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

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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 14% 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.

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.

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. Moreover, the inhibition of renal ACE by various CEI shows a consistently close relation with their effect on blood pressure. 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.

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. Drummer et al have reported chronic administration of the CEI enalapril causes an induction of the carboxypeptidases responsible for this alternate pathway in subcellular fractions of kidney. Johnson et al 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. 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.

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+ 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. 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 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-77 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. 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 52, 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) trifluoroacetic 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 antibody raised against Ang-(1–9), a generous gift from O.H. Drummer (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), and 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-furylacryloyl)-L-phe-nylalanyl-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 phenylmethylsulfonyl 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, 100 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.

**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...
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, 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.celb, which contains the full length rat renin cDNA37 subcloned into pGEM-4, a generous gift from K.R.
Lynch. Globin cRNA was synthesized from plasmid pβ-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,38 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×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.39 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</th>
<th>% Recovery</th>
<th>Plasma</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ang-(1-7)</td>
<td>85±5</td>
<td>62±14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ang II</td>
<td>44±8</td>
<td>41±14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ang-(1-9)</td>
<td>61±6</td>
<td>51±12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ang I</td>
<td>43±13</td>
<td>38±10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ang-(2-7)</td>
<td>50±8</td>
<td>42±9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ang III</td>
<td>45±15</td>
<td>48±11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ang-(2-9)</td>
<td>37±4</td>
<td>37±11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ang-(2-10)</td>
<td>36±7</td>
<td>34±12</td>
<td></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>Peptide</th>
<th>Minimum detectable (fmol/ml plasma or fmol/g kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ang-(1-7)</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Ang II</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>Ang-(1-9)</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Ang I</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Ang-(2-7)</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Ang III</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>Ang-(2-9)</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>Ang-(2-10)</td>
<td>4.7</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).
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>
<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>5±2</td>
<td>47±17</td>
<td>&lt;1.4</td>
<td>100±35</td>
<td>5±2</td>
<td>29±8</td>
<td>&lt;4.6</td>
<td>34±12</td>
</tr>
<tr>
<td>Perindopril (1.4 mg/kg)</td>
<td></td>
<td>61±11*</td>
<td>16±4</td>
<td>3±1*</td>
<td>866±129*</td>
<td>43±5*</td>
<td>17±4</td>
<td>&lt;4.6</td>
<td>378±36*</td>
</tr>
<tr>
<td>Perindopril (4.2 mg/kg)</td>
<td></td>
<td>124±16*</td>
<td>30±11</td>
<td>2±1</td>
<td>919±59*</td>
<td>53±7*</td>
<td>18±4</td>
<td>&lt;4.6</td>
<td>442±37*</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>33±8</td>
<td>338±33</td>
<td>113±19</td>
<td>234±33</td>
<td>&lt;20</td>
<td>&lt;15</td>
<td>&lt;19</td>
<td>37±5</td>
</tr>
<tr>
<td>Perindopril (1.4 mg/kg)</td>
<td></td>
<td>38±5</td>
<td>98±9*</td>
<td>46±3*</td>
<td>138±26*</td>
<td>&lt;20</td>
<td>&lt;15</td>
<td>&lt;19</td>
<td>34±9</td>
</tr>
<tr>
<td>Perindopril (4.2 mg/kg)</td>
<td></td>
<td>27±3</td>
<td>48±6*</td>
<td>37±6*</td>
<td>162±18</td>
<td>&lt;20</td>
<td>&lt;15</td>
<td>&lt;19</td>
<td>41±5</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.

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 3). These data indicate a differential regulation of renal renin and angiotensinogen 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).

