Differential Regulation of Angiotensin Peptide Levels in Plasma and Kidney of the Rat

Duncan J. Campbell, Anne C. Lawrence, Amanda Towrie, Athena Kladis, and Anthony J. Valentijn

We compared the effects of the converting enzyme inhibitor perindopril on components of the renin-angiotensin system in plasma and kidney of male Sprague-Dawley rats administered perindopril in their drinking water at two doses (1.4 and 4.2 mg/kg) over 7 days. Eight angiotensin peptides were measured in plasma and kidney: angiotensin-(1-7), angiotensin II, angiotensin-(1-9), angiotensin I, angiotensin-(2-7), angiotensin III, angiotensin-(2-9), and angiotensin-(2-10). In addition, angiotensin converting enzyme activity, renin, and angiotensinogen were measured in plasma, and renin, angiotensinogen, and their respective messenger RNAs were measured in kidney; angiotensinogen messenger RNA was also measured in liver. In plasma, the highest dose of perindopril reduced angiotensin converting enzyme activity to 11% of control, increased renin 200-fold, reduced angiotensinogen to 11% of control, increased angiotensin-(1-7), angiotensin I, angiotensin-(2-7), and angiotensin-(2-10) levels 25-, 9-, 10-, and 13-fold, respectively; angiotensin II levels were not significantly different from control. By contrast, for the kidney, angiotensin-(1-7), angiotensin I, angiotensin-(2-7), and angiotensin-(2-10) levels did not increase; angiotensin II levels fell to 12% of control, and angiotensinogen fell to 12% of control. Kidney renin messenger RNA levels increased 12-fold, but renal renin content and angiotensinogen messenger RNA levels in kidney and liver were not influenced by perindopril treatment. These results demonstrate a differential regulation of angiotensin peptides in plasma and kidney and provide direct support for the proposal that the cardiovascular effects of converting enzyme inhibitors depend on modulation of tissue angiotensin systems. Moreover, the failure of kidney angiotensin I levels to increase with perindopril treatment, taken together with the fall in kidney angiotensinogen levels, suggests that angiotensinogen may be a major rate-limiting determinant of angiotensin peptide levels in the kidney. (Hypertension 1991;18:763-773)
the potencies of CEI for inhibition of ACE in different tissues.6,7,21-24 In support of the first hypothesis, several tissues, including kidney, show a prolonged suppression of ACE activity that parallels the prolonged suppression of blood pressure during and after cessation of CEI administration.6-8 Several lines of evidence suggest that the inhibition of renal ACE may be important for the antihypertensive effect of CEI. Bilateral nephrectomy significantly reduces or abolishes the antihypertensive effect of CEI.5,25,26 Moreover, the inhibition of renal ACE by various CEI shows a consistently close relation with their effect on blood pressure.6,7,21,22 Furthermore, the prolonged reduction in blood pressure of spontaneously hypertensive rats after short-term treatment with CEI is associated with correction of the elevated renal vascular resistance in these rats.27

In support of the second hypothesis, an alternate pathway able to convert Ang I to Ang II by the sequential cleavage of the two C-terminal residues from Ang I has been identified in kidney homogenate.17,18 Drummer et al20 have reported that chronic administration of the CEI enalapril causes an induction of the carboxypeptidases responsible for this alternate pathway in subcellular fractions of kidney. Johnson et al18 have reported angiotensin-(1-9) [Ang-(1-9)] levels to be 34 fmol/ml and 66 fmol/ml in plasma of humans and rats, respectively, thus providing evidence for the operation of such a pathway in vivo. Moreover, these authors have reported increased Ang-(1-9) levels in rats administered the CEI captopril.18 However, data from our laboratory indicate that plasma Ang-(1-9) levels in humans are very low (less than 0.4 fmol/ml) and show only a small increase in hypertensive patients receiving CEI therapy.28

In the present study, we examined these two hypotheses by investigating the effects of the CEI perindopril on components of the renin-angiotensin system in plasma and kidney of rats. With use of high-performance liquid chromatography (HPLC) - based radioimmunoassays (RIA), we quantified levels of eight angiotensin peptides in plasma and kidney: angiotensin-(1-7) [Ang-(1-7)], Ang II, Ang-(1-9), Ang I, angiotensin-(2-7) [Ang-(2-7)], angiotensin III (Ang III), angiotensin-(2-9) [Ang-(2-9)], and angiotensin-(2-10) [Ang-(2-10)]. In addition, we measured renin and angiotensinogen levels in plasma and kidney, renin messenger RNA (mRNA) levels in kidney, and angiotensinogen mRNA levels in kidney and liver.

Methods

Animals

Male Sprague-Dawley rats (~340 g), maintained in a room with a 12-hour light/dark cycle (lights on 6:00 AM to 6:00 PM) were fed a diet of GR 2+1 pellets (Clarke King & Co., Melbourne, Australia). Control rats were given tap water to drink. Perindopril-treated rats received water containing either 16 or 48 µg/ml perindopril. Perindopril was a generous gift from Servier Laboratories, Courbevoie, France, and was freshly prepared each evening. Water intake was approximately 30 ml/rat/24 hr, resulting in a perindopril intake of approximately 1.4 and 4.2 mg/kg, respectively. These doses of perindopril produce near-maximal inhibition of plasma ACE activity and reduction of blood pressure in rats.3,22,24,27 After 7 days, the rats were killed at 10:00 AM by decapitation without prior anesthetic. Three experimental groups of rats were studied. In the first group, trunk blood was collected for measurement of plasma angiotensin peptides, and kidneys were removed for measurement of renal angiotensin peptide levels. In the second group, trunk blood was collected for measurement of plasma levels of ACE, renin, and angiotensinogen, and kidneys were removed for measurement of renal levels of angiotensinogen. In the third group, kidneys were removed for measurement of renal levels of renin and of renin and angiotensinogen mRNA, and a portion of liver was removed for measurement of hepatic levels of angiotensinogen mRNA.

