Renin vs. Angiotensin-Converting Enzyme Inhibition in the Rat: Consequences for Plasma and Renal Tissue Angiotensin

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Accepted for publication July 23, 1997

ABSTRACT

To compare the effects of a potent rat renin inhibitor peptide (RIP) and angiotensin-converting enzyme (ACE) inhibitor on the intrarenal and plasma renin-angiotensin systems, anesthetized Sprague-Dawley rats were treated with an infusion of vehicle, ramipril or graded doses of the rat RIP (acetyl-His-Pro-Phe-Val-statine-Leu-he-NH2) for 30 min. Kidney and plasma samples were processed rapidly, and angiotensin peptides were separated by high-pressure liquid chromatography before measurement by a double-antibody radioimmunoassay. Blood pressure fell identically, by ~15 mm Hg, after either the RIP or ACE inhibitor. Plasma Ang II was 83 ± 20 fmol/ml in vehicle-treated rats and fell to 28 ± 3 fmol/ml with ramipril (10 mg/kg), the dose-response zenith. Plasma Ang II was significantly lower, 9 ± 2 fmol/ml, with the highest RIP dose used. Control renal tissue Ang II was 183 ± 18 fmol/g, fell with ramipril to 56 ± 6 and then fell to a similar level (47 ± 10 fmol/g) after RIP. Ang I/Ang II ratios indicated the expected sharp drop in Ang I conversion after ramipril in plasma and tissue. RIP did not influence conversion rate in plasma but was associated with an unanticipated fall in Ang I conversion in renal tissue, perhaps reflecting local aspartyl protease inhibition, which contributes to normal Ang II formation. Also unanticipated was a rise in tissue Ang I concentration during RIP administration. Renin inhibition is more effective than ACE inhibition in blocking systemic Ang II formation, supporting studies suggesting that quantitatively important non-ACE-dependent pathways participate in Ang II formation.

Pharmacological interruption of the renin system has played a crucial role in the evolution of our understanding of its contribution to both normal processes and disease pathogenesis (Haber, 1976). Because the interaction between renin and its substrate is rate limiting, blockade at this step would be expected to be especially effective in blocking the classical cascade (Reudelhuber et al., 1995). On the other hand, there are non-renin-, non-ACE-dependent pathways for Ang II generation, of which the relative importance remains unclear (Dzau, 1989; Navar et al., 1995; von Thun et al., 1994).

Comparisons of the effect of renin and ACE inhibition at the tissue level, possible only in animal models, have been made rarely because of the remarkable species specificity of renin. Renin inhibitors developed for humans have been relatively ineffective in small animals. We developed an inhibitor directed at rat renin with this in mind (Hui et al., 1988), making this study possible. This study was prompted by both the general considerations outlined above and a number of recent observations. In the rat, we found substantial residual authentic Ang II in the renal tissue and, to a lesser extent, in plasma after treatment with maximal doses of two ACE inhibitors (Allan et al., 1994). Thus, the possibility was raised of either alternative pathways for Ang II generation or limited blockade by ACE inhibition (von Thun et al., 1994). In humans, we found a renal vasodilator response to renin inhibition that exceeded the response to ACE inhibitors, all studied at the top of their respective dose-response range (Hollenberg and Fisher, 1994). This study was designed to address these issues by measuring authentic plasma and renal tissue angiotensin levels in the rat in response to blockade of the system at the renin or ACE step. Our hypothesis was that the role of renin in Ang II formation is crucial, but ACE-independent pathways play a major role in renal tissue Ang II formation, an observation that would explain the above findings. There is abundant evidence of both intrarenal generation and uptake of circulating Ang II by renal tissue (Campbell, 1987; Ichikawa and Harris, 1991; Johnston, 1992; Mitchell and Navar, 1991).

