Endothelium-independent conversion of angiotensin I by vascular smooth muscle cells. *Cell Tissue Res.*, **303**, 227–234.

DANSER, A.H.J., CHOWDURY, S., DE LANNOY, L.M., VAN DER GIJSEN, W.J., SAXENA, P.R. & SCHALEKAMP, M.A.D.H. (1995). Conversion and degradation of [¹²⁵I] labelled angiotensin I in isolated perfused porcine coronary and carotid arteries. *Cardiovasc. Res.*, **29**, 789–795.

DANSER, A.H.J., KONING, M.M.G., ADMIRAAL, P.J.J., DERKX, F.H.M., VERDOUW, P.D. & SCHALEKAMP, M.A.D.H. (1992a). Metabolism of angiotensin I by different tissues in the intact animal. *Am. J. Physiol.*, **263**, H418–H428.

DANSER, A.H.J., KONING, M.M.G., ADMIRAAL, P.J.J., SASSEN, L.M.A., DERKX, F.H.M., VERDOUW, P.D. & SCHALEKAMP, M.A.D.H. (1992b). Production of angiotensins I and II at tissue sites in intact pigs. *Am. J. Physiol.*, **263**, H429–H437.

DE LANNOY, L.M., DANSER, A.H.J., BOUHUIZEN, A.M.B., SAXENA, P.R. & SCHALEKAMP, M.A.D.H. (1998). Localization and production of angiotensin II in the isolated perfused rat heart. *Hypertension*, **31**, 1111–1117.

DE LANNOY, L.M., DANSER, A.H.J., VAN KATS, J.P., SCHOOEMAKER, R.G., SAXENA, P.R. & SCHALEKAMP, M.A.D.H. (1997). Renin-angiotensin system components in the interstitial fluid of the isolated perfused rat heart. Local production of angiotensin I. *Hypertension*, **29**, 1240–1251.

DE LANNOY, L.M., SCHUIJT, M.P., SAXENA, P.R., SCHALEKAMP, M.A.D.H. & DANSER, A.H.J. (2001). Angiotensin-converting enzyme is the main contributor to angiotensin I-II conversion in the interstitium of isolated perfused rat heart. *J. Hypertens.*, **19**, 959–965.

DE LEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.*, **235**, E97–E102.

DELL'ITALIA, L.J., MENG, Q.C., BALCELLS, E., WEI, C.C., PALMER, R., HAGEMAN, G.R., DURAND, J., HANKES, G.H. & OPARIL, S. (1997). Compartmentalization of angiotensin II generation in the dog heart. Evidence for independent mechanisms in intravascular and interstitial spaces. *J. Clin. Invest.*, **100**, 253–258.

DUDLEY, D.T., PANEK, R.L., MAJOR, T.C., LU, G.H., BRUNS, R.F., KLINKEFUS, B.A., HODGES, J.C. & WEISHAAR, R.E. (1990). Subclasses of angiotensin II binding sites and their functional significance. *Mol. Pharmacol.*, **38**, 370–377.

FERRARIO, C.M., CHAPPELL, M.C., TALLANT, E.A., BROSNIHAN, K.B. & DIZ, D.I. (1997). Counterregulatory actions of angiotensin-(1-7). *Hypertension*, **30**, 535–541.

HILGERS, K.F., BINGENER, E., STUMPF, C., MULLER, D.N., SCHMIEDER, R.E. & VEELKEN, R. (1998). Angiotensinases restrict locally generated angiotensin II to the blood vessel wall. *Hypertension*, **31**, 368–372.

HULSMANN, A.R., RAATGEEP, H.R., BONTA, I.L., STIJNEN, T., KERREBIJN, K.F. & DE JONGSTE, J.C. (1992). The perfused human bronchiolar tube characteristics of a new model. *J. Pharmacol. Toxicol. Methods*, **28**, 29–34.

