Renin Inhibitor and Converting Enzyme Inhibitors Suppress Vascular Angiotensin II

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The direct effects of a renin inhibitor, N-acetyl-pepstatin and five angiotensin converting enzyme inhibitors, captopril and the active diacid forms of enalapril, ramipril, cilazapril, and CS-622, on the vascular renin-angiotensin system were examined in isolated perfused rat mesenteric arteries. Vascular renin activity and angiotensin II (Ang II) released into the perfusate were determined. Infusion of N-acetyl-pepstatin (5 x 10^(-8) - 5 x 10^(-6) M) suppressed vascular renin activity and Ang II release dose dependently. Isoproterenol (10^(-6) M) induced a 135 ± 30% increase in Ang II release from the basal value. N-Acetyl-pepstatin (5 x 10^(-6) M) suppressed isoproterenol-induced Ang II release. Infusions of 5 x 10^(-6) M captopril and the diacid forms of enalapril, ramipril, cilazapril, and CS-622 by themselves had little effect on Ang II release, but concomitant infusion of isoproterenol with these angiotensin converting enzyme inhibitors significantly decreased Ang II release (71 ± 21%, 51 ± 40%, 8 ± 21%, 69 ± 24%, and 44 ± 29% increase, respectively, from the basal values). These results indicate that N-acetyl-pepstatin suppresses the vascular renin-angiotensin system. This effect may in part contribute to the hypotensive actions of renin inhibitors. Although angiotensin converting enzyme inhibitors also suppress locally generated Ang II, the mechanism and physiological significance still remain to be clarified. (Hypertension 1989;13:749-753)

Angiotensin II (Ang II), which is a final vasoactive peptide of the renin-angiotensin system (RAS), is known to be important in regulation of blood pressure and in control of water and salt metabolism. Recently, various angiotensin converting enzyme (ACE) inhibitors and renin inhibitors have been developed. The therapeutic goal of ACE inhibitors and renin inhibitors in treatment of high blood pressure is to down-regulate the RAS by reducing Ang II production.

Recent biochemical and molecular biological studies have indicated that essential components of the RAS are present in vascular tissue independently of classical circulating RAS.1-4 In previous work, we detected a small measurable amount of Ang II in the perfusate from rat mesenteric arteries and found that locally generated Ang II is released by β-adrenergic receptor activation and may regulate vascular tone directly by inducing contraction of vascular smooth muscle or indirectly by augmenting sympathetic nerve activity.5

Several investigations have shown that the hypotensive mechanism of ACE inhibitors and renin inhibitors may be mediated not only through suppression of the circulating RAS but also by suppression of the tissue RAS, including the vascular RAS. However, there is little direct evidence for the interaction between these inhibitors and the vascular RAS. In the present study, we compared the direct acute effects of a renin inhibitor, N-acetyl-pepstatin and five different ACE inhibitors, captopril and the active diacid forms of enalapril, ramipril, cilazapril, and CS-622, on the vascular RAS in isolated perfused rat mesenteric arteries.

Materials and Methods

Mesenteric Artery Preparation

Male Sprague-Dawley rats maintained on standard rat chow and weighing 250–300 g were used in this study. Mesenteric arteries were prepared for perfusion as described previously. Briefly, the rats were anesthetized with pentobarbital sodium (30 mg/kg i.p.) and treated with heparin (1,300 units/kg i.v.). The superior mesenteric artery was cannulated with PE-90 tubing and flushed with 15 ml Krebs-Ringer solution. The whole preparation was placed in a container with a water jacket maintained...
at 37°C and perfused with Krebs-Ringer solution aerated with a mixture of 5% CO₂-95% O₂ to obtain a pH of 7.4. The tissues were perfused at a constant flow rate of 4.5 ml/min with a peristaltic pump (model MP-6001, Tokyo Rika, Tokyo, Japan). Test drugs were infused with a microinfusion pump (model 501B, ATOM, Tokyo, Japan). The perfusion pressure was recorded with a pressure transducer connected to a polygraph (model RM-6000, Nihonkohden Electronic Company, Tokyo, Japan). Experiments were started after perfusion for an equilibration period of 30 minutes. Each experiment consisted of a 30-minute perfusion period to determine the baseline and two 30-minute periods of drug infusion.

