Local Generation and Release of Angiotensin II in Peripheral Vascular Tissue

KENJI MIZUNO, MITSUAKI NAKAMARU, KOICHI HIGASHIMORI, AND TADASHI INAGAMI

SUMMARY Isolated rat hindlegs were perfused with Krebs-Ringer solution, and immunoreactive angiotensin II (irAng II) released into the perfusate was directly determined using a Sep-Pak C18 cartridge connected to the perfusion system. High performance liquid chromatography clearly demonstrated the presence of angiotensin I (Ang I), angiotensin II (Ang II), and a small amount of angiotensin III. The spontaneous release of irAng II was as high as about 600 pg/30 min, which was stable up to 3 hours. Captopril added to the perfusion medium (10^-9 to 10^-6 M) suppressed irAng II release in a dose-dependent manner (p<0.001), and it (10^-6 M) caused a reciprocal increase of irAng I release (p<0.05). Oral pretreatment of captopril (50 mg/kg/day) for 1 week suppressed the irAng II release by 31% (p<0.02). The same treatment with SA446, a highly lipophilic angiotensin converting enzyme inhibitor, inhibited the irAng II release by 63% (p<0.001). On the other hand, the two inhibitors suppressed the plasma irAng II to very similar extents. Pretreatment with SA446 plus nephrectomy did not cause any further change in irAng II release as compared with that with SA446 alone. These results provide direct proof for local generation and subsequent secretion of Ang II by peripheral vascular tissue. (Hypertension 11: 223-229, 1988)

KEY WORDS • renin-angiotensin system • vascular tissue • captopril • SA446 • angiotensin II • hindleg

THE presence of various components of the renin-angiotensin system in vascular tissue has been reported.1-5 Although the precise origin of each component of the vascular renin-angiotensin system is not completely known, recent findings that 1) vascular smooth muscle cells or endothelial cells in culture synthesize renin enzyme6 and 2) messenger RNA for angiotensinogen could be detected in arterial tissue4 provide strong support for the local production of renin and subsequent generation of angiotensin in vascular tissue. On the other hand, angiotensin converting enzyme (ACE) inhibition is known to induce a fall in blood pressure in various types of human hypertension6 as well as in experimental hypertensive models7-9 whose plasma renin activity is normal or even low. These findings suggest that the antihypertensive response to ACE inhibition cannot be explained solely by the suppression of the circulating renin-angiotensin system, and this raises an important question of whether the hypotensive effect of ACE inhibitors may be due to the inhibition of local angiotensin II (Ang II) formation. Recently, intravenous administration of renin inhibitor was found to reduce blood pressure in a dose-dependent fashion in dogs with completely suppressed plasma renin.10 More recently, Okamura et al.11 demonstrated that the ACE inhibitor enalapril and Ang II antagonist [Sar^2,Ile^8]Ang II lowered the blood pressure of rats in the chronic stage of two-kidney, one clip hypertension when plasma renin activity was almost normal. These findings raise an intriguing hypothesis that the vascular renin-angiotensin system plays an important role in the maintenance of hypertension, though direct evidence is lacking.

The main purpose of the present study was to obtain direct evidence for the generation and subsequent secretion of Ang II by vascular tissue and then to address the possible mechanism by which Ang II is generated. Based on our previous observations,12-14 it appears that Ang II can be produced intracellularly in vascular beds. To further address this possibility, we examined the effect of two ACE inhibitors (captopril and SA446) on immunoreactive Ang II (irAng II) release. These two inhibitors were compared in view of the much greater (120 times) lipophilicity of SA44615 than captopril to explain the possible uptake of the former by vascular tissues and ensuing action in these tissues.
Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) weighing 180 to 220 g were used in this study. The rats were maintained on a regular Purina chow diet (St. Louis, MO, USA) containing 0.39% sodium and 0.9% potassium and allowed free access to tap water.

Treatment with ACE Inhibitors

The ACE inhibitors captopril (E. R. Squibb, Princeton, NJ, USA) and SA 446 (Santen Pharmaceutical, Osaka, Japan) were dissolved in 50 mM carbonate-bicarbonate buffer, pH 8.5, and given orally to the rats at a dose of 50 mg/kg/day at 1600. The drug solutions were made up freshly every day. After 1 week of the treatment, the rats were anesthetized with pentobarbital sodium (30 mg/kg i.p.) and hindleg preparations were made as described in the next section. Before the preparations were made, blood (2 ml) from the inferior caval vein was collected into tubes containing EDTA disodium salt (5 mM, final concentration) for the determination of plasma renin activity. For determination of plasma irAng II, blood (1.5 ml) was collected into tubes containing Na2EDTA and phenylmethylsulfonyl fluoride (PMSF) to make the final concentrations, 5 mM and 2.5 mM, respectively, by aortic puncture from the identical rats.

