Plasma Angiotensins and Blood Pressure During Converting Enzyme Inhibition

PETER F. MENTO AND BARRY M. WILKES

SUMMARY The relationship between plasma angiotensin and the reduction of blood pressure with the angiotensin converting enzyme inhibitor enalapril was studied in rats. Blood pressure was measured in conscious rats with indwelling arterial catheters. To measure angiotensin II, plasma was analyzed by physical separation of angiotensins using high performance liquid chromatography followed by radioimmunoassay. The effects of both single (acute) and long-term (chronic) dosages of enalapril were measured. After a single oral dose of enalapril (10 mg/kg), mean arterial pressure fell from 111 ± 3 to 86 ± 3 mm Hg (p<0.005). Despite the blood pressure reduction, plasma angiotensin II was unaffected (control, 9.9 ± 1.8 vs 9.7 ±1.1 pg/ml). After a higher single oral dose of enalapril (30 mg/kg), there was a reduction in both mean arterial pressure (81 ± 5 mm Hg, p<0.005) and plasma angiotensin II concentration (2.3 ± 0.6 pg/ml, p<0.01). The chronic effects of converting enzyme inhibition were evaluated in rats given enalapril in their drinking water (30 mg/kg/24 hr) for 1 week or 2 months. Mean arterial pressure remained equally low after chronic administration (for 1 week or 2 months), but plasma angiotensin II concentration increased above normal (after 1 week, 28.9 ± 8.7, p<0.01 vs control; after 2 months, 43.1 ± 16.2 pg/ml, p<0.05 vs control). Although plasma angiotensin converting enzyme activity was undetectable at any time after enalapril administration, there was a partial return of the angiotensin I pressor response with chronic administration. The data are most compatible with actions of converting enzyme inhibitors independent of the blockade of plasma angiotensin II formation. (Hypertension 9 [Suppl III]: III-42-III-48, 1987)

KEY WORDS • angiotensin II • converting enzyme inhibition • high performance liquid chromatography • radioimmunoassay • enalapril

ANGIOTENSIN converting enzyme (ACE) inhibitors were designed to lower blood pressure by blocking the formation of the potent vasopressor, angiotensin II (ANG II).1,2 Evidence from many studies seems to confirm this mechanism of ACE inhibitor action. The acute administration of ACE inhibitors lowers blood pressure, decreases levels of immunoreactive ANG II in plasma,3-5 and prevents blood pressure rise in the two-kidney, one clip Goldblatt model.6

Several recent observations suggest that the mechanism of action of ACE inhibitors is more complex than was initially thought. First, ACE inhibitors interact with other vasoregulatory systems, including kinins7-10 and prostaglandins.11-14 Second, restoration of blood pressure with ANG II following ACE inhibition requires supranormal levels of plasma ANG II.7 Third, chronic ACE inhibition increases immunoreactive plasma ANG II above control levels in sodium-depleted rats.15

The development of an assay for ANG II and its fragments that combines physical separation and radioimmunoassay provides an important tool for further study of the mechanism of action of ACE inhibitors.16 The experiments described here were designed to test the hypothesis that blood pressure lowering by chronic ACE inhibition could be dissociated from inhibition of plasma ANG II formation.

Materials and Methods

All chemicals were of the purest commercial grade available. Angiotensin I (ANG I), [Ile1]angiotensin II (ANG II), [des-Asp1] ANG I, and angiotensin III (ANG III) were purchased from Sigma (St. Louis,
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MO, USA). Chromatographically pure angiotensin-(3-8)hexapeptide, angiotensin-(4-8)pentapeptide, and angiotensin-(5-8)tetrapeptide were generously supplied by Dr. Mahesh Khosla of the Cleveland Clinic (Cleveland, OH, USA). Enalapril maleate was provided by Dr. Charles Sweet of the Merck Institute for Research (West Point, PA, USA).