Extraction and Radioimmunoassay of Angiotensin Peptides From Rat Plasma

Trunk blood (5-6 ml) was rapidly collected into tubes containing 0.5 ml inhibitor solution (100 µM H-77, 50 mM 1,10-phenanthroline, 125 mM ethylenediaminetetraacetate [EDTA], 2 g/l neomycin sulfate, and 2% ethanol in water) at 4°C. The renin inhibitor H-7729 was a generous gift from Dr. M. Szelke, Ferring Research Institute, Southampton, UK. The final plasma concentration of H-77 (10-20 µM) was sufficient to cause complete inhibition of rat renin (Reference 30 and unpublished data from our laboratory). The blood was centrifuged and the plasma (~3 ml) was immediately extracted with Sep-Pak C18 cartridges (Waters, Milford, Mass.). The method of extraction, acetylation, HPLC, and RIA of angiotensin peptides is described in detail elsewhere.28 Angiotensin peptides were acetylated before HPLC, and after reconstitution in assay buffer, each HPLC fraction was assayed in duplicate with two different RIA. Antibody 41, raised against N-acetyl-AspArgValTyrIleHisProPheLys (AcAng-II-K), measured AcAng-(1-7), AcAng II, AcAng-(1-9), and AcAng I. Antibody S2, raised against N-acetyl-ArgValTyrIleHisProPheLys (AcAng-III-K), measured AcAng-(2-7), AcAng III, AcAng-(2-9), and AcAng-(2-10).

For recovery estimation, plasma from two control rats was pooled and then divided into two equal portions, to one of which was immediately added 50 µl of a peptide cocktail [500 fmol each of Ang(1-7), Ang II, Ang(1-9), Ang I, Ang(2-7), Ang III, Ang(2-9), and Ang(2-10)]; thus the concentration of each peptide in the 3-ml plasma sample was ~167 fmol/ml. The two plasma samples were then processed in parallel, recoveries were calculated by subtracting the endogenous peptide levels, and the result was expressed as a percentage of the amount added.
Extraction and Radioimmunoassay of Angiotensin Peptides From Rat Kidney

The left kidney (1.2–1.7 g wet wt) was removed immediately after collection of blood and homogenized in 20 ml 4 M guanidine thiocyanate (GTC), 1% (vol/vol) trifluoracetic acid (TFA) in water. The time delay between decapitation and homogenization of tissue was approximately 60 seconds. Tissue homogenates were sonicated briefly and then centrifuged at 5,000g for 20 minutes. Half (10 ml) of the supernatant from each homogenate was extracted on a Sep-Pak C18 cartridge, and the extract was evaporated to dryness under vacuum. Each extract was then dissolved in 1 ml 1 M HCl and was extracted twice with 1 ml diethyl ether. The extracts were then evaporated to dryness again, acetylated, and run on HPLC before measurement of angiotensin peptides by RIA as described for plasma.

To determine the recovery of angiotensin peptides from kidney homogenates, the 20-ml homogenates from control rats were divided into two equal portions, to one of which was added 50 μl of the peptide cocktail described above. The samples were then processed in parallel as described above, and the peptide recoveries were calculated as described for plasma.

Validation of Measurement of Angiotensin Peptides in Rat Kidney

The measurement of angiotensin peptides in kidney was validated by several approaches. The identities of the Ang II and Ang I peaks on HPLC were confirmed using the C-terminal-directed antisera A30 and A16, respectively (data not shown). In addition, the Ang-(1–9) peak was confirmed using a C-terminal-directed antibody raised against Ang-(1–9), a generous gift from O.H. Drummer (data not shown).

To determine the stability of angiotensin peptides in the GTC/TFA homogenate, the 20-ml homogenates from both kidneys of four control rats were combined, from which three replicate 10-ml aliquots were obtained; to one replicate was added Ang II (~1,100 fmol/g kidney) and to another was added Ang I (~1,280 fmol/g kidney). These samples were then processed as described above.

The possible change in angiotensin peptide levels in kidney before homogenization was examined by study of four control rats, the left and right kidneys of which were homogenized 30 seconds and 90 seconds after decapitation, respectively, and then were processed as described above.

Measurement of Angiotensin Converting Enzyme, Renin, and Angiotensinogen in Plasma

For measurement of plasma ACE, renin, and angiotensinogen, trunk blood was collected into heparinized tubes at room temperature. After centrifugation at 2,000g for 10 minutes, 0.9-ml aliquots of plasma were transferred to tubes containing 0.1 ml of 300 mM sodium phosphate, 25 mM N-ethylmaleimide, 100 mM benzamidine, pH 7.4 (to prevent cryoactivation of inactive renin), then were rapidly frozen in dry ice and stored at −30°C until assay. Plasma ACE activity was measured by spectrophotometric assay using 2 mM 3-(2-furylcarbonyl)-1-phenylalanyl-glycyl-glycine as substrate.