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>
<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>Control</td>
<td></td>
<td>33±7</td>
<td>254±26</td>
<td>65±9</td>
<td>100±14</td>
<td>&lt;20</td>
<td>&lt;15</td>
<td>&lt;19</td>
<td>&lt;21</td>
</tr>
<tr>
<td>Replicate plus 1,280 fmol Ang II/1 g kidney</td>
<td></td>
<td>37±8</td>
<td>246±34</td>
<td>59±9</td>
<td>992±78*</td>
<td>&lt;20</td>
<td>&lt;15</td>
<td>&lt;19</td>
<td>&lt;21</td>
</tr>
<tr>
<td>Replicate plus 1,100 fmol Ang II/1 g kidney</td>
<td></td>
<td>23±5</td>
<td>1,547±105*</td>
<td>80±19</td>
<td>142±9*</td>
<td>&lt;20</td>
<td>&lt;15</td>
<td>&lt;21</td>
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</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.
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 perindopril administration, despite a large increase in circulating renin levels. Morphological studies indicate that most renin-secreting cells of the kidney are on the outer aspect of the vascular wall,\textsuperscript{42} supporting the view that renin is secreted mainly into the interstitium of the kidney.\textsuperscript{43-45} 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.\textsuperscript{46-48} However, Matsushima et al\textsuperscript{48} have reported much higher levels of Ang I in kidney than found in the present study. These authors 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 kidney.\textsuperscript{16,41} The present data relate to peptide levels in whole kidney, and it should be noted that much higher peptide concentrations will exist in specific compartments of the kidney, as demonstrated for Ang II by Seikaly et al\textsuperscript{49} 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,\textsuperscript{12} chronic administration is associated with normal plasma Ang II levels,\textsuperscript{13} as shown in the present study, or elevated plasma Ang II levels.\textsuperscript{12} Similarly, in humans, plasma Ang II

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

<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>Left kidney homogenized 30 seconds after decapitation</td>
<td>17±6</td>
</tr>
<tr>
<td>Right kidney homogenized 90 seconds after decapitation</td>
<td>25±5</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM; n=6. 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

<table>
<thead>
<tr>
<th>Rat group</th>
<th>Plasma (pmol/ml)</th>
<th>Total (pmol/g)</th>
<th>Corrected* (pmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>576±22</td>
<td>151±13</td>
<td>114±15</td>
</tr>
<tr>
<td>Perindopril (1.4 mg/kg)</td>
<td>193±17†</td>
<td>72±12†</td>
<td>65±14†</td>
</tr>
<tr>
<td>Perindopril (4.2 mg/kg)</td>
<td>64±17†</td>
<td>17±3†</td>
<td>13±2†</td>
</tr>
</tbody>
</table>

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 (102μl plasma/g) and for the blood space of the kidney (195μl/g). These estimates of plasma space and blood space of the kidney are taken from Dellenback and Muelheims,\textsuperscript{59} based on a red blood cell volume of 92.8μl/g and a hematocrit (corrected for trapping factor of 0.96) of 47.7.
Plasma

<table>
<thead>
<tr>
<th>Control</th>
<th>Perindopril 1.4 mg/kg</th>
<th>Perindopril 4.2 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang II</td>
<td>80 ± 20</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>Ang I</td>
<td>1000 ± 100</td>
<td>800 ± 80</td>
</tr>
<tr>
<td>Ang II/Ang I ratio</td>
<td>0.8 ± 0.05</td>
<td>0.6 ± 0.04</td>
</tr>
</tbody>
</table>

Kidney

<table>
<thead>
<tr>
<th>Control</th>
<th>Perindopril 1.4 mg/kg</th>
<th>Perindopril 4.2 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang II</td>
<td>400 ± 40</td>
<td>100 ± 20</td>
</tr>
<tr>
<td>Ang I</td>
<td>300 ± 30</td>
<td>200 ± 20</td>
</tr>
<tr>
<td>Ang II/Ang I ratio</td>
<td>2.0 ± 0.2</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
</table>

**FIGURE 2.** Line graphs show angiotensin II (Ang II), angiotensin I (Ang I), and Ang II/Ang I ratios for plasma and kidney of control rats and rats administered either 1.4 or 4.2 mg/kg perindopril in their drinking water for 7 days. Each point represents the mean ± SEM (n=6). *p<0.05 compared with control.

Levels return to normal within 24 hours of CEI administration despite a maintained inhibition of ACE. 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. Jackson et al 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. Mendelsohn 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-
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.

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.51 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,49 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.52 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.53

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.54-55 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.56 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.55-57-58 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.59-60

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,28 and similar results have recently been reported for the dog.64 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.62

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

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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

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