ABBREVIATIONS: RIP, renin inhibitor peptide; PRA, plasma renin activity; ACE, angiotensin-converting enzyme; Ang, angiotensin; MAP, mean arterial blood pressure; HPLC, high-pressure liquid chromatography.
Methods

The details of our methodology have been published previously (Allan et al., 1994). In brief, 21 Sprague-Dawley rats weighing 250 to 320 g were studied after an 18-hr fast. For pentobarbital anesthesia and insertion of a tracheal tube, jugular venous catheter and carotid arterial lines, the rats were allowed to stabilize for 30 min. They then were given 0.3 ml of vehicle (D3W, n = 5), ACE inhibitor (10 mg/kg ramipril, n = 5) administered over 5 min or the renin inhibitor (10–1000 μg/kg/min) administered as a constant infusion for 30 min. The low-dose group (n = 5) received 10 μg/kg/min. An intermediate-dose group received either 300 (n = 3) or 100 (n = 1) μg/kg/min. The high-dose group received 1000 μg/kg/min (n = 5). At 30 min later, an abdominal incision was made, both renal arteries were clamped, the right kidney was removed within 20 sec and blood was collected through the carotid artery line before the rat was killed with intravenous pentobarbital.

Sample processing and angiotensin separation. Eight molar urea was used as a chaotropic agent during the processing of the plasma and renal tissue samples, which were homogenized immediately and then prepared for solid-phase extraction using SepPak C18 cartridges as described in detail (Allan et al., 1994). After HPLC separation, authentic Ang I and Ang II concentrations were then measured by a double-antibody RIA. The method was adopted from earlier descriptions by Kifor et al. (1991).

The eluted peptides were dried overnight before HPLC was performed. Briefly, the dried samples were reconstituted in 550 μl of sample solvent (10 mM sodium acetate, 10 mM tetrathylammonium, 5% methanol, 0.15 M NaH2PO4, 10 ml of assay buffer (500 ml) and injected into a Merck Sharp and Dohme 3-μm C18 15 cm × 4 mm column. HPLC equipment consisted of an LKB 2150 pump with a dynamic mixer, a 2152 LKB controller and a 2211 LKB fraction collector. Solvent A contained 10 mM tetrathylammonium and 10 mM sodium acetate adjusted to a pH of 6.2 before mixing with methanol to a final concentration of 30%. Solvent B was prepared similarly except it contained 80% methanol. The following gradient was used: 0% B, 5 min; 14% B, 30% B, 35% B, 20% B, 50% B, 65% B, 54% B and 80% B at 50 μl/min. The flow rate was 0.55 ml/min. The 1-min fractions were collected in test tubes containing 50 μl of 10% glycercol and 150 μl of 50% assay buffer and subsequently dried overnight. Angiotensin peptide elution times and their standard deviation was determined by repeated injections of 4-nmol aliquots of the synthetic peptides into the HPLC apparatus and checked periodically using the acidified peptide samples. The acidified peptide samples were eluted 2 min ahead of the Ang I standard with this gradient. HPLC recovery was analyzed using five duplicate HPLC-purified, tritiated Ang II samples. Eluates contained 94 ± 3% of the β activity seen in their duplicate counterparts.

Synthesis of RIPS. All commercial amino acids were obtained from Peninsula Laboratories (San Carlos, CA). The side-chain protecting group was tosyl for histidine. Other reagents were dichloromethane (Dow Chemical, Midland, MI), N,N'-dicyclo-hexylcarbodiimide (Fluka, Ronkonkoma, NY), trifluoroacetic acid and N,N-diisopropylethylamine (Aldrich, Milwaukee, WI), both distilled before use, acetic anhydride (Fisher Chemical, Fairlawn, NJ), HF (Matheson, Secaucus, NJ), HPLC-grade acetonitrile (Baker, Phillipsburg, NJ) and p-methylbenzhydrolamine resin hydrochloride (United States Biochemical, Cleveland, OH); N-Boc-4-(S)-amino-(3)-hydroxy-6-methylheptanoic acid (Boe-statine) was either synthesized according to Hui et al. (1987) and Rich et al. (1978) or purchased from Advanced Chemtech (Louisville, KY).