The concentration of active renin in plasma was determined by incubation with rat angiotensinogen (~2,700 pmol/ml, final concentration). The plasma (20 μl), serially diluted in 100 mM sodium phosphate, 1 mM EDTA, 0.1% human serum albumin, 0.25 mM thimerosal, pH 7.4 (renin diluent), was incubated at 37°C for 30 minutes in a total volume of 105 μl, containing 50 μl nephrectomized rat plasma, 30 μl 300 mM sodium phosphate, 20 mM EDTA, 25 mM N-ethylmaleimide, 100 mM benzamidine, pH 7.4, and 5 μl 144 mM phenylmethysulfonfluryl fluoride (PMSF) (in ethanol). Ang I generation was quantified by RIA. A parallel series of incubations was performed at 4°C to provide a control for "nonspecific" interference in the RIA for Ang I.

For measurement of angiotensinogen, 10 μl plasma (serially diluted in 100 mM sodium phosphate, 10 mM EDTA, 150 mM sodium chloride, 1 g/l casein, 1 g/l sodium azide, pH 8.0) was incubated in a total volume of 505 μl, containing 100 μl mouse submandibular gland renin, 300 μl 200 mM sodium phosphate, 100 mM citric acid, 50 mM EDTA, 1 g/l sodium azide, pH 5.7, and 5 μl 144 mM PMSF (in ethanol). After incubation at 37°C for 60 minutes, Ang I generation was quantified by RIA.

Measurement of Renin in Kidney

All steps were performed at 4°C. Immediately after decapitation, the left kidney was removed and homogenized in 20 ml 100 mM sodium phosphate, 10 mM EDTA, 12.5 mM N-ethylmaleimide, 2 mM benzamidine, pH 7.4, to which were added 200 μl 144 mM PMSF, 10 mM phenylmercuric acetate (PMA) (in ethanol). The homogenate was sonicated briefly and then was centrifuged at 5,000g for 20 minutes. The supernatant was then serially diluted in renin diluent. The diluted supernatant (40 μl) was incubated in a total volume of 205 μl, containing 100 μl nephrectomized rat plasma (~2,700 pmol/ml angiotensinogen, final concentration), 60 μl 300 mM sodium phosphate, 20 mM EDTA, 25 mM N-ethylmaleimide, 100 mM benzamidine, pH 7.4, and 5 μl 144 mM PMSF, 10 mM PMA (in ethanol). After incubation at 37°C for 60 minutes, Ang I generation was quantified by RIA. A parallel series of incubations was performed at 4°C to provide a control for nonspecific interference in the RIA for Ang I.

Measurement of Angiotensinogen in Kidney

This method was based, in part, on that described by Wallis and Printz for the measurement of angiotensinogen in brain. All steps were performed at 4°C. Immediately after collection of blood, each kidney was homogenized in 10 ml 1.5 M ammonium sulfate, 100 mM sodium phosphate, 10 mM EDTA, 12.5 mM
Figure 1. Line graphs show characterization of angiotensin peptides in plasma (panels A and B) and kidney (panels C and D) after extraction, acetylation, high-performance liquid chromatography, and radioimmunoassay with antibody 41 (panels A and C) and antibody 52 (panels B and D). All separations were performed on a 100 x 4.6 mm Brownlee RP-18 Spheri-5 column preceded by a 15 x 3.2 mm RP-18 guard column (Applied Biosystems, Foster City, Calif.). Solvent A was 0.1% trifluoroacetic acid (TFA) and 0.15 M NaCl in water; solvent B was 0.1% TFA and 90% acetonitrile in water. Peptides were eluted by a linearly increasing gradient of 18–35% solvent B over 60 minutes. The flow rate was 1 ml/min and 1-minute fractions were collected. Values, expressed as femtomoles per fraction, are not corrected for antibody cross-reactivity or peptide recovery. AcAng-(1-7), N-acetylated angiotensin-(1-7); AcAng-(1-9), N-acetylated angiotensin-(1-9); AcAng II, N-acetylated angiotensin II; AcAng I, N-acetylated angiotensin I; AcAng-(2-7), N-acetylated angiotensin-(2-7); AcAng-(2-9), N-acetylated angiotensin-(2-9); AcAng III, N-acetylated angiotensin III; AcAng-(2-10), N-acetylated angiotensin-(2-10).

N-ethylmaleimide, 2 mM benzamidine, pH 6.5, to which were added 1 ml 1.5 mM pepstatin (in ethanol) and 100 μl 144 mM PMSF, 10 mM PMA (in ethanol). The left kidney was homogenized as described, and the right kidney homogenized in homogenization solution containing 100 μl nephrectomized rat plasma (~570 pmol angiotensinogen) to provide a measure of recovery of angiotensinogen. The homogenate was centrifuged at 5,000g for 15 minutes, and the supernatant was decanted. To precipitate angiotensinogen, the ammonium sulfate concentration of the supernatant was adjusted to 2.5 M by addition of 4 M ammonium sulfate and then was centrifuged as before. The ammonium sulfate precipitate was resuspended in 10 ml water, and 100 μl was incubated in a total volume of 505 μl, containing 100 μl mouse submandibular gland renin, 300 μl 200 mM sodium phosphate, 100 mM citric acid, 50 mM EDTA, 0.1% sodium azide, pH 5.7, and 5 μl 144 mM PMSF, 10 mM PMA (in ethanol). After incubation at 37°C for 60 minutes, 0.5 ml 1% TFA was added; each incubation was extracted with a Sep-Pak C18 cartridge, the eluate was dried under vacuum, and Ang I was quantified by RIA. A parallel series of incubations was performed at 4°C to provide a control for non-specific interference in the RIA for Ang I. It was necessary to extract the incubations with Sep-Pak C18 cartridges before RIA to remove a component of the incubation that interfered with the RIA for Ang I.