Experiments were performed on 153 adult male Sprague-Dawley rats weighing 251 to 300 g at the start of the protocols. All protocols were in accordance with institutional guidelines for the care and handling of laboratory animals. The rats were given access ad libitum to standard Purina Rat Chow (0.42% sodium ash content) (Ralston Purina, St. Louis, MO, USA) and tap water. Blood pressure studies were performed on conscious rats at least 48 hours after the insertion of arterial and venous catheters under pentobarbital anesthesia (40 mg/kg, i.v.). The femoral artery catheter was formed by cementing a 2-cm segment of 0.28-mm i.d. x 0.61 mm o.d. vinyl tubing (Dural Plastics, Dural, Australia) to 0.51-mm i.d. x 1.5-mm o.d. microbore tubing (Tygon, Norton Performance Plastics, Akron, OH, USA). The femoral vein catheter was also microbore tubing. Catheters entered the body at the interscapular region and were flushed daily with sodium heparin (250 U/ml in saline). Blood pressure was measured with a transducer (Model P23 ID, Statham, Oxnard, CA, USA) connected to a polygraph (Model 7, Grass, Quincy, MA, USA).

To measure acute ACE inhibition, enalapril was suspended in 3% corn starch and administered by gavage in a single dose (1 ml/kg of body weight). To measure chronic ACE inhibition, enalapril (300 mg/L) was added to the rats' drinking water. Given in the water, the daily dose of enalapril was 38.0 ±3.1 mg/kg/24 hr after 2 months (n = 6; NS).

Rats were killed by rapid decapitation and blood was collected (for measurement of angiotensins and plasma renin activity [PRA]) into heparin-coated beakers containing EDTA (14 mg/100 µl). About 0.5 ml of blood was collected separately to obtain serum for measurement of ACE activity, which was measured with a commercial kit (ACE activity test set, Ventrex Corporation, Portland, ME, USA). After centrifugation, 0.5 ml of plasma was frozen and assayed for PRA by the method of Sealey and Laragh.17 Thirty microliters of 5% di-isopropyl fluorophosphate in isopropyl alcohol was added to the remaining plasma.

Plasma was extracted with phenylsilyl-silica columns (Bond Elut, Analytichem International, Harbor City, CA, USA). Columns were conditioned with methanol (1 ml) and water (1 ml), samples were applied (2 ml), and the loaded columns washed with water (1 ml) and eluted with methanol (0.5 ml). The extract was filtered through 0.45-µm filters (Acrodisc, Gelman, Ann Arbor, MI, USA), and dried.

Before angiotensins were separated by high performance liquid chromatography (HPLC), the extracted residue was dissolved in 50 µl of 0.1 M acetic acid. Angiotensins were separated using a 250 x 4.6-mm, 10-µm column (Hyperchrome C18, Innovativ-Labor, Adliswil, Switzerland) connected to a pump system (Series 4, Perkin-Elmer, Norwalk, CT, USA) fitted with a manual injection valve (Model 7125, Rheodyne, Cotali, CA, USA). Elution was isocratic at 45°C with a mobile phase of methanol/0.085% phosphoric acid (33.5:66.5) and flow rate of 1 ml/min. Fractions (0.4 ml) were collected into 12 x 75-mm glass test tubes and dried. The positions of angiotensin peaks were determined by injection of approximately 1 µg of ANG I, ANG II, and ANG III, (3-8)hexapeptide, and (4-8)pentapeptide, and monitored by absorption at 210 nm to determine the position of the peptides.

Plasma angiotensins were measured on the appropriate fractions by radioimmunoassay. ANG II and angiotensin C-terminal fragments were measured, with minor modifications, as previously described.16 Several antibodies were raised, and one (No. 71084) was chosen that had high sensitivity in the radioimmunoassay and high cross-reactivity with angiotensin fragments. In brief, dried extracts were reconstituted with 0.5 ml of assay diluent, antibody was added, and the tubes were incubated at 37°C for 6 hours. Approximately 1 pg of 125I-ANG II was added and incubated for an additional 18 hours at 4°C. Bound and free hormones were separated using dextran-coated charcoal in Tris buffer, and free hormone was counted in an autogamma spectrometer (Packard, Downers Grove, IL, USA).

Statistics
The data are expressed as the mean ± SE as the index of dispersion. Data were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls method for multiple comparisons. Differences were considered significant for p < 0.05.

Protocols
In the first protocol, the effects of acute administration of the ACE inhibitor enalapril on mean arterial pressure (MAP), responsiveness of ANG I, and blood components of the renin-angiotensin system were studied. Two days before MAP measurement, the catheters were inserted and the rats were allowed to recover from surgery. On the day of the study, MAP was measured in conscious rats in the basal state and following a single injection of ANG I (1 µg/kg, i.v.). Three hours after a single oral dose of enalapril (10 or 30 mg/kg), the response to a second injection of ANG I was studied. For measurements of the renin-angiotensin system, rats without catheters were given enalapril or vehicle alone and the blood collected 3 hours later.