Acetyl-His-Pro-Phe-Val-statine-Leu-he-NEH2 (IC50 against rat plasma renin of 30 nM, pH 7.4) proved to be a potent hypotensive agent and a potentially useful probe for the study of the renin-angiotensin system in rats. This RIP was synthesized by the Merrifield solid-phase method (Barany and Merrifield, 1979) in a stepwise manner according to the general procedure of Stewart and Young (1984). The crude product extracted after HF cleavage showed 70% to 90% purity as determined by analytical reverse-phase HPLC. Purification by preparative reverse-phase HPLC under isocratic conditions yielded a product of >99% purity. Purified RIP had a retention time identical to an original authentic sample (Hui et al., 1988) when analyzed on analytical reversed phase HPLC.

Inhibition of rat PRA. Peptide stock solutions were prepared by dissolving the peptide in Tris buffer (1.0 M, pH 7.4, 0.02% azide) containing 50% dimethylsulfoxide. These stock solutions were then subjected to a series of 1:10 dilutions with Tris buffer containing 25% dimethylsulfoxide. Blood from ether anesthetized rats was collected via a carotid cannula into tubes containing EDTA chilled at 0°C. The blood samples were centrifuged at 4°C to separate the plasma. Before the assay, 5 mM phenylmethylsulfonyl fluoride (0.3 M in ethanol), 3 mM 8-hydroxyquinoline sulfate and 5 mM additional EDTA were added. PRA assays (at zero concentration of peptide inhibitor) showed an average activity of 10 ng of Ang I/ml/hr.

Renin activity was determined by a radioimmunoassay for Ang I (Hui et al., 1988). For the in vitro evaluation of the inhibitory potency of the peptides, plasma samples (200 μl, pH 7.4) were mixed with 10 μl of serial concentrations of peptide inhibitors. The mixtures were divided into two portions for incubation at 37° and 0°C for 1 hr. The renin enzymatic reaction was quenched at pH 8.5 by adding saturated Tris solution (−2 μl/10 μl of mixture) and the solution was then diluted with an equal volume of 0.1 M Tris, pH 8.5, 0.025% azide) were incubated in tubes coated with rabbit anti-Ang I antibody (Travenol-Genentech Diagnostics, Cambridge, MA). The renin activity value was obtained from a standard curve generated by a parallel experiment with known quantities of Ang I. To determine the renin activity of rat plasma obtained from the infusion studies, 100 to 150 μl of plasma was directly assayed without additional peptide.

Preliminary in vivo rat studies. The in vitro inhibition assays showed that RIP was a potent rat renin inhibitor, with an IC50 value of 30 nM when assayed at neutral pH. To evaluate its hypotensive potency in vivo in association with their renin inhibitory activity, RIP in the dose range used in this study (10–1000 μg/kg/min) was infused over 6 min into seven sodium-depleted, anesthetized rats. MAP fell acutely, over several minutes, by 10 to 20 mm Hg, confirming in vivo activity and our earlier observations (Hui et al., 1988). On discontinuing the RIP infusion BP recovery was very rapid, with a half-time of ~1 min, confirming rapid degradation of the RIP.

Results

The rats treated with vehicle remained stable throughout the experimental procedure with no change in blood pressure, whereas treatment with both ramipril and high-dose RIP lowered blood pressure significantly (table 1). The baseline blood pressure for the rats given a ramipril dose of 10 mg/kg was 108 ± 3 mm Hg. In minutes after ramipril administration, MAP reached a nadir of 73 ± 10 mm Hg and slowly climbed to 93 ± 4 mm Hg by the 30-min postinfusion time period, a fall of 15 ± 4 mm Hg. Because of its very short half-life, the renin inhibitor peptide had to be given as a constant infusion; a dose-dependent fall in blood pressure