**Chemicals**

A soluble acid protease inhibitor, N-acetyl-valyl-valyl-4-amino-3-amino-hydroxy-6-methylheptyl-alanyl-4-amino-3-amino-hydroxy-6-methylheptanic acid (N-acetyl-pepstatin), found by Murao and Satoi,6,7 was used as a renin inhibitor. This compound was dissolved in 0.1 M phosphate buffer (pH 7.4) and diluted with Krebs-Ringer solution before use.

The following five ACE inhibitors were kindly donated: captopril (Squibb Institute, Princeton, New Jersey) and the active diacid forms of enalapril (Merck Institute, West Point, Pennsylvania), ramipril (Hoechst Aktiengesellschaft, Frankfurt, FRG), cilazapril (F. Hoffmann-La Roche, Basel, Switzerland), and CS-622 (Sankyo Company, Tokyo, Japan). These drugs and 1-isoproterenol bitartrate (Sigma Chemical Company, St. Louis, Missouri) were dissolved in isotonic saline at concentrations of 1 mM and then diluted to appropriate concentrations with Krebs-Ringer solution (pH 7.4).

**Measurement of Immunoreactive Angiotensin II**

For determination of the small amounts of Ang II released from the vasculature, the peptide in the perfusate was concentrated in a Sep-Pak C18 cartridge (Waters Associates, Milford, Massachusetts) connected to the bottom of the perfusion system.3 The cartridge was first washed with 3 ml 100% methanol and 10 ml Krebs-Ringer solution, and then the perfusate was passed through it to trap Ang II. Cartridges were exchanged at 30-minute intervals. After perfusion, the cartridges were washed with 10 ml 0.1% trifluoroacetic acid (TFA) in water and 3 ml 10% methanol, and then peptides were eluted with 3 ml 80% methanol. The eluate was dried in a vacuum evaporator, and the residue was dissolved in 1 ml 0.1 M Tris-acetate buffer containing 2.6 mM EDTA-2Na, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% bovine serum albumin, pH 7.4, and immunoreactive Ang II was measured by direct radioimmunoassay.8 The amount of Ang II released during perfusion with drugs was calculated as the mean of two consecutive 30-minute samples.

**Measurement of Vascular Renin Activity**

After perfusion of a compound for 1 hour, the mesenteric vascular bed was promptly placed on ice, and surrounding fatty tissue was carefully removed with scissors and cotton wool. The tissue was freeze-thawed five times, suspended in 2 ml 10 mM Tris buffer containing 2 mM EDTA-2Na, 5 mM PMSF, and 5 μg/ml leupeptin, pH 7.4, and minced. The minced tissue was homogenized in a homogenizer (model NS-300, Nichion-i-rika, Osaka, Japan). The crude homogenate was centrifuged at 800g for 10 minutes at 4°C, and the supernatant was used for assay of vascular renin activity. For this, 100 μl extract was incubated with 100 μl plasma from nephrectomized rats in the presence 5 mM EDTA-2Na and 1 mM PMSF. All incubations were performed at 37°C for 12 hours. The Ang I generated was measured by radioimmunoassay. Protein concentration was determined by the method of Lowry et al.9 To exclude the contribution of nonspecific Ang I generating enzymes other than renin, immunosuppressive-specific vascular renin activity was determined with antirenin antibody as described previously.10

**Statistical Analysis**

Values are expressed as mean±SEM. Statistical significances were calculated by ANOVA followed by Dunnett’s multiple-range test for individual comparisons of means, and p values of less than 0.05 were regarded as significant.

