Angiotensinases Restrict Locally Generated Angiotensin II to the Blood Vessel Wall

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Abstract—We tested the hypothesis that angiotensinases limit the spillover of locally formed angiotensin II into the circulation. The release of angiotensin peptides from isolated rat hindquarters perfused with an artificial medium was measured by high-performance liquid chromatography and radioimmunoassay. The spontaneous release of angiotensins was increased by the angiotensinase inhibitors phenanthroline (850±195 versus 95±33 fmol of angiotensin I per 30 minutes in controls, P<0.05, n=5 each) and amastatin (P<0.05, n=5 each). Infusion of renin induced sustained local angiotensin I formation, which was also increased by phenanthroline. Stimulation of local angiotensin formation by renin infusion was compared with infusion of exogenous angiotensin II. Renin caused similar increases of perfusion pressure (111±22 versus 76±19 mm Hg after angiotensin II, P>0.05) despite lower angiotensin II levels in the venous effluent than during infusion of exogenous angiotensin II (65±2 versus 48±233 fmol/mL, P<0.05, n=7 each). Thus, renin must have caused higher angiotensin II tissue levels than indicated by the measurements in the venous effluent. The pressor response to renin was abolished by the type 1 angiotensin II receptor antagonist losartan. We conclude that the major part of locally generated angiotensins is not released into the circulation but degraded by angiotensinases within the tissue compartment. (Hypertension. 1998;31[part 2]:368-372.)

Key Words: angiotensinase • aminopeptidase • angiotensin • renin • vasculature

In vivo studies in several species, including humans, provided indirect evidence for a significant generation of Ang I and II in the vascular tissue of extrarenal blood vessels. In support of this notion, release of Ang I and II from vascular tissue has been demonstrated in vitro. However, very low quantities of both peptides are released, particularly compared to the high concentrations of Ang II required to affect vascular tone in these preparations.

On the other hand, the clearance of Ang I and II by the peripheral vasculature is extensive. In perfused rat hindquarters, approximately 40% to 60% of Ang II and about 85% of Ang I are cleared from the circulation during one pass through the preparation, if peptides are infused. Similar clearance rates for both peptides are observed in vivo in different species, including humans. We speculated that locally formed Ang peptides may be degraded or taken up to an even higher degree than exogenous peptides. In support of this notion, we previously observed that induction of local vascular Ang II formation by renin caused marked vasoconstriction with relatively little Ang II release, if compared with exogenous Ang I or II infusion.

The current study was designed to test the hypothesis that Ang release from vascular tissue is only a minor part of a substantially higher local Ang production. We used two different approaches to test this hypothesis. First, we measured Ang I and II release from isolated, perfused rat hindquarters after blockade of Ang-cleaving enzymes. Second, we studied the effects of (1) infusion of exogenous Ang peptides and (2) induction of endogenous Ang I and II formation by renin infusion. We compared the increase of perfusion pressure and the Ang II levels produced in the venous effluent. The latter approach is based on the assumption that the pressor effects of locally formed Ang II reflect the Ang II concentration at the receptor site, i.e., within vascular tissue.

Materials and Methods

Animals
Male Sprague-Dawley rats (Charles River, Kissing, Germany) weighing 250 to 300 g were housed in a room maintained at 22±2°C and exposed to a 12-hour dark/light cycle. The animals were allowed unlimited access to chow (no 1320, Altromin) and tap water. All procedures performed on animals were done in accordance with guidelines of the American Physiological Society and were approved by the local government authorities (Regierung von Mittelfranken, AZ no. 621-2531 3 A-17/96).

Hindquarter Perfusion
The perfusion was performed as previously described. Briefly, rats underwent median laparotomy under thiobarbiturate anesthesia (60 mg/kg body weight intraperitoneally). After decannulation, an intravenous injection of 500 U of sodium-heparin was given. Five minutes thereafter, the abdominal aorta and the inferior vena cava were cannulated, and the perfusion was begun immediately. The hindquarters were perfused in a nonrecirculating system with modified Tyrode's solution containing 2
Experimental Protocols

Effects of Angiotensinase Inhibitors on the Spontaneous Release of Ang I and Ang II

