Vascular Renin in the Guinea Pig
Suppression by the Renin Inhibitor Remikiren

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Abstract Angiotensin I and II are generated by the vascular wall. Whether this generation depends on renin or on other enzymes is debated. We tested the hypothesis that remikiren, a highly specific inhibitor of human and guinea pig renin, may inhibit the vascular renin-angiotensin system. Isolated hindquarters from guinea pigs were perfused with an artificial medium, and angiotensin I and II release was measured by high-performance liquid chromatography and radioimmunoassay. Guinea pig hindquarters released angiotensin I (23.8±5.6 fmol/30 min; n=13) and angiotensin II (95.2±19 fmol/30 min; n=13) spontaneously. Inhibition of the angiotensin I–converting enzyme by captopril (10 nmol/mL) suppressed angiotensin II by 85% and increased angiotensin I by 352% (n=5, P<.05). Infusion of remikiren (1.6 nmol/mL) in addition to captopril decreased angiotensin I release by 68% (P<.05 versus captopril alone, n=5 each). We conclude that renin generates angiotensin I in an isolated guinea pig resistance vessel bed. Our study demonstrates that renin rather than nonrenin enzymes is responsible for the major part of vascular angiotensin formation. (Hypertension. 1994;23[part 2]:861-864.)

Key Words • angiotensins • kininase II • vascular resistance • renin

The notion that angiotensin I and angiotensin II (Ang I and Ang II, respectively) are formed in isolated, perfused resistance vessel beds is now supported by numerous data from studies in isolated perfused organs. However, nonrenin enzymes could contribute to the local formation of Ang I and Ang II in isolated organs perfused without blood, ie, in the absence of plasma-derived protease inhibitors. We have recently shown that the natural renin substrate angiotensinogen is cleaved in an isolated, perfused resistance vessel bed. These data provide indirect evidence for the presence of renin in resistance vessels, since other enzymes would not form Ang I from angiotensinogen at pH greater than 7. To demonstrate the presence of vascular renin directly, inhibition of vascular angiotensin formation by specific renin inhibitors is necessary. Saito et al have shown that vascular angiotensin formation in rat mesentery can be suppressed by a derivative of pepstatin that inhibits rat renin. However, concentrations of pepstatin that inhibit rat renin also inhibit nonspecific proteases such as cathepsin D. More specific inhibitors for rat renin are not available.

Remikiren (RO 42-5892) is a highly specific renin inhibitor in primates that is 3500 times more potent against renin than against cathepsin D. Furthermore, several in vivo studies suggest that remikiren inhibits renin at tissue sites, eg, vascular renin. Therefore, remikiren appears to be well suited to investigate vascular renin. Remikiren is not active against rat renin but is a specific inhibitor of guinea pig renin.

To test the hypothesis that remikiren inhibits vascular renin, we adapted our rat hindquarter perfusion model 1,2,3 for guinea pigs. We investigated whether perfused guinea pig hindquarters release Ang I and Ang II and tested the effects of Ang I–converting enzyme (ACE) inhibition and remikiren. The renin inhibitor was given in the presence of an ACE inhibitor to increase Ang I formation and hence to sensitize the vasculature to the inhibitory effects of remikiren.

Methods

Animals Male Dunkin-Hartley guinea pigs (weight, 300 to 400 g) and male Sprague-Dawley rats (weight, 300 to 350 g) were purchased from the Zentralinstitut für Versuchstierzucht, Hanover, Germany. Animals were kept in a room at 24±2°C. Guinea pigs were anesthetized with ketamine hydrochloride (Parke-Davis), midazolam hydrochloride (Hoffmann-La-Roche), and buprenorphine hydrochloride (Boehringer Mannheim) intraperitoneally; rats were anesthetized with thiobarbbitone (Byk-Gulden) intraperitoneally. All procedures performed in animals were done in accordance with the guidelines of the American Physiological Society and were approved by the local government (Regierung von Mittelfranken, AZ 211-2531.3.A-8/92 and AZ 211-2531.3.A-7/92).

Hindquarter Preparation and Perfusion The preparation and perfusion of the isolated rat hindquarter were performed as previously described. The same procedure was adapted for guinea pigs. After evisceration and preparation of the abdominal aorta and vena cava, an injection of sodium heparin (500 U IV) was given. Five minutes later, the abdominal aorta and the inferior vena cava were cannulated, and the perfusion was begun immediately. Approximately 20% of eviscerated guinea pigs could not be used for perfusion experiments because of cannulation problems (eg, two small instead of one large abdominal vena cava). However, in the remaining guinea pigs perfusion could be started within 30 seconds after ligation of the aorta, as in rats.

The hindquarters were perfused in a nonrecirculating system with modified Tyrode's solution containing 2 g/L glucose and 40 g/L of the artificial colloid Ficoll 70 (Pharmacia). The perfusate was gassed with O2/CO2 (95%/5%), adjusted to pH 7.4, and maintained at 38°C. The hindquarter perfusion was
performed at a constant flow rate (10 mL/min) using a two-channel peristaltic pump (Harvard Apparatus). All experimental substances were infused into the perfusion system at a rate of 30 μL/min by means of a syringe pump (model 22, Harvard Apparatus). All experimental protocols were begun after an initial perfusion period of 30 minutes (washout period). Samples for measurement of Ang I and II were collected from the venous cannula.