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td><strong>Blood pressure response to RIP and ramipril</strong></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Maximal effect</th>
<th>3-min after dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mm Hg</td>
</tr>
<tr>
<td>Vehicle (n = 5)</td>
<td>112 ± 10</td>
<td>109 ± 10</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>Low-dose RIP (n = 5)</td>
<td>112 ± 8</td>
<td>109 ± 5</td>
<td>114 ± 9</td>
</tr>
<tr>
<td>Intermediate-dose RIP (n = 3)</td>
<td>119 ± 13</td>
<td>108 ± 12</td>
<td>116 ± 13</td>
</tr>
<tr>
<td>High-dose RIP (n = 13)</td>
<td>118 ± 12</td>
<td>102 ± 2</td>
<td>102 ± 2</td>
</tr>
<tr>
<td>Ramipril (n = 5)</td>
<td>108 ± 3</td>
<td>73 ± 10</td>
<td>93 ± 4</td>
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</table>
followed. With the highest dose of the renin inhibitor, a sustained fall in MAP of 16 ± 4 mm Hg was obtained, from 118 ± 12 to 102 ± 2 mm Hg (table 1), which is identical to the depressor response to ramipril. Neither the intermediate nor the low dose of RIP induced a sustained fall in MAP.

Plasma Ang II concentration in the vehicle-treated rats was 83.2 ± 20.1 fmol/ml. These rats had a corresponding plasma Ang I concentration of 273 ± 84 fmol/ml. As anticipated, the ramipril-treated rats had significantly lower plasma Ang II levels (28 ± 2.9 fmol/ml) with a correspondingly higher plasma Ang I level (566 ± 91 fmol/ml; P < .01). The conversion of Ang I to Ang II was greatly decreased by the RIP.

The conversion of Ang I to Ang II was unaffected by the RIP. Nonetheless higher plasma Ang I level (566 ± 91 fmol/ml; P < .001) than the nadir achieved at the top of the ramipril dose response.

The renal tissue Ang II concentration in vehicle-treated rats was 183 ± 18 fmol/g, with a renal tissue Ang I concentration of 93 ± 7 fmol/g. The ramipril-treated group of rats had the anticipated fall in renal tissue Ang II concentration to 56 ± 6 fmol/g. A dose-related fall in renal tissue Ang II concentration also followed the administration of the RIP (table 2). The greatest reduction in tissue Ang II concentration followed high-dose RIP administration. All RIP doses, however, failed to reduce tissue Ang I concentration, and a fall in the index of conversion occurred with each dose (P < .01; table 2).

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

**Discussion**

The hypothesis tested in this study was that pharmacological interruption of the renin cascade would be more effective at the rate-limiting step, the interaction of renin and angiotensinogen, than at the ACE step, especially at the renal tissue level. We exploited recent advances in tissue peptide measurement, using HPLC and RIA to measure authentic Ang I and Ang II concentrations (Allan et al., 1994; Fox et al., 1992; Kifor et al., 1991). Validation of the method for measurements of the tissue level (Allan et al., 1994) included several observations. The use of hypertonic urea as a chaotic agent, totally disrupting both Ang II formation and degradation, was documented in *in vitro* experiments. The method proved effective in assessing anticipated alterations in Ang II at the renal tissue level, provided the first documentation of relation between ACE inhibitor dose and reduction in tissue level and documented the anticipated increase in renal tissue Ang II levels in SHR (Zangen et al., 1996). Measurement in plasma provides a more straightforward technical task, and the method documented the anticipated change with each maneuver in the rat (Allan et al., 1994) and in humans (Fisher et al., 1994). Thus, the method is very likely to have been adequate for the issues addressed in this study.

Interpretation of the biological responses to two agents can only be made with reference to the position of the dose used on the dose-response relationship. In the case of ramipril, we had documented earlier that 10 mg/kg lies at the top of the dose-response relationship for plasma and renal tissue Ang II

