Significance of Vascular Renin for Local Generation of Angiotensins

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The effects of specific renin inhibitors, angiotensin converting enzyme inhibitors, indomethacin, and prostaglandin I\(_2\) analogue on the release of angiotensins from isolated and Krebs-Ringer-perfused rabbit mesenteric arteries were examined. Three different renin inhibitors suppressed release of angiotensins in dose-dependent manners. At the highest concentration (10\(^{-7}\) M), the inhibitors EMD 52,620, EMD 54,388, and EMD 52,742 induced 46\%, 52\%, and 48\% decreases, respectively, in the basal rate of immunoreactive angiotensin II release. These results provide clear evidence that released angiotensins are produced by the specific action of vascular renin and that the renin inhibitors suppress the vascular renin-angiotensin system as well as the circulating renin-angiotensin system and appear to provide a useful mode for the treatment of hypertension. Nonsulfhydryl angiotensin converting enzyme inhibitors cilazapril and delapril were more effective than captopril, and ramipril was equipotent to captopril, suggesting that the effectiveness of angiotensin converting enzyme inhibitors on the vascular renin-angiotensin system cannot be explained only by its inhibitory effect on angiotensin converting enzyme. Indomethacin, which was reported to suppress angiotensin II release from rat hind limbs, elicited a dose-dependent increase of angiotensin release from rabbit mesenteric arteries. These results suggest that a difference exists in the regulatory mechanisms in the release of angiotensins from diverse vascular beds. (Hypertension 1991;17:270–277)

The renin-angiotensin system plays an important role in blood pressure regulation as well as in fluid and electrolyte balance. Angiotensin converting enzyme (ACE) inhibitors effectively block the renin-angiotensin system and have become successful therapeutic agents in the treatment of hypertension and congestive heart failure.\(^1\)-\(^3\) However, the effect of ACE inhibitors is not limited to the renin-angiotensin system; they also affect the kallikrein-kinin system\(^4\) and the prostaglandin system.\(^5\) Thus, specific inhibition of the renin-angiotensin system at its initial step has become an important strategy in treating various cardiovascular disorders. Potent renin inhibitors that function as substrate analogues have recently been developed.\(^6\)-\(^8\)

The circulating renin and angiotensins are not the only renin-angiotensin system. The presence of essential components of the renin-angiotensin system in vascular tissue has been reported,\(^9\)-\(^19\) and several investigators have provided evidence that the vascular renin-angiotensin system may be related to the pathogenesis of hypertension.\(^20\)-\(^22\) Recently, we reported direct evidence of local generation and subsequent secretion of angiotensin II (Ang II) by peripheral vascular tissue.\(^24\)-\(^27\) However, there is no definitive evidence that specific renin in vascular tissue is responsible for the generation of angiotensins. Furthermore, we demonstrated that Ang II release from isolated perfused rat mesenteric arteries was mediated by \(\beta\)-adrenergic receptor activation\(^24\) and that the Ang II release from rat hind limbs was modulated by prostaglandins.\(^27\) These data suggest that the mechanisms or stimuli responsible for the regulation of vascular Ang II release differ among diverse vascular beds.

The main purpose of the present study was to elucidate the effects of new specific and potent renin inhibitors on Ang II release from isolated rabbit mesenteric arteries in comparison with ACE inhibitors. To further evaluate the differences in the regulation of vascular Ang II release among diverse vascular beds, we examined the effect of indomethacin and the stable analogue of prostaglandin I\(_2\) (PGI\(_2\)) on Ang II release.
Methods

Animals

Male New Zealand White rabbits weighing 3.5–3.8 kg were used. The rabbits were maintained on a high fiber diet (Ralston-Purina, St. Louis) containing 0.32% sodium and 1.4% potassium, and they were allowed ad libitum access to tap water.