These experiments addressed the question whether or not inhibition of angiotensinase activity would increase the spontaneous release of Ang peptides from isolated, perfused rat hindquarter preparations. The peptides were collected from the perfusate for two consecutive 30-minute periods by "on-line" peptide extraction. A prepared SepPak cartridge (Waters Associates) was connected to the venous perfusion cannula immediately after collection as previously described. During the second 30-minute period of collection, the angiotensinase inhibitor amastatin (0.1 μmol/mL, n=5) or the angiotensinase inhibitor phenanthroline (0.5 μmol/mL, n=5) was infused. In vitro studies using cultured cells or tissue membranes have shown that amastatin inhibition of angiotensinase A is maximal at 0.1 μmol/mL. Further, 0.5 μmol/mL of the metallo-chelator phenanthroline completely abolishes angiotensinase activity of angiotensinases, carboxypeptidases, and neutral endopeptidases.

Effects of Phenanthroline on Renin-Induced Ang I Release

These experiments addressed the question whether or not inhibition of angiotensinase activity would increase the vascular release of Ang peptides in response to renin infusion. We and others have previously shown that infusion of renin into isolated, perfused rat hindquarters stimulates the release of Ang peptides. Porcine renin was prepared from hog kidneys at the Department of Pharmacology, University of Heidelberg, Germany, and infused Ang II during one pass through the hindquarter vasculature. Ang II was infused at two concentrations: 1 μmol/mL for 15 minutes, followed by 10 μmol/mL for another 15 minutes, either in the presence or the absence of 0.5 μmol/mL phenanthroline (n=6 each). Ang II was infused into perfused rat hindquarters and into a second perfusion channel without hindquarter perfused in parallel (blank channel). Samples for Ang II measurement were taken at 5-minute intervals from both channels. The disappearance rate (as a percentage) was calculated as follows:

Disappearance = 100 × [(Ang IIblank channel − Ang IIhindquarter)/Ang IIblank channel]

Effects of Renin, Ang I, and Ang II on Perfusion Pressure and Ang II Concentration in Venous Effluent

These experiments were designed to compare local Ang II formation with exogenous Ang II infusion with regard to vasocostriction and venous Ang II levels. After 20 minutes of washout perfusion with methoxamine (10 mM/mL) was added to the perfusate. Methoxamine increases the perfusion pressure and stimulates hindquarter preparations for the pressor action of Ang II. Ang I, Ang II, or renin was infused 30 minutes after the addition of methoxamine, when perfusion pressure had stabilized. Hindquarter preparations received Ang I (1 μmol/mL final dilution in the perfusate), Ang II (1 μmol/mL), or renin (100 μU/mL) (n=7 each). All substances were given for 15 minutes. Samples for Ang II measurement were taken at 5-minute intervals. Identical experiments (n=6 per group) were performed in the presence of the Ang II type 1 receptor antagonist losartan (1 mM/mL). Perfusion pressure was monitored continuously.

Measurement of Ang I and Ang II

Samples from protocol 1 (spontaneous Ang release) underwent HPLC separation before quantitation of peptides by radiommunoassay (RIA) as previously described. For reverse-phase HPLC (Gradient Pump 2249, Pharmacia LKB), samples (redissolved in 500 μL of saline) were loaded onto a 7-μm Nucleosil C18 column (Merck-Hager & Co.), guarded by an ODS C18 guard column (Guard-Pak, Waters Associates), and eluted using a methanol gradient. These fractions were redissolved in 250 μL of 1 mM/mL Tris-acetate buffer, and peptides were quantitated by RIA for Ang I and II. Synthetic angiotensins were used to calibrate the HPLC column and to determine the recovery, which was 61% to 79% for Ang I and 85% to 92% for Ang II. Measurements were corrected for recovery.