In the second protocol, rats were given enalapril in their drinking water (300 mg/L) for either 1 week or 2 months prior to the study. Age-matched rats given tap water served as controls. Two days before the study, femoral artery and vein catheters were implanted.

In rats studied at 1 week, the pressor response to ANG I (1 µg/kg, i.v.) was recorded and a dose-response curve to ANG II was constructed using increasing doses of ANG II (1–300 ng/kg). In rats studied at 2 months, the
responses to a single dose of ANG I (1 μg/kg, i.v.) and ANG II (100 ng/kg, i.v.) were studied. Blood was taken from the same rats used for blood pressure measurements after a 24-hour recovery period.

Results

HPLC Separation and Radioimmunoassay

Figure 1 is a representative chromatogram depicting separation of angiotensins by HPLC. Retention times (in minutes) were: ANG I (14.34), ANG II (6.69), ANG III (5.38), (3-8)hexapeptide (8.43), (4-8)pentapeptide (5.14). Peak positions were very reproducible, and 0.4-ml fractions were collected between 2.8 and 9.6 minutes. ANG III was not separated from (4-8)pentapeptide, and concentrations for these peptides are reported as the combined value. ANG I was not measured, but did not interfere with the other measurements because of its complete separation from other angiotensins.

Extraction efficiency was determined using 123I-ANGII as a marker. Recovery from the extraction step was 88.8 ± 4.7% and recovery through the filtration step was 80.4 ± 3.0%. Total recovery of each of the peptides through the entire procedure was determined by separately spiking pooled normal plasma with 100 pg of each of the peptides and subtracting the amount of peptide in unspiked plasma. Recoveries were: ANG n, 48.2 ± 2.2%; ANG m, 38.0 ± 9.4%; (3-8)hexapeptide, 35.3 ± 1.3%; and (4-8)pentapeptide, 36.8 ± 2.6%. All reported values are corrected for recovery.

The lower limit of sensitivity of the angiotensin radioimmunoassay was 0.6 pg of ANG II. Values less than 0.6 pg were taken to be 0 for calculations. Cross-reactivity of the anti-ANG II antibody to ANG III, (3-8)hexapeptide, and (4-8)pentapeptide was 100% (Figure 2). The (5-8)tetrapeptide did not cross-react to any measurable extent. Therefore, taking recovery into account, the minimum amounts that could be detected in 2 ml of plasma were: ANG II, 0.6 pg; ANG III, 0.8 pg; (3-8)hexapeptide, 0.8 pg; (4-8)pentapeptide, 0.8 pg. The assay characteristics were similar to those reported by others.16

Effects of Enalapril

Following the administration of a single low dose of enalapril (10 mg/kg), PRA rose from 5.6 ± 1.2 to 26.5 ± 2.0 ng/ml/hr (p<0.005), and ACE fell from 215 ± 12 to <15 nmol/ml/min (Table 1). MAP fell from a control of 111 ± 3 to 86 ± 3 mm Hg (p<0.005; Figure 3). The ANG I pressor response was inhibited by 93.1%. There was, however, no fall in plasma ANG II (control, 9.9 ± 1.8; low-dose enalapril, 9.7 ± 1.1 pg/ml).

The effects of a single higher dose of enalapril (30 mg/kg) on blood pressure, inhibition of ACE activity, blockade of the ANG I pressor response, and stimulation of PRA were similar to effects of the single low dose (see Table 1 and Figure 3). At the higher dose, however, there was a marked reduction in plasma ANG II concentration (2.3 ± 0.6 pg/ml; p<0.01 vs control).

When enalapril was added to the rats’ drinking water for 1 week or 2 months (30 mg/kg/24 hr), MAP and plasma ACE values were comparable to values for rats given acute doses (Figure 4; see Table 1). There were, however, dramatic temporal changes in PRA, the pressor response to ANG I, and plasma ANG II. PRA, which was elevated 4.3-fold following acute administration of enalapril, returned toward control levels (see Table 1). Plasma ANG II, which was suppressed following acute dosage, became elevated at both 1 week
TABLE 1. Components of the Renin-Angiotensin System During CEI