Mesenteric Artery Preparation

Rabbits were anesthetized with pentobarbital sodium (24 mg/kg i.v.) and administered heparin (1,000 units/kg i.v.). The superior mesenteric artery was cannulated with PE-90 tubing (0.86 mm i.d.), flushed with 180 ml of Krebs-Ringer solution to eliminate blood, and quickly removed by cutting around the intestinal borders of the mesentery. The entire preparation was placed into a water-jacketed container maintained at 37°C and perfused with Krebs-Ringer solution of the following composition (mM): NaCl 120, KCl 5.0, CaCl₂ 2.4, NaH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, EDTA disodium 0.027, and D- (+)-glucose 11.0. This solution was maintained at 37°C and aerated with a 5% CO₂-95% O₂ mixture to obtain pH 7.4. The tissues were perfused at a constant flow rate of 4.5 ml/min with a roller pump (Polysaltic Pump, Buchler, Fort Lee, N.J.). The perfusion pressure was monitored by a pressure transducer (model P231D, Statham, Oxnard, Calif.) connected to a polygraph (model 7D, Grass Instrument Co., Quincy, Mass.). During perfusion of the mesenteric artery, the pressure was approximately 18–23 mm Hg. To avoid the contamination of plasma angiotensins, the preparations were thoroughly washed with the Krebs-Ringer solution for at least 30 minutes before the experiments were started.

Determination of Angiotensins

Angiotensins that were released from isolated perfused mesenteric arteries were trapped by Sep-Pak C-18 cartridges (Waters Associates, Milford, Mass.) as described previously. The cartridge was connected to the water-jacketed container to allow perfusate to pass through the cartridge. The cartridges were prewashed with 3 ml methanol and then with 10 ml Krebs-Ringer solution just before use and were exchanged at a 30-minute interval during which 135 ml perfusate passed through the cartridge. After the cartridge was washed with 10 ml of 0.1% trifluoroacetic acid (TFA) in distilled water, trapped peptides were eluted with 3 ml methanol-H₂O-TFA (80:19, 9:0.1, vol:vol). The eluate was dried in a vacuum centrifuge (Speed Vac, Savant, Hicksville, N.Y.); resultant residues were dissolved in 0.1 M Tris-acetate buffer (pH 7.4) containing 2 mM EDTA disodium, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5% bovine serum albumin and subjected to radioimmunoassay. The Ang II antiserum showed less than 1% cross-reactivity with Ang I but 100% cross-reactivity with Ang III (angiotensin-(2–8) heptapeptide, angiotensin-(3–8) hexapeptide, and angiotensin-(4–8) pentapeptide). The detectability of the immunoreactive (ir) Ang I and irAng II was 1 pg per tube.

The identities of the angiotensinlike materials in the perfusate were examined using high-performance liquid chromatography as previously described. After extraction of the perfusate by the Sep-Pak C-18 cartridges, the eluted and dried residues were dissolved in 250 μl of 0.1% TFA and chromatographed on a Vydac C-18 reverse-phase column (0.46×25 cm, The Separation Group, Hesperia, Calif.). The elution was performed with an exponential gradient of methanol from 28% to 80% in 10 mM Na acetate buffer (pH 5.6) during 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, Calif.) were used for the calibration of the column.

The recovery of synthetic Ang I and Ang II (50 pg each), added to the perfusate and then collected on the Sep-Pak cartridge, was 96.5±3.9% (mean±SD; n=5) for Ang I and 95.5±6.2% (mean±SD; n=7) for Ang II.

Treatment With Renin Inhibitor

The renin inhibitor (EMD 52,620; 0.1 mg/kg body wt/day) or vehicle was infused intravenously through an external jugular vein using an osmotic minipump (model 2 ML 1, Alza Corp., Palo Alto, Calif.). After 1 week of the treatment, the rabbit mesenteric artery preparation was made as described before.