Recovery of Ang I was monitored during the incubation and was found to be 102±6% (mean±SD, n=4). Thus, the ammonium sulfate precipitation of angiotensinogen from the kidney homogenate effectively removed angiotensinases. Recovery of angiotensinogen through this procedure was 35±6% (mean±SD, n=18), and the renal level of angiotensinogen for each rat has been corrected for recovery.

Measurement of Angiotensinogen and Renin Messenger RNA

RNA was extracted from kidney and liver, and angiotensinogen and renin mRNA were measured by Northern blot analysis as described previously, except that glyoxalated RNA was transferred to Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, Calif.) using 10 mM sodium hydroxide. Rabbit globin mRNA was used to monitor recovery. Blots were hybridized with complementary RNA (cRNA) probes. Angiotensinogen cRNA was synthesized from plasmid pRANG A3/1, constructed by subcloning the Taq I–EcoRI fragment of plasmid pRANG 22B, (Taq I) 3/2,36 containing ~1,000 bases of the 5' end of the rat angiotensinogen complementary DNA (cDNA), into the Acc I–EcoRI site of plasmid pGEM-4Z (Promega Biotec, Madison, Wis.). Renin cRNA was synthesized from plasmid pREN44.ceb, which contains the full length rat renin cDNA39 subcloned into pGEM-4, a generous gift from K.R.
Angiotensin Peptides in Plasma and Kidney

Lynch. Globin cRNA was synthesized from plasmid β-globin 1/38, constructed by subcloning the HindIII-Bgl II fragment of plasmid pRSV-β-globin (American Type Culture Collection, Rockville, Md.), which contains the rabbit β-globin cDNA, into the HindIII-BamHI site of pGEM-3Z. Blots were hybridized at 65°C in 50% formamide, 45 mM sodium chloride, 1.5 mM sodium phosphate, 1.5 mM EDTA, pH 7.4 (1.5×SSPE), 1% sodium dodecyl sulfate, 0.5% nonfat powdered milk, 0.5 mg/ml salmon sperm DNA. Blots were washed at 65°C in 30 mM sodium chloride, 3 mM sodium citrate, pH 7.0 (0.2×SSC), 0.1% sodium dodecyl sulfate. Autoradiographs were quantified by densitometry, and the tissue levels of mRNA were corrected for losses by normalization to globin mRNA. The data presented have also been normalized to wet weight of tissue; there were no differences between groups in yield of RNA from each tissue (liver, 5.6±1.3 mg/g wet wt [mean±SD, n=18]; kidney, 3.4±0.2 mg/g wet wt).

Statistical Analysis

Comparisons between control and perindopril-treated rats were made by unpaired t test. Differences were termed significant if the t value exceeded the critical value for the 5% level.

Results

Angiotensin Peptides in Plasma and Kidney of Control Rats

The HPLC-based RIA used in this study enabled the precise identification of eight angiotensin peptides in plasma and kidney. Typical HPLC profiles are shown in Figure 1. The elution positions of angiotensin peptides from HPLC were highly reproducible, with peptides usually eluting in no more than two fractions. The recoveries (from extraction, acetylation, and HPLC) of angiotensin peptides from plasma and kidney are shown in Table 1, and all data have been corrected for recovery and cross-reactivity with antisera. The minimum detectable amounts are shown in Table 2.

For control rats, the predominant angiotensin peptide in plasma was Ang I, with Ang II levels being approximately half the levels of Ang I (Table 3). Ang III and Ang-(2–10) levels were approximately half the levels of Ang II and Ang I, respectively; the other peptides measured were of much lower abundance.

In kidney, both the absolute levels and the relative levels of angiotensin peptides were different from plasma (Tables 3–5). The levels of Ang-(1–7), Ang II, Ang-(1–9), and Ang I in kidney were higher than plasma levels. Ang II was the predominant angiotensin peptide in kidney, with Ang I levels approximately 70% of Ang II levels, and the des-Asp-angiotensin peptides were of much lower abundance. Of particular note were the levels of Ang-(1–9) in kidney, which were approximately half the levels of Ang I.

Addition of Ang II and Ang I to kidney homogenates before processing produced no significant changes in peptide levels apart from the expected changes in Ang II and Ang I, and a small but statistically significant increase in Ang I in the sample to which Ang II was added (Table 4). The absence of evidence for metabolism of angiotensin peptides in this experiment indicates that these peptides were stable in the GTC/TFA homogenate of kidney. A 60-second delay in homogenization of the kidney did not result in any significant change in the levels of angiotensin peptides measured (Table 5).