**Results**

The mean basal perfusion pressure was 19.6±1.1 mm Hg and was not significantly changed during perfusion with any drug. The mean basal level of immunoreactive Ang II release was 44±5 pg/30 min (n=8) during the first 30-minute period, and this basal Ang II release remained steady throughout the study.

Infusion of N-acetyl-pepstatin (5×10⁻⁸-5×10⁻⁶ M) for 1 hour produced dose-dependent decreases in Ang II release (Figure 1). β-Adrenoceptor activation by isoproterenol induced a marked increase in Ang II release (p<0.01). N-Acetyl-pepstatin at 5×10⁻⁶ M also decreased this isoproterenol-induced increase in Ang II release (Figure 2). Infusion of N-acetyl-pepstatin (5×10⁻⁸-5×10⁻⁶ M) for 1 hour decreased vascular renin activity dose dependently (Figure 3). Infusion of 5×10⁻⁶ M captopril and the diacid forms of enalapril, ramipril, cilazapril, and CS-622 (n=4) for 1 hour had little effect on Ang II release (-12±9%, -12±6%, -1±7%, -9±15%, and -10±6% change, respectively, from the basal values). On the other hand, isoproterenol-induced Ang II release was significantly decreased by concomitant infusion of these ACE inhibitors, the inhibitory effect of ramipril being the greatest (Figure 4). Figure 5 shows the changes in vascular renin activity induced by ACE inhibitors. Enalapril and ramipril increased vascular renin activity.
slightly but not significantly, whereas other ACE inhibitors did not affect vascular renin activity compared with that of the control.

Discussion

The present study demonstrated that acute administration of N-acetyl-pepstatin and ACE inhibitors suppressed the vascular RAS and that the potencies of inhibition and mechanisms of action of these compounds on the vascular RAS were different. Namely, infusion of N-acetyl-pepstatin caused significant decreases in both unstimulated and isoproterenol-stimulated Ang II release, while infusion of ACE inhibitors reduced isoproterenol-induced Ang II release but had little effect on unstimulated Ang II release. Moreover, vascular renin activity was inhibited by N-acetyl-pepstatin but was not affected by ACE inhibitors.

Several novel renin inhibitors have been synthesized, and these are useful in studies on the RAS. The acute hypotensive effects of these renin inhibitors are associated with complete suppression of plasma renin and Ang II levels, but Wood et al recently reported that after chronic administration of a specific renin inhibitor and a monoclonal anti-

![Figure 1. Bar graph showing effect of N-acetyl-pepstatin (5×10⁻⁸-5×10⁻⁶ M) on angiotensin II (Ang II) release from isolated perfused rat mesenteric arteries. The Ang II response is expressed as percent change from the basal value. Columns and bars represent mean±SEM. **p<0.01 vs. control.](image1)

![Figure 2. Bar graph showing effect of N-acetyl-pepstatin (5×10⁻⁶ M) on isoproterenol (Isop, 10⁻⁶ M)-induced angiotensin II (Ang II) release from isolated perfused rat mesenteric arteries. The Ang II response is expressed as percent change from the basal value. Columns and bars represent mean±SEM. **p<0.01 vs. Isop alone.](image2)

![Figure 3. Bar graph showing effect of N-acetyl-pepstatin (5×10⁻⁸-5×10⁻⁶ M) on vascular renin activity of rat mesenteric arteries. Columns and bars represent mean±SEM. *p<0.05, **p<0.01 vs. control.](image3)

![Figure 4. Bar graph showing effects of various angiotensin converting enzyme inhibitors (5×10⁻⁶ M) on isoproterenol (Isop, 10⁻⁶ M)-induced angiotensin II (Ang II) release from isolated perfused rat mesenteric arteries. The Ang II response is expressed as percent change from the basal value. Columns and bars represent mean±SEM. *p<0.05, **p<0.01 vs. Isop alone.](image4)
The mechanism of the hypotensive effects of ACE inhibitors has been explained by their suppression of the circulating RAS. However, accumulating evidence has indicated that the consequent decrease in circulating level of plasma Ang II is probably not the sole mechanism involved. ACE inhibitors were found to lower the blood pressure not only in normal subjects but also in low-renin hypertensive human and experimental animal models. Furthermore, several groups have reported the absence of a close relation between changes in the plasma Ang II level and in the blood pressure.