Drugs

Tripeptide renin inhibitors EMD 54,338, EMD 52,742, and EMD 52,620, which have high affinity for rabbit renin, were synthesized. Structures of the inhibitors are given in Figure 1. The IC₅₀ values of these inhibitors against renin activity in rabbit plasma were 4×10⁻¹⁰ M for EMD 52,620, 4.9×10⁻¹⁰ M for EMD 52,742, and 8×10⁻¹⁰ M for EMD 54,338. They have no inhibitory actions to cathepsin D at 10⁻⁷ M.

The drugs kindly supplied by manufacturers were captopril (Squibb Institute, Princeton, N.J.), cilazapril (Hoffman-LaRoche, Inc., Nutley, N.J.), ramipril (Hoechst Aktiengesellschaft, Frankfurt, FRG), delapril (Takeda Chemical Industries, Osaka, Japan), and stable PGI₂ analogue OP-41,483 (Ono Pharmaceutical Co., Osaka). Indomethacin, 1-isoproterenol bitartrate, and dl-propranolol were purchased from Sigma Chemical Co., St. Louis. These drugs were dissolved in dimethyl sulfoxide at a concentration of 10⁻² M and diluted to appropriate concentrations with the Krebs-Ringer solution just before use.

Results

Release of Angiotensins From Isolated Perfused Rabbit Mesenteric Arteries

The time course of the spontaneous release of irAng I and irAng II from isolated rabbit mesenteric
arteries was examined for as long as 4 hours. To equilibrate the preparations and eliminate the contamination of plasma angiotensins, the tissues were exhaustively perfused with the Krebs-Ringer solution for 30 minutes before sample collections were started. As shown in Figure 2, the rates of release of angiotensins were stable for as long as 3 hours but tended to decrease toward the end of the 4-hour period. For this reason, all experiments were performed for 2 hours.

To identify the angiotensinlike materials, the perfusate was purified by high-performance liquid chroma-

**Figure 1.** Structures of rabbit renin inhibitors. EMD 54,388: N-[(3S)-4-(N-(N-tert-Butylxycarbonyl-1-phenylalanyl)-L-histidineamido)-5-cyclohexyl-3-hydroxypentanoyl]-L-leucine-4-[(II-tetrazole-5-yl)-anilide, sodium salt, tetrahydrate. EMD 52,742: 2-Amino-5,6-dimethyl-3-[(3S,4S)-4-((2S)-N-tert-butyloxycarbonylphenylalanyl)-3-(3-pyridyl)-alanineamido]-5-cyclohexyl-3-hydroxypentanoyl]-isoleucineamidomethyl]-pyrazine. EMD 52,620: N-[(3S,4S)-4-(N-tert-Butylxycarbonylphenylalanyl)-histidineamido]-5-cyclohexyl-3-hydroxypentanoyl]-leucine-4-(amidosulfonfyl)-anilide.

**Figure 2.** Bar graphs of time courses of immunoreactive (ir) angiotensin (Ang) I (left panel) and irAng II (right panel) release from isolated rabbit mesenteric arteries. Each column shows mean value of release of irANG I or irAng II in 30-minute periods. Experiments were performed with five preparations.
Figure 3. Plot of high-performance liquid chromatography elution profile of perfusate of isolated perfused rabbit mesenteric arteries showing typical results of extracting angiotensin (Ang) I, Ang II, and Ang III from perfusate, which was passed through a Sep-Pak C-18 cartridge. Fractions were subjected to radioimmunoassay of Ang I and Ang II. Elution time of Ang II was 18.1 ± 0.1 minutes (n=5).

Effects of Renin Inhibitors on Release of Angioteins

The effects of three different renin inhibitors on the release of irAng I and irAng II from isolated perfused rabbit mesenteric arteries are shown in Figure 4. Renin inhibitor infusion (10^{-10} to 10^{-7} M) caused significant decreases in irAng I and irAng II release in dose-dependent manners. At the highest concentration used (10^{-7} M), EMD 52,620, EMD 54,388, and EMD 52,742 induced 46%, 52%, and 48% decreases in the basal rates of irAng II release, respectively.