Effect of Perindopril on Plasma Angiotensin Converting Enzyme, Renin, and Angiotensinogen

Control levels of plasma ACE were 274±12 units/l (mean±SEM, n=6); these levels were reduced to 113±8 units/l (p<0.001) and 31±8 units/l (p<0.001) in rats administered 1.4 and 4.2 mg/kg perindopril,

### Table 1. Recovery of Angiotensin Peptides From Rat Plasma and Rat Kidney Homogenate

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peptide (%) recovery</th>
<th>Ang-(1–7)</th>
<th>Ang II</th>
<th>Ang-(1–9)</th>
<th>Ang I</th>
<th>Ang-(2–7)</th>
<th>Ang III</th>
<th>Ang-(2–9)</th>
<th>Ang-(2–10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td>85±5</td>
<td>44±8</td>
<td>61±6</td>
<td>43±13</td>
<td>50±8</td>
<td>45±15</td>
<td>37±4</td>
<td>36±7</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>62±14</td>
<td>41±14</td>
<td>51±12</td>
<td>38±10</td>
<td>42±9</td>
<td>48±11</td>
<td>37±11</td>
<td>34±12</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD; n=6 for plasma, and n=14 for kidney. Ang-(1–7), angiotensin(1–7); Ang II, angiotensin II; Ang-(1–9), angiotensin(1–9); Ang I, angiotensin I; Ang-(2–7), angiotensin(2–7); Ang III, angiotensin III; Ang-(2–9), angiotensin(2–9); Ang-(2–10), angiotensin(2–10).

### Table 2. Minimum Detectable Levels for Angiotensin Peptides in Plasma and Kidney

<table>
<thead>
<tr>
<th>Sample</th>
<th>Minimum detectable (fmol/ml plasma or fmol/g kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1.3, 1.9, 1.4, 2.0, 3.9, 3.8, 4.6, 4.7</td>
</tr>
<tr>
<td>Kidney</td>
<td>11.3, 8.8, 7.0, 9.5, 20, 15, 19, 21</td>
</tr>
</tbody>
</table>

Minimum detectable levels were calculated from the assay detection limit and have been corrected for peptide recovery (Table 1), antibody cross-reactivity, and sample volume. For plasma, the mean volume was approximately 3 ml; for kidney, 10 ml homogenate was extracted, which corresponded to approximately 0.75 g kidney. Ang-(1–7), angiotensin(1–7); Ang II, angiotensin II; Ang-(1–9), angiotensin(1–9); Ang I, angiotensin I; Ang-(2–7), angiotensin(2–7); Ang III, angiotensin III; Ang-(2–9), angiotensin(2–9); Ang-(2–10), angiotensin(2–10).
respectively. Control levels of renin were 10 ±1 pmol/ml/hr (mean±SEM, n=6); these levels increased to 413 ±94 pmol/ml/hr (p <0.01) and 2,003 ±428 pmol/ml/hr (p <0.001) in rats administered 1.4 and 4.2 mg/kg perindopril, respectively. Plasma angiotensinogen levels were reduced to 34% and 11% of control levels in rats administered 1.4 and 4.2 mg/kg perindopril, respectively (Table 6).

**Effect of Perindopril on Angiotensin Peptides in Plasma and Kidney**

Perindopril had markedly different effects on angiotensin peptide levels in plasma and kidney (Table 3). For plasma angiotensin peptides, perindopril caused approximately 10-fold increases in Ang I, Ang-(2-10), and Ang-(2-7). Plasma Ang-(1-7) levels increased 12- and 25-fold in rats administered 1.4 and 4.2 mg/kg perindopril, respectively, and the Ang-(1-7)/Ang I ratio showed a significant 3.5-fold increase for the higher dose of perindopril (p <0.01). Plasma levels of Ang II and Ang III did not show any significant change from control for either dose of perindopril.

In kidney, Ang II levels showed a marked decrease to 14% of control levels for the higher dose of perindopril (Table 3), and Ang-(1-9) also showed a significant decrease. In contrast to plasma, renal levels of Ang I, Ang-(2-10), and Ang-(1-7) did not increase with perindopril administration (Table 3).

These data indicate a differential regulation of angiotensin peptides in plasma and kidney in response to perindopril administration. However, both plasma and kidney showed evidence of inhibition of conversion of Ang I to Ang II, as indicated by the fall in Ang II/Ang I ratio that occurred (Figure 2).

**Effect of Perindopril on Renal Renin and Angiotensinogen**

Perindopril administration had no effect on renal renin levels: 455 ±38, 531 ±129, and 720 ±195 nmol/g/hr (mean±SEM, n=6) for control rats and rats administered 1.4 and 4.2 mg/kg perindopril, respectively. By contrast, renal angiotensinogen levels were reduced to 57% and 12% of control levels in rats administered 1.4 and 4.2 mg/kg perindopril, respectively (Table 6).

**Effect of Perindopril on Renin and Angiotensinogen Messenger RNA**

Perindopril administration had no significant effect on angiotensinogen mRNA levels in either liver or kidney (Figure 3). By contrast, renal renin mRNA levels increased 11- and 12-fold in rats administered 1.4 and 4.2 mg/kg perindopril, respectively.