<table>
<thead>
<tr>
<th>Variable</th>
<th>ACE (nmol/ml/min)</th>
<th>PRA (ng/ml/hr)</th>
<th>ANG II (pg/ml)</th>
<th>ANG III + (4-8)pentapeptide (pg/ml)</th>
<th>(3-8)hexapeptide (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>215 ± 12</td>
<td>5.6 ± 1.2</td>
<td>9.9 ± 1.8</td>
<td>4.6 ± 0.5</td>
<td>2.1 ± 0.8</td>
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<td>(7)</td>
<td>(7)</td>
<td>(8)</td>
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<tr>
<td>Acute administration of enalapril</td>
<td></td>
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<td></td>
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<tr>
<td>10 mg/kg (3 hr)</td>
<td>ND</td>
<td>26.5 ± 2.0*</td>
<td>9.7 ± 1.1</td>
<td>5.3 ± 1.5</td>
<td>5.5 ± 0.8†</td>
</tr>
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<td>(7)</td>
<td>(8)</td>
<td>(7)</td>
<td>(7)</td>
<td>(7)</td>
</tr>
<tr>
<td>30 mg/kg (3 hr)</td>
<td>ND</td>
<td>21.3 ± 4.3*</td>
<td>2.3 ± 0.6†</td>
<td>1.3 ± 0.4†</td>
<td>1.8 ± 0.7</td>
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<td>(6)</td>
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<td>Chronic administration of enalapril</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>30 mg/kg/24 hr (1 wk)</td>
<td>ND</td>
<td>9.9 ± 1.6</td>
<td>28.9 ± 8.7†</td>
<td>4.2 ± 0.9</td>
<td>2.7 ± 1.0</td>
</tr>
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<td>(6)</td>
<td>(6)</td>
<td>(7)</td>
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<td>(7)</td>
</tr>
<tr>
<td>30 mg/kg/24 hr (2 mo)</td>
<td>ND</td>
<td>9.4 ± 1.4</td>
<td>43.1 ± 16.2†</td>
<td>11.5 ± 4.2</td>
<td>6.4 ± 2.7</td>
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<tr>
<td></td>
<td>(11)</td>
<td>(11)</td>
<td>(7)</td>
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</tbody>
</table>

Numbers in parentheses indicate number of rats. ND = not detectable.

* p < .005 compared to control, 1 week, 2 months.
† p < .05 compared to control.
‡ p < .01 compared to control.

(2.9-fold) and 2 months (4.4-fold; see Figure 4). The pressor response to ANG I, which was blocked by 94.1% after 3 hours of acute ACE inhibition, was only blocked by 62.1% at 1 week and by 39.8% at 2 months (Figure 5). Responsiveness to ANG II was unchanged after 1 week of enalapril treatment (Figure 6).

Concentration of Angiotensin Fragments During ACE Inhibition

In control rats, angiotensin fragments accounted for 40.0 ± 3.3% of total angiotensins (see Table 1). During acute ACE inhibition with enalapril (30 mg/kg), angiotensin fragments accounted for 58.0 ± 7.0%
Figure 5. The effects of acute and chronic enalapril administration on the pressor response to ANG I (1 μg/kg, i.v.). Mean arterial pressure (MAP) was measured intraarterially in conscious rats. The pressor response was inhibited by 94.1%, 62.1%, and 39.8% at 3 hours, 1 week, and 2 months, respectively. Numbers within vertical bars indicate number of rats. Single (p<0.005) and double (p<0.001) asterisks indicate significant difference compared to control.

Figure 6. The pressor response to ANG II during chronic enalapril administration. The data in the figure were obtained from rats given enalapril (30 mg/kg/24 hr) for 1 week and from their controls. In rats on the same protocol for 2 months (not shown) there were no differences in the pressor response to ANG II (100 ng/kg, i.v.). A II = ANG II.

(p<0.025), and during chronic ACE inhibition (1 week and 2 months) they accounted for 25.5 ± 3.6% of total angiotensins (p<0.005). The differences in the ratios of angiotensin fragments to total angiotensins were caused by decreased ANG II with acute ACE inhibition and increased ANG II concentrations with chronic ACE inhibition.

Discussion

The antihypertensive effects of acute doses of enalapril were independent of suppression of plasma ANG II. Blood pressure was reduced equally at a single dose of 10 or 30 mg/kg, but plasma ANG II was only suppressed at the higher dose. There was also temporal dissociation between the hypotensive effect of enalapril and suppression of plasma ANG II: at 3 hours (30 mg/kg), plasma ANG II was inhibited, but with prolonged administration (30 mg/kg/24 hr; 1 week or 2 months) plasma ANG II rose to supranormal levels (see Table 1). The data cannot be attributed to cross-reactivity of ANG II antisera with other angiotensins, since the concentrations of ANG II were measured by radioimmunoassay after physical separation from ANG I and other angiotensins. Therefore, these experiments showed a dissociation between the hypotensive effects of enalapril and plasma ANG II.