Thus, the hypotensive actions of ACE inhibitors have been thought to involve suppression of the circulating RAS and effects on other vasoregulating systems including the vascular RAS, bradykinin, and prostaglandins. Unger et al. observed that treatment with ACE inhibitors caused significant decrease in ACE in tissues including the vascular wall, the kidney, the adrenal gland, the heart, and the brain. Thus, the suppression of tissue ACE by these inhibitors is probably important in the mechanism of their hypotensive actions.

In this study, ACE inhibitors did not change either vascular renin or Ang II release, and the only change observed was in the isoproterenol-induced Ang II release. Short-term administration of ACE inhibitors may not have a potent effect on the vascular RAS compared with N-acetyl-pepstatin. In isolated rat hindlegs, we found that infusion of captopril or SA 446 significantly suppressed vascular Ang II release. Because the amounts of Ang II released from mesenteric arteries were small, we were not able to detect significant change in Ang II release in the unstimulated condition.

A β-adrenoceptor agonist is well known to stimulate the release of renin from juxtaglomerular cells with consequent increase in the plasma Ang II level. On the other hand, our previous studies showed that isoproterenol caused considerable increase in Ang II release but not in renin release from the vasculature into the perfusate. The mechanism of this isoproterenol-induced Ang II release from the vascular wall is unknown. One possible explanation is as follows: the increase in cyclic adenosine 5′-monophosphate (cAMP) caused by stimulation of adenylate cyclase by isoproterenol may activate vascular renin and increase the intracellular concentration of Ang I. This Ang I will then be secreted into the extracellular space and converted to Ang II by ACE, which is present on the surface of endothelium, or will be converted to Ang II by intracellular ACE and then secreted as Ang II into the extracellular space. Another possible explanation is that activation of ACE activity by isoproterenol may result in increase in Ang II release. cAMP related agents like dibutylcAMP, isoproterenol, and theophylline stimulate the production of ACE in cultured human vascular endothelial cells. Because ACE is a final key enzyme in the vascular RAS, the suppression of Ang II release by ACE inhibitors may become more prominent on treatment with isoproterenol.

Many ACE inhibitors have been developed for the treatment of hypertension, and their potencies and durations of inhibition of the circulating RAS have been examined and compared in various experimental models in vivo and in vitro. Ramipril is reported to have more effect than captopril or enalapril on rat plasma ACE in vitro. Of the five different ACE inhibitors used in this study, ramipril had the most potent inhibitory action on isoproterenol-induced Ang II release, suggesting that on an equimolecular base, ramipril has greater effect than the other ACE inhibitors on vascular ACE. In addition to differences in their potencies of...
inhibition of classical ACE, differences in other characteristics like lipid solubility may contribute to the differences in inhibition of tissue ACE by these compounds. 25

In conclusion, the present results showed that the addition of N-acetyl-pepstatin to the perfusate of isolated perfused rat mesenteric arteries directly suppressed Ang II release, suggesting the inhibitory effect of renin inhibitors on the vascular RAS. On the other hand, administration of ACE inhibitors decreased isoproterenol-induced Ang II release but had little effect on unstimulated Ang II release. Further studies are needed to clarify the role of ACE inhibitors in the suppression of the vascular RAS.

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Key Words: angiotensin II • renin inhibitors • captopril • converting enzyme inhibitors • renin-angiotensin system • enalapril • rat mesenteric arteries
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Hypertension. 1989;13:749-753
doi: 10.1161/01.HYP.13.6.749

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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