### Table 3. Angiotensin Peptide Concentrations in Plasma and Kidney of Control Rats and Rats Treated With Either 1.4 mg/kg or 4.2 mg/kg Perindopril for 7 Days

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peptide (fmol/ml plasma or fmol/g kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ang-(1-7)</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5±2</td>
</tr>
<tr>
<td>Perindopril (1.4 mg/kg)</td>
<td>61±11*</td>
</tr>
<tr>
<td>Perindopril (4.2 mg/kg)</td>
<td>124±16*</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>33±8</td>
</tr>
<tr>
<td>Perindopril (1.4 mg/kg)</td>
<td>38±5</td>
</tr>
<tr>
<td>Perindopril (4.2 mg/kg)</td>
<td>27±3</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM; n=6. Ang-(1-7), angiotensin-(1-7); Ang II, angiotensin II; Ang-(1-9), angiotensin-(1-9); Ang I, angiotensin I; Ang-(2-7), angiotensin-(2-7); Ang III, angiotensin III; Ang-(2-9), angiotensin-(2-9); Ang-(2-10), angiotensin-(2-10).

*p <0.05 compared with control using t test.

---

### Table 4. Metabolism of Angiotensin I and II During Processing of Kidney Homogenate for Assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peptide (fmol/g kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ang-(1-7)</td>
</tr>
<tr>
<td>Control</td>
<td>33±7</td>
</tr>
<tr>
<td>Replicate plus 1,280 fmol Ang I/g kidney</td>
<td>37±8</td>
</tr>
<tr>
<td>Replicate plus 1,100 fmol Ang II/g kidney</td>
<td>23±5</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM; n=4. Angiotensin peptides are for replicate 10-ml aliquots of four separate pooled kidney homogenates; to one replicate was added 1,280 fmol Ang I, and to the other was added 1,100 fmol Ang II. Ang-(1-7), angiotensin-(1-7); Ang II, angiotensin II; Ang-(1-9), angiotensin-(1-9); Ang I, angiotensin I; Ang-(2-7), angiotensin-(2-7); Ang III, angiotensin III; Ang-(2-9), angiotensin-(2-9); Ang-(2-10), angiotensin-(2-10).

*p <0.05 compared with control, using paired t test.
by guest on December 25, 2017 http://hyper.ahajournals.org/ Downloaded from

trapping factor of 0.96) of 47.7.

Kidney are taken from Dellenback and Muelheims, 63 based on a

sinogen (102195)

the contribution of plasma to the measured amount of angioten-

Perindopril (4.2 mg/kg) 64±7f 17±3t 13±2t

Perindopril (1.4 mg/kg) 193±17f 72±12t 65±14f

Control 576±22 151±13 114±15

4.2 mg/kg Perindopril for 7 Days

TABLE

Control Rats and Rats Treated With Either 1.4 mg/kg or

Kidney levels on circulating angioten-

discussion

The use of HPLC and specific RIA in the present

study enabled the precise identification and measure-ment of eight angiotensin peptides in plasma and kidney. We have shown marked differences in the

levels and relative proportions of these eight peptides in these two tissues, and we have demonstrated a
differential regulation of these peptides in plasma and kidney of rats administered perindopril. Partic-

ular care was taken to validate the measurement of angiotensin peptides in kidneys. In agreement with

the present study, Mendelsohn40 has previously shown a lack of effect of a 60-second delay in processing of the kidneys on the measured levels of

Ang II. This does not indicate that renal angiotensin peptides are protected from metabolism, which is

very rapid in kidney, at least in vivo.1641 Rather, the relatively constant level of angiotensin peptides dur-

ing the delay before processing indicates that during this time peptide metabolism approximates the rate

of production of these peptides in kidneys.

The data presented here for renal levels of angio-
tensin peptides, renin, and angiotensinogen are open
to more than one interpretation. We have chosen to
interpret the angiotensin peptide levels measured in

kidney as being largely extracellular angiotensins
generated by the action of extracellular renin on

extracellular angiotensinogen. As discussed below,
several lines of evidence indicate a dependence of
renal angiotensinogen levels on circulating angioten-

sinogen and thus support our proposal that renal angiotensinogen is largely extracellular. With regard
to renin, it should be noted that the renal renin levels measured represent, in large part, intracellular stores of renin, which were relatively constant with perin-
dopril administration, despite a large increase in cir-culating renin levels. Morphological studies indi-
cate that most renin-secreting cells of the kidney are

on the outer aspect of the vascular wall,42 supporting

the view that renin is secreted mainly into the

interstitium of the kidney.41–45 We consider that

extracellular renin levels in the kidney would have
increased markedly with perindopril administration,
in parallel with the 200-fold increase in circulating renin levels and, by inference, a similar increase in
renin secretion rate.

The present estimates of Ang II levels in rat kidney
are in close agreement with previous studies using
alternative methodology, including the use of HPLC
or thin-layer chromatography to separate angiotensin peptides before RIA.46–48 However, Matsushima et
al48 have reported much higher levels of Ang I in
kidney than found in the present study. These au-
thors collected kidneys into liquid nitrogen, and the
high renal levels of Ang I reported may reflect a
failure to prevent Ang I generation during freezing
and thawing of the tissue. These authors have also
reported much higher plasma levels of Ang I than
found in the present study, which may also be due to
failure to prevent Ang I generation during sample
processing, given that these authors did not collect
blood into a renin inhibitor solution. In the present
study, Ang I generation in plasma was prevented by
use of the renin inhibitor H-77.