KATZ, S.A., OPSAHL, J.A., LUNZER, M.M., FORBIS, L.M. & HIRSCH, A.T. (1997). Effect of bilateral nephrectomy on active renin, angiotensinogen, and renin glycoforms in plasma and myocardium. *Hypertension*, **30**, 259–266.

LI, P., FUKUHARA, M., DIZ, D.I., FERRARIO, C.M. & BROSNIHAN, K.B. (2000). Novel angiotensin II AT1 antagonist irbesartan prevents thromboxane A2-induced vasoconstriction in canine coronary arteries and human platelet aggregation. *J. Pharmacol. Exp. Ther.*, **292**, 238–246.

LI, Q., ZHANG, L., PFAFFENDORF, M. & VAN ZWIETEN, P.A. (1995). Comparative effects of angiotensin II and its degradation products angiotensin III and angiotensin IV in rat aorta. *Br. J. Pharmacol.*, **116**, 2963–2970.

LINDE, B. & CHISOLM, G. (1975). The interstitial space of adipose tissue as determined by single injection and equilibration techniques. *Acta Physiol. Scand.*, **95**, 383–390.

MAASSENVANDENBRINK, A., DE VRIES, R., SAXENA, P.R., SCHALEKAMP, M.A.D.H. & DANSER, A.H.J. (1999). Vasoconstriction by in-situ formed angiotensin II: role of ACE and chymase. *Cardiovasc. Res.*, **44**, 407–415.

MATSUBARA, H. (1998). Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. *Circ. Res.*, **83**, 1182–1191.

MULLER, C., ENDLICH, K. & HELWIG, J. (1998). AT2 antagonist-sensitive potentiation of angiotensin II-induced constriction by NO blockade and its dependence on endothelium and P450 eicosanoids in rat renal vasculature. *Br. J. Pharmacol.*, **124**, 946–952.

NUSSBERGER, J., BRUNNER, D.B., WAEBER, B. & BRUNNER, H.R. (1986). Specific measurement of angiotensin metabolites and in vitro generated angiotensin II in plasma. *Hypertension*, **8**, 476–482.

PLANK, B., RABERGER, G., BRUGGER, G. & ADLER-KASTNER, L. (1976). The determination of the myocardial extracellular space in the cat *in vivo*: a comparative methodological study. *Basic Res. Cardiol.*, **71**, 173–178.

REED, R.K. & WIIG, H. (1984). Compliance of the interstitial space in rats. III. Contribution of skin and skeletal muscle interstitial fluid volume to changes in total extracellular fluid volume. *Acta Physiol. Scand.*, **121**, 57–63.

SARIS, J.J., VAN DIJK, M.A., KROON, I., SCHALEKAMP, M.A.D.H. & DANSER, A.H.J. (2000). Functional importance of angiotensin-converting enzyme-dependent in situ angiotensin II generation in the human forearm. *Hypertension*, **35**, 764–768.

SCHUIJT, M.P., VAN KATS, J.P., DE ZEEUW, S., DUNCKER, D.J., VERDOUW, P.D., SCHALEKAMP, M.A.D.H. & DANSER, A.H.J. (1999). Cardiac interstitial fluid levels of angiotensin I and II in the pig. *J. Hypertens.*, **17**, 1885–1891.

SCHUNKERT, H., INGELFINGER, J.R., HIRSCH, A.T., PINTO, Y., REMME, W.J., JACOB, H. & DZAU, V.J. (1993). Feedback regulation of angiotensin converting enzyme activity and mRNA levels by angiotensin II. *Circ. Res.*, **72**, 312–318.

SIRAGY, H.M., HOWELL, N.L., RAGSDALE, N.V. & CAREY, R.M. (1995). Renal interstitial fluid angiotensin. Modulation by anesthesia, epinephrine, sodium depletion, and renin inhibition. *Hypertension*, **25**, 1021–1024.