<table>
<thead>
<tr>
<th>Dose</th>
<th>Ang I</th>
<th>Ang II</th>
<th>CI</th>
<th>Ang I</th>
<th>Ang II</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fmol/ml</td>
<td>fmol/g</td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>0</td>
<td>273 ± 84</td>
<td>83 ± 20</td>
<td>0.23</td>
<td>93 ± 7</td>
<td>183 ± 18</td>
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<tr>
<td>Low-dose RIP</td>
<td>10 μg/kg/min</td>
<td>127 ± 42</td>
<td>40 ± 13</td>
<td>0.24</td>
<td>185 ± 35</td>
<td>128 ± 30</td>
</tr>
<tr>
<td>Intermediate-dose RIP</td>
<td>100–300 μg/kg/min</td>
<td>103 ± 70</td>
<td>28 ± 9</td>
<td>0.21</td>
<td>115 ± 17</td>
<td>83 ± 25</td>
</tr>
<tr>
<td>High-dose RIP</td>
<td>1000 μg/kg/min</td>
<td>29 ± 6</td>
<td>9 ± 2</td>
<td>0.21</td>
<td>112 ± 13</td>
<td>47 ± 10</td>
</tr>
<tr>
<td>Ramipril 10 mg/kg</td>
<td>566 ± 91</td>
<td>28 ± 3</td>
<td>0.05</td>
<td>243 ± 52</td>
<td>56 ± 6</td>
<td>0.19</td>
</tr>
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Values are given as mean ± S.E.M.

CI = conversion index: (Ang II)/(Ang I + Ang II).
concentration (Allan et al., 1994). In the case of the top dose of the renin inhibitor, on the other hand, substantially less information is available. Delineation of the relation between RIP dose and response was limited by several factors. The limited solubility of the agent limited the maximum rate of administration, and its very rapid degradation necessitated continuous infusion. Finally, there were limited supplies of the agent. The blood pressure response to the renin inhibitor was similar to the response to ramipril, which satisfied one of our goals in this comparison. These issues will not be resolved until a more potent or long-lasting renin inhibitor specific for rat renin can be found. Despite these limitations, the substantially larger fall in plasma Ang II concentration with renin inhibition than ACE inhibition supports our premise that blockade at the rate-limiting step is likely to be more effective. The issue of blockade in renal tissue is substantially more complicated. The reduction in renal tissue Ang II concentration was only slightly, not significantly, greater after high-dose RIP than ramipril.

Comparison of the early blood pressure fall with ramipril and the renin inhibitor should be tempered by the fact that ramipril was easily administered as a bolus, whereas the renin inhibitor, because of its limited solubility, had to be administered more gradually. At 30 min after ramipril administration and after initiation of the administration of the renin inhibitor, there was an essentially identical blood pressure response to ramipril and the high-dose RIP, despite a substantially larger fall in plasma Ang II levels after high-dose RIP. Moreover, the lower dose of the renin inhibitor induced an unambiguous and large fall in plasma Ang II level without influencing blood pressure. Indeed, the fall with the intermediate dose of the RIP was identical to that induced by ramipril. Because authentic plasma Ang II was measured, the data provide further support for arguments that changes in the plasma compartment are quantitatively less important for blood pressure homeostasis during renin system blockade than tissue activity.

Renin, an aspartyl protease enzyme, plays a vital rate-limiting role in the systemic RAS hormonal system (Reudelhuber et al., 1995). Renal tissue Ang II production may not parallel renin level as evidence suggesting the existence of non-renin-dependent pathways accumulates (Campbell et al., 1993; Dzau, 1989; Miura et al., 1994; Navar et al., 1995; von Thun et al., 1994; Urata et al., 1994). In this study, we used the pharmacological blockade provided by a novel renin inhibitor to modify plasma and tissue renin. This study verifies that renin does play a major role in rat renal tissue Ang II production but also supports the intriguing possibility that measurable non-renin-dependent Ang II formation occurs in renal tissue. Non-renin-dependent pathways have been described involving serine proteases such as trypsin (Boucher et al., 1974), human neutrophil protease (Wintroub et al., 1981) or cathepsin G (Klickstein et al., 1982). More recently, Miura et al. (1994) demonstrated that a serine protease inhibitor, nafamostat, partially blocked the exercise-induced increase in Ang II seen in humans treated with captopril. Campbell et al. (1993) demonstrated the persistence of Ang II in plasma and tissues of anephric rats and suggested that elevated plasma angiotensinogen levels after nephrectomy may play a role in enhancing non-renin-dependent pathways.