Experimental Protocols

To investigate whether angiotensin release from guinea pig hindquarters was comparable to that from rat hindquarters, perfusate was collected for 30 minutes from perfused hindquarters of 16 rats and 13 guinea pigs of comparable body weight. In addition, perfusate was also collected from eight perfusion channels without hindquarter ("blank" channel) to exclude a contamination of the perfusion system. To test whether angiotensin release from guinea pig hind limb was constant over time, perfusate was collected for three consecutive 30-minute periods from 5 guinea pig hindquarters. To evaluate the effects of ACE inhibition on angiotensin release, perfusate was collected for two consecutive 30-minute periods from 10 guinea pig hindquarter preparations. During the second sampling period, captopril (10 nmol/mL) was infused to 5 preparations, while the remaining 5 hindquarters received vehicle (0.9% NaCl). The renin inhibitor remikiren (1 μg, mL=1.6 nmol/mL) was given in addition to captopril during the second 30-minute sampling period in 5 additional hindquarter preparations.

Measurement of Angiotensin I and II

ODS C18 cartridges for peptide extraction were connected on-line to the vena cava cannula. The on-line connection was "open" to exclude an increase of venous pressure, and the effluent was passed through cartridges by means of a second pump adjusted to the flow rate of the perfusion pump. Peptides were eluted from cartridges as described previously. Eluates from cartridges were lyophilized, and the dry residues were analyzed by high-performance liquid chromatography (HPLC) and subsequent radioimmunoassay (RIA) for Ang I and II as described in detail elsewhere. For reverse-phase HPLC (gradient pump 2249, Pharmacia LKB), samples were loaded onto a 7-μm Nucleosil C18 column (Macherey Nagel & Co), guarded by an ODS C18 guard column (Guard-Pak, Waters Assoc), and eluted using a methanol gradient (35% for 10 minutes, then increasing to 80% during a period of 35 minutes) in a 10-mmol/L ammonium acetate buffer, pH 5.4, at a flow rate of 1 mL/min and a temperature of 40°C. Peptides in HPLC fractions were quantified by RIA for Ang I and II as described in detail elsewhere. For reverse-phase HPLC (gradient pump 2249, Pharmacia LKB), samples were loaded onto a 7-μm Nucleosil C18 column (Macherey Nagel & Co), guarded by an ODS C18 guard column (Guard-Pak, Waters Assoc), and eluted using a methanol gradient (35% for 10 minutes, then increasing to 80% during a period of 35 minutes) in a 10-mmol/L ammonium acetate buffer, pH 5.4, at a flow rate of 1 mL/min and a temperature of 40°C. Peptides in HPLC fractions were quantified by RIA for Ang I and II (sensitivity, 1 pg per tube). The cross-reactivity of the Ang I antibody was 0.05% for Ang II. The cross-reactivity of the Ang II antibody was 100% for both the Ang-(2-8) heptapeptide (Ang III) and the Ang-(3-8) hexapeptide. Recovery from Sep-Pak cartridges and HPLC was 71% for Ang I and 92% for Ang II; values shown were corrected for recovery.

Statistical Analysis

All data are expressed as mean±SEM. The significance of differences was assessed by ANOVA and subsequent Newman-Keuls test. Statistical analysis was performed using a CSS statistical software package (StatSoft Inc). A value of P<.05 was considered significant.

Results

Ang I (23.8±5.6 fmol/30 min) and Ang II (95.2±12 fmol/30 min, n=13 each) were released from isolated, perfused guinea pig hindquarters. Perfused rat hindquarters released somewhat higher amounts of Ang I (76.2±1.9 fmol/30 min) and Ang II (145.6±7.7 fmol/30 min; n=16 each). No Ang I or II could be detected in blank perfusion channels without hindquarter. Release of Ang I and II from guinea pig hindquarters remained constant during three consecutive 30-minute sampling periods (Fig 1). The HPLC elution times for Ang I and II in guinea pig samples were identical to the elution times of synthetic Ang I and II (Fig 2). Ang II-immunoreactive peptides were not detectable in the absence (top) or presence (bottom) of captopril. Open bars indicate angiotensin I (ANG I) immunoreactivity; filled bars, angiotensin II (ANG II) immunoreactivity. The line indicates the methanol gradient. The immunoreactivity peaks of angiotensin I and II coeluted with the respective synthetic peptides. Note that angiotensin II was still detectable in the presence of captopril.
peptides other than Ang II were not detected (Fig 2). The identification of Ang I peaks by HPLC in guinea pig samples was more difficult than in rat perfusate samples because of a higher background immunoreactivity (Fig 2). However, when Ang I release was increased by ACE inhibition, sharp Ang I peaks were detected (Fig 2). In some but not all samples from guinea pig hindquarters, Ang I-immunoreactive material eluting 1 to 2 minutes after Ang I was detected (data not shown). The amount of this material was not sufficient to allow further characterization.