Single doses of enalapril, whether low or high, resulted in a 4.3-fold elevation of PRA. The elevation of PRA was not sustained with chronic administration: PRA returned toward normal despite continued suppression of ACE activity and increased plasma ANG II levels. The pressor response to ANG I gradually returned with increasing duration of ACE inhibition despite a steady level of blood pressure reduction. The data suggest that blood pressure lowering by ACE inhibition is independent of plasma ANG II and the inhibition of the ANG I pressor response.

The finding of increased plasma ANG II during chronic ACE inhibition suggests either increased production or decreased metabolism. We measured the concentration of ANG III, (3–8)hexapeptide, and (4–8)pentapeptide as an index of ANG II metabolism. When plasma ANG II was elevated during chronic ACE inhibition, the concentration of fragments relative to ANG II was decreased, suggesting that the rate of degradation did not match the rate of production. The possibility that alternate pathways for ANG II formation were induced by ACE inhibition was not addressed in this study. However, Lanzillo et al. have reported conversion of ANG I to ANG II by an endothelial cell peptidyl dipeptidase from cultured bovine pulmonary artery by an enzyme other than ACE. It seems likely that ANG I conversion in the presence of ACE inhibition occurs by the induction of one or more alternative enzymes.

Factors other than angiotensin inhibition are important for the actions of ACE inhibitors. Swartz et al. reported that in essential hypertensive patients treated with teprotide, ANG II concentrations had to be raised 45 pg/ml above normal to return blood pressure to pretreatment levels. The effectiveness of ACE inhibitors in lowering blood pressure showed no relationship to pretreatment PRA in hypertensive patients. Biolaz et al. reported that long-term treatment of hypertensive patients with enalapril controlled blood pressure, although plasma ANG II concentrations returned to baseline levels 12 to 16 hours after the dose was given. In animal models of hypertension, Sweet reported a dissociation between the antihypertensive action of single doses of enalapril and the inhibition of the pressor response to ANG I.

A number of studies have tested the role of prostaglandins and kinins in the hypotensive effects of ACE inhibitors. Reports of the effects of ACE inhibition on prostaglandins have been conflicting. Increased urinary prostaglandin E production in rats and increased
prostaglandin E-metabolite in normal humans have been reported.24 Moore et al.25 reported that indomethacin blunted the hypotensive response to captopril in some patients with essential hypertension. Hui et al.26 reported potentiation of the depressor response to arachidonic acid by ACE inhibitors in rats. ACE inhibitors directly stimulated prostaglandin synthesis in isolated rat glomeruli and aorta.12,27 Renal blood flow became prostaglandin-dependent during chronic enalapril treatment in sodium-depleted rats, but blood pressure lowering was not affected by inhibition of prostaglandin synthesis.11 Several reports, however, indicate that prostaglandins do not change with ACE inhibition in rats,28,29 normal human subjects,30,31 or hypertensive patients.32-34 The elucidation of the role of prostaglandins, if any, in the mechanism of action of ACE inhibition awaits further study.

ACE inhibitors may lower blood pressure by reducing kinin degradation. Some investigators have reported that ACE inhibition causes increased bradykinin levels,7-10 while others have reported no effect.29-31,35,36 The reasons for the discrepancies are not completely clear, but discrepancies may be due to difficulties in measuring kinins and the lack of specific kinin inhibitors.

Interactions between ACE inhibitors and catecholamines have also been reported. Kohlmann et al.37 showed decreased turnover of norepinephrine in the heart and brainstem of normal rats treated with enalapril. ACE inhibition has also been reported to decrease the response to norepinephrine in mesenteric arteries protected from ACE inhibition. Possible alternative mechanisms of blood pressure lowering by enalapril include prostaglandin production, decreased kinin degradation, interactions with catecholamines, or the production of other vasodilator substances by the kidney. ANG II formation during chronic ACE inhibition may occur via enzymes that are not affected by ACE inhibitors. ANG II formed in this way does not interfere with blood pressure lowering by ACE inhibition.

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