In the ramipril-treated rats, the remaining residual Ang II in the tissue may represent formation from non-ACE-dependent pathways, by either serine protease or sequential carboxyl peptidase activity (Campbell et al., 1993), as previously described to occur in the heart and vascular wall (Okunishi et al., 1984, 1987; Urata et al., 1990). Urata et al. identified and characterized a neutral serine protease from the left ventricle of the human heart that may play a significant role in Ang II formation in this tissue. Whether this in vitro finding is significant in vivo is debated. In accord, Okunishi et al. (1987) found that the conversion of Ang I to Ang II in the vascular wall was diminished with the use of an ACE inhibitor but abolished only by the combination of an ACE inhibitor with chymostatin. The postulated enzyme belongs to the chymotrypsin family but had a different pH optimum than cathepsin G, also previously described to cleave Ang I to Ang II (Thibault and Genest, 1981).

Measurements of plasma Ang I, Ang II and the index of conversion provided no surprise. Treatment with the ACE inhibitor induced the anticipated fall in Ang II, reactive rise in Ang I and a striking fall in the conversion index. The renin inhibitor, conversely, induced a parallel fall in plasma Ang I and Ang II, with no influence on the apparent rate of conversion, also as anticipated from the pharmacology of a renin inhibitor. In renal tissue, on the other hand, the findings were less straightforward. Ramipril induced the anticipated increase in Ang I and reduction in conversion index. Renin inhibition, on the other hand, did not influence Ang I as anticipated: Indeed, tissue levels of Ang I rose rather than falling. The possibility of laboratory error cannot be ignored, but these observations were consistent and nested in a series of observations that conform with current understanding. The possibility that a peptide designed to be a renin inhibitor also has affinity for converting enzyme cannot be ignored, but it is very unlikely: a parallel influence on plasma and tissue Ang I would have been anticipated. The explanation is probably multifactorial. One element might involve the striking influence that Ang has on renin release. Although the measurement of plasma or tissue renin activity is complicated in the presence of a renin inhibitor, antisera specific for active renin mass have documented an extraordinary rise in active renin in response to renin inhibition (Menard et al., 1991). Indeed, the resultant plasma renin level was far in excess of the rise associated with known potent stimuli, such as the combination of a low-salt diet and upright posture for hours. Intrarenal renin release in response to the fall in local Ang II concentration would lead to a reactive increase in local renin concentration, which could overcome, in part, the limited quantities of renin inhibitor. An alternative interpretation would question the specificity of our aspartyl protease inhibitor for renin. A series of RIP analogs (Hui et al., 1992; Hui and Siragy, 1990) were found to inhibit effectively the aspartyl protease of human immunodeficiency virus. Thus, it is possible that RIP and analogs possess a broader spectrum of inhibitor activities against other aspartyl proteases, especially those of mammalian origin. This possibility would provide an explanation for our observation of a dose-related inhibition of renal tissue Ang II formation (table 2) and suggests that an aspartyl protease other than renin participates in the conversion of Ang I to Ang II in the kidney. It is possible that non-ACE-dependent pathways for Ang II generation become more important, quantitatively, when ACE is inhibited (Mento et al., 1989). A similar logic can be applied to the contribution of alternative pathways when renin is
inhibited. The possibility that aspartyl proteases in tissue contribute to Ang II degradation further complicates the relationships. None of the data in this study would allow us to choose from among this array of possibilities, so the interpretation of the findings will remain a subject of debate until further information is obtained. To achieve that goal, we need more efficient methods for measuring authentic angiotensin peptides in tissue, especially at low levels, and more effective pharmacological probes for work in vivo.

Taken in all, this comparative study provides strong support for the concept that Ang II in plasma derives largely from the classic renin cascade and production is more effectively limited by inhibition at the rate-limiting step than by ACE inhibition. Our findings also suggest that Ang II production in renal tissue is more complex and may well involve non-ACE- and non-renin-dependent pathways. The current availability of Ang II blockers now becomes even more important. Resolution will await the development of more potent and more long-acting renin inhibitors specific for rat renin.

Acknowledgments

We are grateful to Ms. Diana Capone for her assistance in manuscript preparation and submission.

References