Renal levels of Ang-(1–7), Ang II, Ang-(1–9), and
Ang I in control rats were all higher than circulating
levels, consistent with their local production in kid-

nies.1641 The present data relate to peptide levels in whole kid-
ney, and it should be noted that much higher peptide concentrations will exist in specific compart-
ments of the kidney, as demonstrated for

Ang II by Seikaly et al49 for star vessel plasma,
glomerular filtrate, and proximal tubular fluid.

Although acute administration of CEI causes a fall
in plasma Ang II levels in rats,12 chronic administra-

tion is associated with normal plasma Ang II levels,13
as shown in the present study, or elevated plasma
Ang II levels.12 Similarly, in human, plasma Ang II

TABLE 5. Effect of Time Delay Before Homogenization on Angiotensin Peptide Levels in Rat Kidney

Sample  Peptide (fmol/g kidney)

Ang-(1–7)  Ang II  Ang-(1–9)  Ang I  Ang-(2–7)  Ang III  Ang-(2–9)  Ang-(2–10)

Left kidney homogenized 30 seconds after decapitation 17±6 140±20 37±13 103±21 <20 <15 <19 <21

Right kidney homogenized 90 seconds after decapitation 25±5 156±20 29±4 122±21 <20 <15 <19 <21

Data are presented as mean±SEM; n=4. For each of four rats, the left kidney was homogenized 30 seconds after decapitation and the right kidney homogenized after a time delay of 60 seconds (i.e., 90 seconds after decapitation). Ang-(1–7), angiotensin-(1–7); Ang II, angiotensin II; Ang-(1–9), angiotensin-(1–9); Ang I, angiotensin I; Ang-(2–7), angiotensin-(2–7); Ang III, angiotensin III; Ang-(2–9), angiotensin-(2–9); Ang-(2–10), angiotensin-(2–10).

TABLE 6. Levels of Angiotensinogen in Plasma and Kidney of Control Rats and Rats Treated With Either 1.4 mg/kg or 4.2 mg/kg Perindopril for 7 Days

Rat group  Plasma (pmol/ml)  Total (pmol/g)  Corrected* (pmol/g)

Control  576±22 151±13 114±15

Perindopril (1.4 mg/kg)  193±17† 72±12† 65±14†

Perindopril (4.2 mg/kg)  64±7† 17±3† 13±2†

Data are presented as mean±SEM; n=6.

*Data for renal angiotensinogen levels have been corrected for the contribution of plasma to the measured amount of angiotensinogen (102 μl plasma/g) and for the blood space of the kidney (195 μl/g). These estimates of plasma space and blood space of the kidney are taken from Dellenback and Muelheims,49 based on a red blood cell volume of 9.28 μl/g and a hematocrit (corrected for trapping factor of 0.96) of 47.7.

†p<0.05 compared with control.
levels return to normal within 24 hours of CEI administration despite a maintained inhibition of ACE.9-11 The return of plasma Ang II levels to normal despite continued inhibition of ACE can be accounted for by the marked elevation of circulating renin and Ang I levels, sufficient to overcome the effects of CEI.11 In the present study, rats were killed several hours after they had ingested perindopril in their drinking water, given that rats drink mostly in the evening hours. Thus, the plasma and tissue concentrations of perindopril may have been less than maximal, and plasma Ang II levels may have been suppressed if measurements had been made closer to the time of ingestion of perindopril. The doses of perindopril administered have been shown to produce near-maximal inhibition of plasma ACE (confirmed in this study by measurement of both ACE activity and Ang II/Ang I ratio) and reduction of blood pressure in spontaneously hypertensive rats.4,22,24,27 We found no evidence for a carboxypeptidase-mediated alternate pathway for conversion of Ang I to Ang II, in that plasma Ang-(1-9) levels were very low, even in rats receiving perindopril. However, the present data cannot exclude the possible existence of an alternate pathway of direct conversion of Ang I to Ang II, such as may be mediated by a serine protease.50

Jackson et al24 have shown that the doses of perindopril used in this study produce equivalent inhibition of ACE activity in plasma and kidney. The present results confirm this by showing a similar reduction in Ang II/Ang I ratio in plasma and kidney with perindopril administration. The response of individual angiotensin peptides to perindopril administration was very different in kidney and plasma, in that renal Ang II levels fell to 14% of control and renal Ang I levels did not increase. Mendelsohn40 has previously described a significant reduction in renal Ang II levels after acute CEI administration. The marked reduction of renal Ang II levels indicates that at least 86% of renal Ang II is produced by an ACE-mediated pathway. The significant levels of Ang-(1-9) in kidney clearly indicate a role for carboxypeptidases in renal metabolism of Ang I. However, no increase in Ang-(1-9) formation was observed with perindopril administration, and the marked fall in Ang II levels indicates that Ang-(1-9) makes little, if any, contribution to Ang II formation in the kidney.

The failure of renal Ang I levels to increase with perindopril administration is of considerable importance, given that circulating renin levels, and thus renal renin secretion rate, were increased 200-fold. Analogous to the argument proposed for the main-
tenance of plasma Ang II levels during perindopril administration, it can be argued that the failure of renal Ang I levels to increase was a critical determinant of the degree to which renal Ang II levels fell with perindopril administration. These data provide direct support for the hypothesis that the hypotensive action of CEI is dependent on an effect on Ang II levels in tissue rather than in plasma and also provide support for the proposal that inhibition of renal ACE is important for the antihypertensive effect of CEI.