In another group of rats, SA 446 was given at the same dose as captopril for 1 week. Some of the rats were then nephrectomized under pentobarbital anesthesia (30 mg/kg i.p.), and they further received the same amount of the drug for an additional 2 days after nephrectomy. Preparations of hindlegs and blood collection were performed in these rats as described 2 days after the nephrectomy.

Hindleg Preparation

Rats were anesthetized with pentobarbital sodium (30 mg/kg i.p.) and given heparin (1300 IU/kg i.v.). The aorta was ligated at sites distal to the renal artery, and a cannula (PE-90; inside diameter, 0.86 mm) was inserted in the aorta. The hindleg was then flushed with 60 ml of Krebs-Ringer solution to eliminate blood. After ligation of the contralateral femoral artery, a hindleg was quickly isolated by sectioning the artery, a hindleg was quickly isolated by sectioning the

Determination of Angiotensins and Plasma Renin Activity

Angiotensins that were released from isolated perfused hindlegs were isolated by extraction with Sep-Pak C18 cartridges (Waters Associates, Milford, MA, USA) as described previously.16,17 In brief, the cartridge was connected to the water-jacketed container to allow the perfusate to pass through the cartridge. Cartridges, which were exchanged at 30-minute intervals, were moistened with 3 ml of methanol and prewashed with 10 ml of Krebs-Ringer solution just before use. Angiotensins released during perfusion could be trapped by these cartridges. After the cartridge was washed with 10 ml of 0.1% trifluoroacetic acid (TFA) in distilled water, peptides were eluted with 3 ml of methanol/water/TFA (80:19.9:0.1, vol/vol). The extracts were dried in a vacuum centrifuge (Speed Vac, Savant, Hicksville, NY, USA) and resultant residues were dissolved in 0.1 M Tris acetate buffer containing 2.6 mM Na2EDTA, 1 mM PMSF, and 0.1% bovine serum albumin, pH 7.4, and subjected to radioimmunoassay. The Ang II antiserum showed less than 1% cross-reactivity with angiotensin I (Ang I) but 100% cross-reactivity with angiotensin III (Ang III; Ang-(2-8) heptapeptide), Ang-(3-8) hexapeptide, and Ang-(4-8) pentapeptide (Protein Research Foundation, Osaka, Japan). The detectability of the irAng II determination was 1 pg/tube. The blank values of irAng I and irAng II were less than 1 pg/tube.

The identity of the angiotensinlike materials in the perfusate was examined using high performance liquid chromatography (HPLC) as previously described.16,17 After extraction of the perfusate by the Sep-Pak C18 cartridges, the eluted and dried materials were dissolved in 250 μl of 0.1% TFA and chromatographed on a Vydac C18 reverse-phase column (0.46 × 25 cm. The Separation Group, Hesperia, CA, USA). The elution was performed with an exponential gradient of methanol from 28 to 80% in 10 mM sodium acetate buffer, pH 5.6, over a period of 23 minutes at a flow rate of 1 ml/min, and 200-μl fractions were collected and dried in a vacuum centrifuge. Samples were redissolved in 0.1 M Tris buffer, pH 7.4, and subjected to radioimmunoassay of Ang I and Ang II. Synthetic Ang I, Ang II, and Ang III (Peninsula Laboratories, Belmont, CA, USA) were used for the calibration of the column. The recovery of Ang I and Ang II (50 pg each) added to the Sep-Pak cartridge was 96.5 ± 3.9% (mean ± SD, n = 5) for Ang I and 95.5 ± 6.2% (n = 7) for Ang II.