Compared with the vehicle control, ACE inhibition with captopril decreased Ang II release significantly by 85.2% (Figs 2 and 3). Ang II release was still detectable (Fig 2). Captopril induced a significant, 3.5-fold increase of Ang I release (Figs 2 and 3). The renin inhibitor remikiren combined with captopril decreased Ang I release significantly by 67.8% compared with captopril alone; there was no further reduction of Ang II release (Fig 3).

**Discussion**

This study demonstrates the formation of Ang I and II in an isolated, skeletal muscle resistance vessel bed of guinea pigs. The amounts of released Ang I and II were comparable to angiotensin release from perfused rat hindquarters. Vascular angiotensin formation was sensitive to ACE inhibition. Remikiren, a highly specific inhibitor of human and guinea pig renin, suppressed local Ang I formation. These data show that renin is active in the guinea pig resistance vessel bed.

Results of measurements of Ang I and II in arterial and venous plasma in vivo have provided indirect evidence for vascular angiotensin production in several species, including humans, pigs, sheep, rabbits, and rats. Angiotensin release from isolated vessels and the presence of renin mRNA in the blood vessel wall have been demonstrated in human tissue. However, the bulk of direct evidence for vascular angiotensin production has been derived from studies in rats. Our results show that there is a vascular production of Ang I and II in another species, the guinea pig. The suppression of Ang I release by remikiren provides direct evidence for vascular renin in guinea pig resistance vessels. The specificity of remikiren for guinea pig renin was not tested with cathepsin D or other proteases from the same species. However, remikiren is 3500-fold more potent against guinea pig renin than against bovine cathepsin D. The observation that bilateral nephrectomy blunts the hypertensive effect of remikiren in guinea pigs is also consistent with the notion that this inhibitor is specific for renin.

The local formation of Ang I was suppressed but not completely abolished by remikiren. The remaining Ang I generation could be due to an Ang I-forming enzyme other than renin. Alternatively, the inhibition of vascular renin by remikiren may have been incomplete, since we used a rather moderate perfusion dose of remikiren (a total of 480 nmol in 300 mL of perfusate given over 30 minutes). Generating a dose-response curve for remikiren in a preparation like ours is probably not feasible, since remikiren rapidly accumulates in guinea pig vascular tissue (W.F., unpublished data, 1993). Perfusion with higher doses might lead to local remikiren concentrations, which would inhibit nonrenin enzymes. In contrast, this problem could not occur with the dose used in our study, even if all of the perfused remikiren were trapped in the hindquarter.

Our results do not allow us to determine whether renin and angiotensinogen in guinea pig vessels are synthesized locally or taken up from plasma. In rats, synthesis of angiotensinogen within the blood vessel wall has been demonstrated. In contrast, the question of how much of vascular renin is synthesized locally or taken up from the bloodstream is still a matter of debate. Kidney-derived renin as a source of vascular renin activity was not ruled out in our study. Our data provide evidence for the physiological interaction of renin and angiotensinogen within the vascular system to form substantial amounts of Ang I, regardless of whether renin and angiotensinogen may be produced locally or derived from plasma.

Melli and Turker have estimated rates of Ang I conversion and angiotensin degradation in guinea pig hindquarter vasculature that closely resemble the respective rates measured in perfused rat hindquarters by us and others. The local formation of Ang II in guinea pig vessels observed in the present study was suppressed but not completely abolished by captopril, in contrast to previous studies in rats. Since we did not use higher doses of captopril, we cannot exclude the possibility that other concentrations of the drug are required in guinea pigs than in rats. The part of Ang II formation that was resistant to ACE inhibition could also be due to cleavage of Ang I by a chymase-like enzyme, which is absent in rats, or to nonrenin enzymes that cleave Ang II from angiotensinogen. We are not aware of any report addressing these enzymes in the guinea pig. However, the similarity of the hemodynamic effects of renin inhibition, ACE inhibition, and Ang II receptor blockade in guinea pigs argues against a major role for nonrenin or chymase-like enzymes.

Previous studies suggested that remikiren may exert its effects on blood pressure by inhibiting renin at tissue sites, eg, the vascular wall. In normotensive primates, the effects on blood pressure are much more prolonged than those on plasma renin. Furthermore, in cyclosporine-induced hypertensive monkeys, remikiren but not renin-neutralizing antibodies reduced blood pressure, even though both interventions led to dramatic reductions in plasma Ang II. Our results support these
previous studies by providing direct evidence for inhibition of vascular renin by remikiren.

In summary, our data show that a functional renin-angiotensin system is active in the blood vessel wall of guinea pigs. Since vascular angiotensin formation can be suppressed by remikiren, our results support the notion that renin rather than nonrenin angiotensin-forming enzymes mediates vascular angiotensin production. In light of the results of previous in vivo studies, remikiren may be a valuable tool to investigate the relative contribution of tissue renin-angiotensin systems in models of hypertension.

*Note added in proof.* The present data and our previous results clearly invalidate recently published assumptions that vascular angiotensin formation in isolated perfused tissues may be due to lysosomal proteases rather than renin.\(^\text{25}\)

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**References**

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