CEI administration is associated with marked increases in circulating renin, with consequent consumption of angiotensinogen and accumulation of des-Ang I-angiotensinogen. The fall in plasma levels of angiotensinogen has an important rate-limiting effect on Ang I production as illustrated in the present study, in which plasma Ang I levels increased only 10-fold, compared with a 200-fold increase in circulating renin levels. The failure of renal Ang I levels to increase with perindopril administration led us to propose that renal levels of angiotensinogen may have a similar role in limiting Ang I production in the kidney. In the present study, we measured renal levels of angiotensinogen, taking particular care to control for nonspecific interference in the assay and to correct for metabolism of angiotensinogen during tissue processing. For control rats, the levels of angiotensinogen in kidney were 20% of plasma levels, which is consistent with a concentration in interstitial fluid approximating that of plasma. We measured only the angiotensinogen content of whole kidney. Given that different compartments of the kidney have very different concentrations of renin and Ang II, those compartments with high renin levels would be predicted to show more marked depletion of angiotensinogen, which would thus more severely impair Ang I generation in kidney than is apparent in plasma. Previous studies have demonstrated a dependence of renal angiotensin production on circulating angiotensinogen. Further evidence for the importance of angiotensinogen in determining renal angiotensin peptide levels is the increased renal vascular resistance that occurs in subjects with raised plasma angiotensinogen levels due to oral contraceptive therapy.

Renal renin mRNA levels increased 11- to 12-fold with perindopril administration, in contrast to the 200-fold increase in circulating renin that occurred. This result is in agreement with the previous demonstration of nonproportional changes in plasma renin and renal renin mRNA levels. Perindopril administration was associated with no significant change in hepatic or renal angiotensinogen mRNA levels, in agreement with a previous study by Gomez et al. By contrast, several authors have reported a reduction in hepatic and renal angiotensinogen mRNA levels in response to either acute or chronic administration of CEI. The failure of hepatic and renal synthesis of angiotensinogen to increase in response to the marked depletion of angiotensinogen during converting enzyme inhibition is consistent with the proposal that basal levels of Ang II exert a tonic stimulation of angiotensinogen synthesis in these two tissues.

Plasma levels of Ang-(1–7) increased 25-fold with perindopril administration. We have previously observed a 9.4-fold increase in plasma Ang-(1–7) levels in hypertensive subjects receiving CEI therapy, and similar results have recently been reported for the dog. Whereas Ang-(1–7) was present in significant levels in kidney, these levels did not change in response to perindopril. These data indicate the importance of this endopeptidase-mediated alternate pathway for Ang I metabolism in both plasma and kidney. Moreover, the 3.5-fold increase in Ang-(1–7)/Ang I ratio in plasma is consistent with the hypothesis that CEI may increase Ang-(1–7)–generating endopeptidase levels in a manner similar to the increase in plasma ACE that occurs with CEI administration.

The present data examine the apparent dissociation between the 200-fold increase in circulating

![Figure 3. Bar graphs show renin messenger RNA (mRNA) levels in kidney (panel A) and angiotensinogen mRNA levels in kidney (panel B) and liver (panel C) of control rats and rats administered either 1.4 or 4.2 mg/kg perindopril in their drinking water for 7 days. Data for mRNA (mean ± SEM, n=6) are expressed as ratio to control and have been corrected for mRNA recovery and normalized to wet weight of tissue. *p<0.05 compared with control.](http://hyper.ahajournals.org/cover)
Renin levels and the normal levels of circulating Ang II during perindopril administration. Although other effects of CEI, such as reduced blood pressure, may contribute, the present data suggest that the major stimulus to the marked increase in renin secretion is the reduction in renal levels of Ang II. Inasmuch as renal levels of Ang II are much higher than circulating levels in the basal state, such an interpretation is consistent with the hypothesis that renal levels of Ang II are more important than circulating Ang II levels in the regulation of renin secretion.

In conclusion, our demonstration of the differential regulation of angiotensin peptides in plasma and kidney provides direct support for the hypothesis that the hypertensive effect of CEI is dependent on an effect on Ang II levels in tissue rather than in plasma and also provides support for the proposal that inhibition of renal ACE is important for the antihypertensive effect of CEI. Moreover, the failure of kidney Ang I levels to increase with perindopril treatment, taken together with the fall in kidney angiotensinogen levels, suggests that angiotensinogen may be a major rate-limiting determinant of angiotensin peptide levels in the kidney. Furthermore, we have confirmed the importance of the Ang-(1-7) pathway in Ang I metabolism in both plasma and kidney. Although Ang-(1-9) levels in kidney were significant, we found no evidence for an important role for a carboxypeptidase-mediated alternate pathway in the conversion of Ang I to Ang II in either plasma or kidney.

Acknowledgment

We are grateful to Thaddeus P. Gorski for performing the assays for plasma ACE.

References

Differential regulation of angiotensin peptide levels in plasma and kidney of the rat.
D J Campbell, A C Lawrence, A Towrie, A Kladis and A J Valentijn

Hypertension. 1991;18:763-773
doi: 10.1161/01.HYP.18.6.763

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/18/6/763

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in
Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial
Office. Once the online version of the published article for which permission is being requested is located, click
Request Permissions in the middle column of the Web page under Services. Further information about this
process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/