Perfusate samples from protocols 2 to 4 (1 mL) were collected in tubes containing 50 μL of a mixture of ortho-phenanthroline (26 mM/mL) and Na-EDTA (125 mM/mL), chilled, immediately snap frozen on dry ice, and kept at −26°C until they were assayed for Ang I and II in perfusate were measured by RIA. The sensitivity of the Ang I and Ang II RIAs was 1 pg/tube. The cross-reactivity of the Ang I antibody (K18) was 0.05% for Ang II. Ang II in perfusate samples was measured using an antibody that does not cross-react with any Ang II metabolite. The cross-reactivity with Ang I was 0.25% Ang II in HPLC fractions was measured with an antibody Celine III). That exhibits 1% cross-reactivity Ang I and 100% with Ang III, Ang IV, and Ang-(4–8), respectively. For all antibodies, cross-reactivity with Ang-(1–9) and Ang-(1–7) was below 1% RIAs were performed as previously described. All samples were estimated in duplicate.

Statistical Analysis

Results are expressed as mean and SEM. Two-way ANOVA with subsequent Newman-Keuls's test for posthoc analysis was used to assess significant differences between groups. P<0.05 was considered signifi-
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**Results**

Ang I and II were released from isolated, perfused rat hindquarters (Fig 1). Amastatin approximately doubled the release of both peptides whereas phenanthroline caused a more than 10-fold increase of Ang I release but did not change Ang II levels (Fig 1). Fig 2 shows examples of HPLC elution profiles with or without amastatin. Immunoreactive metabolites other than Ang I and II were detectable in some but not all experiments; if detectable, the levels of these metabolites were consistently elevated by amastatin, as shown in Fig 2. In the presence of phenanthroline, immunoreactive metabolites other than Ang I and II were undetectable.

Infusion of renin stimulated Ang I formation; a prolonged Ang I release continued after cessation of renin infusion (Fig 3). Phenanthroline showed little effect during renin infusion but significantly enhanced the sustained Ang I release after cessation of renin infusion (Fig 3). Furthermore, phenanthroline inhibited the disappearance of exogenously administered Ang II. During infusion of 1 pmol/mL Ang II, the disappearance rate during one pass was 35.7 ± 2.9% in the presence of phenanthroline and 53.1 ± 4.6% in control experiments (P < 0.05). During infusion of 10 pmol/mL Ang II, the disappearance rate was 41.2 ± 3.6% in the presence of phenanthroline and 59.8 ± 6.3% in control experiments (P < 0.05).

Fig 4 shows the effects of renin, Ang I, and Ang II on venous effluent Ang II levels and perfusion pressure after 15 minutes of infusion. Renin caused lower Ang II concentrations in the venous effluent than Ang I and II but equal or higher pressor responses (Fig 4). Within 5 minutes after cessation of Ang I and II infusion, the perfusion pressure returned to levels not significantly different from baseline. In contrast, perfusion pressure remained 7 to 10 mm Hg above baseline for the entire observation period (15 minutes) after cessation of the renin infusion. The pressor effects of all three substances were completely prevented by the Ang II receptor antagonist losartan (data not shown).

**Discussion**

We tested the hypothesis that Ang release from vascular tissue is only a minor part of a substantially higher local Ang production. Inhibition of angiotensinase activity markedly increased spontaneous and renin-induced Ang release. Moreover, a comparison between the pressor effects and the venous
Ang II levels elicited by infusion of renin (to stimulate local Ang formation) and Ang peptides also indicated higher local Ang II formation than measured by the peptide concentrations in the effluent. The pressor effects of renin were prevented by losartan, in agreement with our previous results that ACE inhibition, renin inhibition, and nonspecific Ang II receptor blockade all abolished renin-induced vasoconstriction. Thus, our data demonstrate that angiotensinas together restrict locally generated Ang II to the tissue compartment. Only a minor fraction of locally formed Ang II is released into the circulation.

The products generated by the enzymatic cleavage of Ang II are not merely metabolites but bioactive peptides. Independent effects mediated by specific receptors have been ascribed to Ang-(1-7) and Ang IV. Some authors described a vasoconstrictor response to Ang IV, which was not confirmed by others. Further, Ang III is an agonist of Ang II receptors. Our study did not address the biological activity of these Ang peptides, and our methods were not suited to detect Ang-(1-7) or Ang-(1-9). We did not directly measure Ang-metabolizing enzymes but instead relied on estimates indirectly derived from measurements of Ang II in venous effluent and Ang II-dependent pressor effects. However, we mainly focused on vascular Ang II production and used angiotensinas inhibitors primarily as a tool to obtain a better estimate of locally generated Ang II. It is impossible to give a precise calculation of the relation between local Ang II formation and its release into the lumen, but our data suggest that Ang II release underestimates the actual generation of the peptide by a factor of at least 2 (as suggested by the amastatin experiments) and possibly as high as 10 (as suggested by approximately equipressor doses of Ang II and renin).