Plasma irAng II was measured by radioimmunoassay after extraction with Sep-Pak C18 cartridges. Blood was centrifuged for 30 minutes at 900 g at 4°C, and plasma (0.5 to 0.8 ml) was applied to the cartridges, which had been washed with methanol (3 ml) followed
by 0.1 M Tris acetate buffer, pH 7.4 (10 ml). After washing with distilled water (10 ml), irAng II was eluted with a mixture (3 ml) of methanol/water/TFA (90:9:0.1, vol/vol). The extracts were dried in a vacuum centrifuge and redissolved in 0.8 ml of 0.1 M Tris acetate buffer containing 2.6 mM Na₂EDTA, 1 mM PMSF, and 0.1% bovine serum albumin, pH 7.4. To each tube was added 0.1 ml of antiserum against irAng II, which had been dissolved in the Tris buffer to give a final dilution of 1:30,000, and 0.1 ml of [¹²³I]Ang II (equivalent to about 12,000 cpm, New England Nuclear, Boston, MA, USA; specific activity, 1000–1500 μCi/μg). The tubes were then incubated for 20 hours at 4°C, after which bound and free [¹²³I]Ang II was separated by polyethylene glycol (PEG; type 8000, Sigma Chemicals, St. Louis, MO, USA). To each tube 50 μl of 4.2% bovine gamma globulin in the Tris buffer and 1 ml of 25% PEG in distilled water were added and mixed, and the tube was centrifuged for 30 minutes at 1200 g at 4°C. The supernatant was carefully aspirated, and the radioactivity in the precipitate was counted in a gamma counter. The lowest concentration of irAng II detected was 1 pg/tube, and the 50% intercept was at 25 pg/tube. The blank values were less than 1 pg/tube. Interassay variation was 7.8% (n = 6). The recovery of Ang II (25, 50, 100, 500, and 1000 pg) added to five different rat plasma samples ranged from 88 to 95, with an average of 93%. Plasma renin activity was measured by radioimmunoassay.18

Statistical Analysis

The results of the experiments are expressed as the means ± SE. Statistical analysis of data was performed by a one-way analysis of variance and Student's t test as appropriate, and significance was defined as a p value of less than 0.05.

Results

Release of Angiotensins from Isolated Perfused Rat Hindlegs

In isolated rat hindlegs perfused with Krebs-Ringer solution at a constant flow rate of 4.0 ml/min, the mean basal perfusion pressure was 59.1 ± 2.2 mm Hg (n = 32), which remained stable for 3 hours; the pressure was 58.6 ± 3.9 mm Hg (n = 32) at the end of the perfusion.

The time course of the spontaneous release of irAng I and irAng II from isolated perfused hindlegs was examined up to 3 hours. To equilibrate the preparations and to eliminate the contamination of plasma angiotensins, the tissue was thoroughly washed with Krebs-Ringer solution (about 300 ml) before sample collections were started.

As shown in Figures 1 and 2, the rate of release of angiotensins was as high as 647.8 ± 75.4 and 612.5 ± 36.1 pg (n = 8) for irAng I (see Figure 1) and irAng II (see Figure 2), respectively, during the first infusion period of 30 minutes. The release of irAng I and irAng II was constant up to 3 hours.

To identify the angiotensin-like materials, the perfusate was purified by HPLC after extraction using Sep-Pak C₁₈ cartridges. The chromatography revealed distinct peaks of Ang I, Ang II, and Ang III corresponding to the established elution profiles of synthetic Ang I, Ang II, and Ang III (Figure 3). The peak of Ang III was small compared with those for Ang I and Ang II.

Effects of Captopril on Release of Angiotensins

The effect of captopril, added to the perfusion medium, on the release of irAng II from isolated perfused rat hindlegs is shown in Figure 4. Captopril infusion (10⁻⁸ to 10⁻⁶ M) caused a significant decrease of irAng II release in a dose-dependent manner (p < 0.001). At the highest concentration of captopril (10⁻⁶ M), it induced an approximately 44% decrease in the basal rate of release. To confirm if this reduction in irAng II release resulted from ACE inhibition, we further examined the effect of captopril (10⁻⁹ M) on the release of irAng II as well as irAng I (Figure 5). After the sample for determination of the basal release of peptides was collected, captopril was immediately added to the perfusion medium and then infused for a further period of 30 minutes. Captopril caused a significant increase in irAng I release, from a control value of 626.6 ± 22.1 to 867.8 ± 89.5 pg/30 min (p < 0.05), with the simultaneous reduction of irAng II release, from a control value of 580.3 ± 21.3 to 347.8 ± 30.5 pg/30 min (p < 0.001). After cessation of captopril

**FIGURE 1.** Time course of immunoreactive Ang I release from isolated perfused rat hindlegs. Each column shows mean value of release of immunoreactive Ang I for 30 minutes. Experiments were performed with eight preparations.

**FIGURE 2.** Time course of immunoreactive Ang II release from isolated perfused rat hindlegs. Each column shows mean value of release of immunoreactive Ang II for 30 minutes. Experiments were performed with eight preparations.
injection, release of the two peptides tended to return toward their basal levels.