Pre-treatment with EMD 52,620 for 1 week decreased spontaneous release of irAng I to 65.4±3.4 pg/30 min compared with a control level of 82.4±3.5 pg/30 min (p<0.05) and of irAng II to 15.2±0.7 pg/30 min compared with a control level of 21.4±0.5 pg/30 min (p<0.001) (Figure 5). Renin inhibitor perfusion (10^{-7} M) caused further decreases in irAng I release to 45.3±2.0 pg/30 min from a basal level of 65.4±3.4 pg/30 min (p<0.01) and in irAng II release to 11.4±0.4 pg/30 min from a basal level of 15.2±0.7 pg/30 min (p<0.01).

Effects of Angiotensin Converting Enzyme Inhibitors on Release of Angiotensins

The effects of four ACE inhibitors on the release of angiotensins from isolated rabbit mesenteric arteries are shown in Figure 6. Ramipril, cilazapril, delapril, and captopril used at a level of 10^{-7} M caused 16%, 38%, 38%, and 19% decreases in basal rates of irAng II release, respectively. On the other hand, cilazapril, delapril, and captopril induced 15%, 31%, and 14% increases in the basal rates of irAng I release, respectively.

Effects of Indomethacin, OP-41,483, Isoproterenol, and Propranolol on Release of Angiotensins

The effects of indomethacin on the release of irAng I and irAng II are shown in Figure 7. This cyclooxygenase inhibitor (10^{-8} to 10^{-6} M) caused significant increases of irAng I and irAng II release in dose-dependent manners. Similar results were obtained with meclofenamate (data not shown). However, the stable prostacyclin analogue OP-41,483 (10^{-5} M) did not cause significant changes in the release of angiotensin.
(Figure 8). Isoproterenol (10^{-6} to 10^{-5} M) and propranolol (10^{-6} M) showed no effect.

Discussion

A series of recent studies indicated that rat mesenteric and hind limb vascular beds perfused with Krebs-Ringer buffer that is free from any components of renin or angiotensinogen release Ang II into the perfusate. Although the amount of Ang II secreted from the vascular beds is rather small, it was reported to exert a significant effect in regulating the vascular tone under electrical stimulation, presumably because it is produced in the immediate vicinity of vascular receptors. In view of the presence of renin and angiotensinogen in vascular tissues, it has been tacitly assumed that renin in these tissues is responsible for the generation of angiotensins. However, no definitive evidence for the action of renin was available. Although acetylpepstatin added to perfusion mixture suppressed Ang II production significantly, pepstatin is not a specific inhibitor of renin. The use of specific and potent inhibitors of rabbit renin and marked suppression of the release of Ang II from the perfused rabbit mesenteric artery clearly established that at least the suppressed portion of Ang II is produced by the specific action of renin in the vascular tissue.

In recent years, several renin inhibitors whose structures resemble renin substrate have been synthesized and reported to induce plasma renin inhibition and concomitant blood pressure reduction in salt-depleted animals. More recently, Kleinert et al reported that renin inhibitor could lower blood pressure not only in salt-depleted monkeys but also in normal monkeys and even in anephric monkeys in which plasma renin activity was maximally suppressed. These observations suggested that the renin inhibitor might suppress the extrarenal renin-angio-
tensin system, including the vascular renin-angiotensin system as well as the circulating renin-angiotensin system. In the present study, three different renin inhibitors were shown to suppress the releases of irAng I and irAng II from isolated rabbit mesenteric arteries in dose-dependent manners, which provides clear evidence that the renin inhibitor suppresses the vascular renin-angiotensin system and suggests that the antihypertensive effect of the renin inhibitor may be, at least in part, a result of the inhibition of local Ang II formation.