STOLL, M., STECKELINGS, U.M., PAUL, M., BOTTARI, S.P., METZGER, R. & UNGER, T. (1995). The angiotensin AT2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J. Clin. Invest.*, **95**, 651–657.

TOM, B., DE VRIES, R., SAXENA, P.R. & DANSER, A.H.J. (2001). Bradykinin potentiation by angiotensin-(1-7) and angiotensin-converting enzyme (ACE) inhibitors correlates with ACE C- and N-domain blockade. *Hypertension*, **38**, 95–99.

URATA, H., BOEHM, K.D., PHILIP, A., KINOSHITA, A., GABROVSEK, J., BUMPUS, F.M. & HUSAIN, A. (1993). Cellular localization and regional distribution of an angiotensin II-forming chymase in the heart. *J. Clin. Invest.*, **91**, 1269–1281.

URATA, H., HEALY, B., STEWART, R.W., BUMPUS, F.M. & HUSAIN, A. (1990). Angiotensin II-forming pathways in normal and failing human hearts. *Circ. Res.*, **66**, 883–890.

VAN KATS, J.P., DUNCKER, D.J., HAITSMA, D.B., SCHUIJT, M.P., NIEBUUR, R., STUBENITSKY, R., BOOMSMA, F., SCHALEKAMP, M.A.D.H., VERDOUW, P.D. & DANSER, A.H.J. (2000). Angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade prevent cardiac remodeling in pigs after myocardial infarction: role of tissue angiotensin II. *Circulation*, **102**, 1556–1563.

VAN KATS, J.P., VAN MEEGEN, J.R., VERDOUW, P.D., DUNCKER, D.J., SCHALEKAMP, M.A.D.H. & DANSER, A.H.J. (2001). Subcellular localization of angiotensin II in kidney and adrenal. *J. Hypertens.*, **19**, 583–589.

VAN KESTEREN, C.A.M., VAN HEUGTEN, H.A.A., LAMERS, J.M.J., SAXENA, P.R., SCHALEKAMP, M.A.D.H. & DANSER, A.H.J. (1997). Angiotensin II-mediated growth and antiproliferative effects in cultured neonatal rat cardiac myocytes and fibroblasts. *J. Mol. Cell. Cardiol.*, **29**, 2147–2157.

VOORS, A.A., PINTO, Y.M., BUIKEMA, H., URATA, H., OOSTERGA, M., ROOKS, G., GRANDJEAN, J.G., GANTEN, D. & VAN GILST, W.H. (1998). Dual pathway for angiotensin II formation in human internal mammary arteries. *Br. J. Pharmacol.*, **125**, 1028–1032.

WEI, C.C., MENG, Q.C., PALMER, R., HAGEMAN, G.R., DURAND, J., BRADLEY, W.E., FARRELL, D.M., HANKES, G.H., OPARIL, S. & DELL'ITALIA, L.J. (1999). Evidence for angiotensin-converting enzyme- and chymase-mediated angiotensin II formation in the interstitial fluid space of the dog heart *In vivo*. *Circulation*, **99**, 2583–2589.

WHITEBREAD, S., MELE, M., KAMBER, B. & DE GASPARO, M. (1989). Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.*, **163**, 284–291.

WILLEMS, E.W., HEILIGERS, J.P.C., DE VRIES, P., TOM, B., KAPOOR, K., VILLALON, C.M. & SAXENA, P.R. (2001). A61603-induced vasoconstriction in porcine carotid vasculature: involvement of a non-adrenergic mechanism. *Eur. J. Pharmacol.*, **417**, 195–201.

YAMADA, T., HORIUCHI, M. & DZAU, V.J. (1996). Angiotensin II type 2 receptor mediates programmed cell death. *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 156–160.

ZWART, A.S., DAVIS, E.A. & WIDDOP, R.E. (1998). Modulation of AT1 receptor-mediated contraction of rat uterine artery by AT2 receptors. *Br. J. Pharmacol.*, **125**, 1429–1436.

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