**Effect of Oral Administration of Captopril and SA446 on Angiotensin Release and Plasma Renin-Angiotensin System**

As shown in Figure 6, pretreatment with captopril for 1 week decreased irAng II release (425.1 ± 46.1 pg/30 min; control, 612.4 ± 36.1 pg/30 min; p < 0.02) whereas it increased irAng I release (1376.5 ± 201.5 pg/30 min; control, 647.8 ± 76.2 pg/30 min; p < 0.01). In the SA446-treated rats, on the other hand, a marked decrease of irAng II release was observed after 1 week of treatment (224.9 ± 27.4 pg/30 min; p < 0.001 compared with control; p < 0.01 compared with captopril-treated rats). In these rats irAng I release was increased (1660.4 ± 64.7 pg/30 min; p < 0.001 compared with control), but the difference from the captopril-treated group was insignificant (0.05 < p < 0.1). Treatment with SA446 plus nephrectomy caused no further changes in irAng I and irAng II release compared with the SA446-treated group. The total amount of peptide release (irAng I + irAng II) was approximately 1.02, 1.39, 1.40, and 1.32 pmol for control, captopril-treated, SA446-treated, and SA446-treated, nephrectomized groups, respectively.

The two ACE inhibitors produced almost the same changes in plasma renin activity and irAng II levels (Table 1). Treatment with captopril increased plasma...
renin activity ($p < 0.01$), but it reduced plasma irAng II by approximately 80%. Similar effects were observed in the treatment with SA446 (see Table 1). However, plasma renin activity and plasma irAng II were markedly decreased by the treatment with SA446 plus nephrectomy (see Table 1).

**Discussion**

The notable findings from the present study are that 1) much irAng II could be detected in the perfusate from isolated perfused rat hindlegs, 2) ACE inhibitor captopril, when added to the perfusion medium, decreased irAng II release in a dose-dependent fashion, whereas it caused an apparent reciprocal increment in irAng I release, and 3) oral pretreatment with SA446, a potently lipophilic ACE inhibitor, was more effective in inhibiting irAng II release than was captopril.

Although several studies have demonstrated the presence of Ang II–like immunoreactivity in various tissues, the present studies using a Sep-Pak C$_{18}$ cartridge that was connected to the perfusion system permitted us to directly detect sizable amounts of irAng II released from the isolated perfused rat hindlegs in vitro. In addition, HPLC coupled with radioimmunoassay clearly demonstrated the presence of Ang I, Ang II, and Ang III in the perfusate. Special care was taken to avoid the contamination of plasma angiotensins in this series of experiments: The hindleg preparations were thoroughly washed with about 300 ml of Krebs-Ringer solution for at least 60 minutes before experiments were started. Under these conditions, the basal release of irAng II was constant up to 3 hours. Further, in a previous study we found that nephrectomy did not cause any significant change in irAng II release from the hindleg. Thus, the possibility that the irAng II determined in this study was due to contamination of circulating plasma peptide is negligible.

The amount of basal release of irAng II was 50-fold to 60-fold of that released from isolated perfused rat mesenteric arteries on which we reported previously. Although we do not know whether the amount of irAng II release from vascular tissue depends simply on surface area of the respective vascular beds, the finding that much irAng II could be detected enabled us to gain insight into the mechanism of the generation of Ang II in the vascular beds.

While some studies have demonstrated biochemically and immunohistochemically the presence of immunoreactive renin in vascular tissue, it is still controversial whether the renin is endogenous or taken up from plasma renin by the vascular wall. Vascular smooth muscle cells in tissue culture synthesize renin, strongly supporting the local synthesis of the enzyme in vascular smooth muscle. On the one hand, the findings that vascular reninlike activity disappeared after nephrectomy in parallel with plasma renin activity$^{24}$ support the concept that renin activity in vascular tissue is due to a contamination by plasma renin of renal origin. As to angiotensinogen, its messenger RNA has recently been detected in rat aorta, providing evidence for local synthesis of angiotensinogen in the vascular tissue. Thus, whatever the source of the vascular renin or reninlike activity may be, the renin-angiotensinogen reaction can occur locally in the vascular tissue to produce angiotensin. Our results provide experimental evidence for such a mechanism since irAng I and irAng II were produced continuously in the absence of angiotensinogen in the perfusion medium.