We did not address the origin of vascular renin in the current study, but previous data by others and us indicate that uptake of renin from the circulation is the main source of vascular Ang I-forming activity, at least in the perfused hindquarter of Sprague-Dawley rats. Our current data reinforce the notion that renin is taken up into vascular tissue, and Renn led to prolonged Ang release and Ang II-dependent vasoconstriction, as described previously. We do not know why phenanthroline affected only the sustained Ang I release after renin infusion but not the initial Ang I release. We cannot exclude the possibility that phenanthroline might affect uptake and/or stability of renin. However, such an effect would not explain the action of phenanthroline on spontaneous Ang release (which shows little variation over time) or the pronounced Ang II pressor effect of renin despite relatively little Ang II release. We speculate that renin causes luminal and tissue Ang I formation during infusion of the enzyme but mainly tissue Ang I generation after cessation of the renin infusion. The relatively small effect of phenanthroline on the clearance of luminal Ang peptides might explain why the inhibitor increases Ang I release only during the sustained phase after renin infusion.

The enzymes that metabolize Ang peptides have been studied most extensively in neuronal tissue or cultured neuronal cells but recent studies provided valuable information about Ang cleaving enzymes in vascular endothelial and smooth muscle cells. According to Mentlem and Roos, the most important angiotensinases in vascular smooth muscle are aminopeptidase A, aminopeptidase N (EC 3.4.11.2, sometimes referred to as aminopeptidase M13,27), neutral endopeptidases, and carboxypeptidase P, the enzyme that forms Ang-(1-7) and Amastatin is often considered as a specific inhibitor of aminopeptidase A, which generates Ang III from Ang II, but some authors reported that amastatin is an even better inhibitor of aminopeptidase N, which cleaves amino-terminal amino acids from Ang III and Ang IV. Our data are in agreement with the latter notion because the levels of Ang II, Ang III, Ang IV, and Ang-(4-8) all tended to increase in the presence of amastatin, consistent with inhibition of both aminopeptidases A and N.

In contrast to amastatin, phenanthroline inhibits all known pathways of Ang cleavage, which explains its more pronounced effect in our study (see Figure 1). Because phenanthroline also inhibited ACE, only Ang I release was increased. In view of these marked effects of phenanthroline, we were surprised that the inhibitor affected the disappearance of exogenously infused Ang II only moderately (by approximately 20%). We cannot exclude the possibility that angiotensinas inhibition by phenanthroline was incomplete, although we used a concentration that was highly effective in cell culture. More likely, Ang II may be cleared from the circulation by nonenzymatic mechanisms. Receptor-mediated uptake of Ang II into the cells is one possible mechanism. However, skeletal muscle tissue shows relatively little Ang II uptake, and studies using receptor blockers indicated unaltered Ang II disappearance both in vivo and in the perfused rat hindquarter. Finally, Ang II may be distributed in the intestinal fluid, as described in the perfused rat heart. In any case, the moderate effect of phenanthroline on the disappearance of exog-
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Ang II may indicate that our calculations still underestimate the amount of locally generated Ang peptides.

Studies on Ang II degradation are relatively sparse, if compared with the enormous body of research devoted to Ang II formation. However, the turnover of Ang II in vivo is fast, 1-13, 21, 25 and angiotensinases could quite conceivably play a role in regulating the effects of Ang II. 17 Little information exists regarding angiotensinases in pathological situations. Several studies reported diminished angiotensinase activity in spontaneously hypertensive rats. 3-5 In kidney failure, angiotensinase A is increased in kidney glomeruli, 3, 5, 6 but may be diminished in other organs. 3, 5 In agreement with these notions, we previously observed diminished Ang clearance by perfused rat hindquarters of spontaneously hypertensive rats 3, 6 and rats with renal failure. 17 For future research concerning the role of angiotensinases, it should be kept in mind that angiotensinases may particularly affect tissue concentrations of locally generated Ang II.

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References

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