Although the question is still debated as to whether the "vascular renin" is synthesized in the vascular tissue or taken up from plasma by the vascular wall, several reports have accumulated in favor of the local production of renin and subsequent generation of angiotensins in vascular tissues. In the present study, we demonstrated continuous and steady release of Ang I and Ang II from isolated rabbit mesenteric arteries that were perfused in the absence of angiotensinogen, and the angiotensin release was inhibited by renin inhibitors with small molecular sizes. These results provide additional evidence for local (or intracellular) synthesis of renin and angiotensins. The present observation that renin inhibitors (with IC₅₀ ranging from 4x10⁻¹⁰ to 8x10⁻¹⁰ M) used at a high concentration of 10⁻⁷ M failed to completely suppress the release of Ang II may suggest the possibility that nonrenin protease (such as cathepsins D or G or tonin), which can hydrolyze angiotensinogen directly to Ang II, are in part responsible for generating Ang II in vascular tissues.

An alternative possibility is that renin in the vascular tissue is present in more than one compartment and that only renin in a more accessible compartment such as on the outer surfaces of vascular cell membranes may be inhibited. It is interesting to note that the larger inhibitor acetylpepstatin, which appears to be less discriminating than the present inhibitors, was also effective in partially inhibiting Ang II release from perfused rat mesenteric artery.

ACE inhibitors are known to lower blood pressure in many hypertensive patients and in various types of animal models of hypertension in which plasma renin activity is normal or even low. However, the mechanism of its antihypertensive effect has not been completely clarified because of its relatively nonspecific nature. The present observations confirm our previous findings that ACE inhibitors suppressed

**Figure 7.** Bar graphs of effects of indomethacin on immunoreactive (ir) angiotensin (Ang) I (left panel) and irAng II (right panel) release from isolated rabbit mesenteric arteries (n=5). *p<0.05, **p<0.01 compared with basal values.

**Figure 8.** Bar graphs of effects of OP-41,483 on immunoreactive (ir) angiotensin (Ang) I (left panel) and irAng II (right panel) release from isolated rabbit mesenteric arteries (n=5).
irAng II release and increased irAng I release from isolated vascular beds, which may explain one of the antihypertensive mechanisms of ACE inhibitors.

Converting enzyme inhibitors are highly effective drugs in the treatment of certain types of essential hypertension.37–39 In the present study, we examined the effects of three nonsulfhydryl ACE inhibitors and of captopril on the release of angiotensins from isolated rabbit mesenteric arteries. Cilazapril and delapril were more effective than captopril; however, ramipril, which was reported to be more effective than captopril on rat plasma ACE,38 was equipotent to captopril, suggesting that the effectiveness of ACE inhibitors on the vascular renin-angiotensin system is determined not only by its inhibitory effect on circulating ACE but also by other characteristics, such as membrane permeability.

We previously reported that prostaglandin synthesis inhibitors suppressed Ang II release from rat hind limbs.27 However, indomethacin caused increase of the release of angiotensins from rabbit mesenteric arteries. The mechanism of regulation of angiotensin release by prostaglandins is not clear. Prostaglandins participate in the local control of renin release from the kidney,40 and inhibition of prostaglandin synthesis decreased renin release by the kidney.41 Thus, it is possible that indomethacin suppresses renin release by the vascular tissue and increases renin content in vasculature, which consequently induces the increase of local Ang II formation. Also, it is not clear which prostaglandins are responsible for the regulation of angiotensin release from vasculature. PG\(_1\) is the major component of prostaglandins in blood vessels; however, failure to stimulate Ang II release with a stable PG\(_1\) analogue eliminates the possibility that PG\(_1\) is an effective candidate for such a role. These results suggest that different mechanisms are responsible for the regulation of release of angiotensins in diverse vascular beds and different species.

In conclusion, specific renin inhibitors suppress the local formation of Ang II; this inhibitory capacity may also contribute to its hypotensive action. However, physiological roles of the vascular renin-angiotensin system are not clear, and the mechanisms of its regulation appear to require systemic studies on different species and different vascular beds.

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References

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