In the present study, ACE inhibitors suppressed irAng II release from the hindlegs. Captopril, when added to the perfusion medium, decreased irAng II release in a dose-dependent fashion (see Figure 4), and more important, it caused an apparent reciprocal increase of irAng I release (see Figure 5). Further, the total amounts of released irAng I and irAng II altered after the treatment with captopril, SA446 and SA446 plus nephrectomy were very close to each other. Therefore, the data clearly indicate that Ang I is converted to Ang II either intracellularly or extracellularly, since ACE is localized not only in the luminal surface of the vascular endothelium but also in the subcellular particles of various tissues including arterial wall.$^{28}$

However, on the basis of recent findings demonstrating biochemically and also immunohistochemically the coexistence of renin and Ang II in the same cell of various tissues, we postulated that most Ang II might be produced intracellularly by the vascular tissue to be secreted to the extracellular space. To test this hypothesis, the effect on irAng II release of oral pretreatment with a potently lipophilic ACE inhibitor, SA446, was examined. The hypothesis seems to be supported at least partially by our observations that SA446 inhibited irAng II release more effectively than did captopril, whereas the two inhibitors suppressed the plasma irAng II to similar extents. This effect of SA446 was also confirmed by our results that pretreatment with SA446 plus nephrectomy failed to cause any further change of irAng II release as compared with the treatment with SA446 alone. The present study thus indicates that the intracellular mechanism is most likely responsible for the generation of Ang II in the vascular tissue.

**Table 1. Effect of ACE Inhibitor on Changes in Plasma Renin-Angiotensin System**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 6)</th>
<th>Captopril (n = 5)</th>
<th>SA446 (n = 5)</th>
<th>SA446 + nephrectomy (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA (ng Ang I/ml/hr)</td>
<td>13.97 ± 1.11</td>
<td>26.10 ± 3.10*</td>
<td>29.51 ± 2.58†</td>
<td>0.22 ± 0.09†</td>
</tr>
<tr>
<td>irAng II (pg/ml)</td>
<td>85.52 ± 15.90</td>
<td>15.42 ± 5.52*</td>
<td>23.36 ± 2.44*</td>
<td>2.90 ± 0.42†</td>
</tr>
</tbody>
</table>

Values are means ± SE. PRA = plasma renin activity; irAng II = immunoreactive Ang II.

*p < 0.01, †p < 0.001, compared with control values.
Despite the finding that a greater decrease of irAng II release was evoked by the treatment with SA446, no difference was observed between SA446 and captopril treatment in the increase of irAng I release (see Figure 6). The reason for this discrepancy is difficult to explain. One possible explanation might be that Ang I released from the vascular tissue is taken up readily by the endothelial or smooth muscle cells to be degraded intracellularly by the tissue angiotensinase system. Further study is needed for the resolution of this point.

In the current study using isolated perfused rat hindlegs, we did not address the role of the vascular renin-angiotensin system in blood pressure regulation. We did find, however, that it is Ang II that is secreted from the vascular tissue. It is possible that secreted Ang II binds to receptors on the same or neighboring cells in an autocrine or paracrine fashion and then exerts its biological effects, such as stimulation of prosta glandin biosynthesis, activation of vascular smooth muscle tone, and potentiation of sympathetic activity. Indeed, in a previous study we presented data suggesting that the isoproterenol-induced facilitation of vascular noradrenergic neurotransmission is due to the activation of the vascular renin-angiotensin system. In support of this physiological relevance, it was demonstrated that captopril resulted in prejunctional and postjunctional inhibition of vascular sympathetic function in spontaneously hypertensive rats. These findings suggest that Ang II released from the vascular tissue has an important influence on blood pressure control.

Finally, we provide direct evidence for the first time (to our knowledge) that ACE inhibitors can inhibit irAng II release by vascular tissue. Recently, Okamura et al. demonstrated, though indirectly, that vascular renin-angiotensin activity was increased during the chronic stage of rat two-kidney, one clip hypertension, when plasma renin activity was almost normal. It was also observed that ACE inhibitor enalapril and Ang II receptor antagonist [Sar',Ile8]Ang II significantly lowered the blood pressure of these hypertensive rats. Therefore, the results of the present study, together with these findings, strongly support the idea that an abnormally activated vascular angiotensin system is involved in the maintenance of high blood pressure, and also could explain the effectiveness of ACE inhibitors in lowering blood pressure in forms of hypertension with little renin activity in the blood. Apparently, further work is required to establish, in addition to the resolution of its regulatory mechanism, if and to what extent the vascular renin-angiotensin system is responsible for the pathogenesis of diverse types of animal and human hypertension.

Acknowledgment
We thank Edward Price, Jr., for his technical assistance.

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Hypertension. 1988;11:223-229
doi: 10.1161/01.HYP.11.3.223

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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