Effects of Converting Enzyme Inhibitors on Angiotensin and Bradykinin Peptides

Duncan J. Campbell, Athena Kladis, Ann-Marie Duncan

Abstract We examined the dose-related effects of angiotensin-converting enzyme inhibitors on circulating and tissue levels of angiotensin and bradykinin peptides by administering perindopril or lisinopril to rats in drinking water for 7 days. A reduction in the ratio of plasma angiotensin II (Ang II) to Ang I was seen for 0.006 mg/kg per day perindopril, with an increase in plasma renin and Ang I at 0.017 mg/kg per day. Plasma Ang II levels did not decrease until 1.4 mg/kg per day perindopril, at which dose plasma Ang I levels reached a plateau of an approximate 25-fold increase. The effects of perindopril on Ang II and Ang I levels in heart, lung, aorta, and brown adipose tissue were parallel to those observed for plasma. By contrast, renal Ang I levels did not increase, and renal Ang II levels decreased by 40% at 0.017 mg/kg per day, the same threshold seen for the increase in plasma renin. Perindopril increased circulating bradykinin-(1-9) levels approximately eightfold, with a threshold dose of 0.052 mg/kg per day, and increased bradykinin-(1-9) levels in kidney, heart, and lung in parallel with the changes observed for plasma. By contrast, aortic and brown adipose tissue bradykinin-(1-9) and bradykinin-(1-7) levels increased severalfold for perindopril doses as low as 0.006 mg/kg per day. Lisinopril also increased aortic bradykinin-(1-9) and bradykinin-(1-7) levels at doses below the threshold for the decrease in the ratio of Ang II to Ang I. These data indicate that renal Ang II levels and vascular bradykinin-(1-9) levels respond to low doses of converting enzyme inhibitor and may be important mediators of the effects of these compounds. The parallel increases in bradykinin-(1-9) and bradykinin-(1-7) levels in aorta and brown adipose tissue, at inhibitor doses below the threshold for inhibition of Ang I conversion, may result from a mechanism different from inhibition of "classic" angiotensin-converting enzyme. (Hypertension. 1994;23:439-449.)

Key Words • angiotensins • radioimmunoassay • bradykinins • chromatography, high performance liquid

Angiotensin-converting enzyme (ACE, kininase II, dipeptidyl carboxypeptidase, peptidyl dipeptide hydrolase; EC 3.4.15.1) has many different substrates. These include angiotensin I (Ang I), which is converted to Ang II, a potent vasoconstrictor with a major role in the regulation of blood pressure, fluid, and electrolyte homeostasis, and bradykinin-(1-9) [BK-(1-9)], a potent vasodilator that is converted to BK-(1-7), an inactive metabolite.

ACE inhibitors are valuable therapeutic agents for the management of hypertension and cardiac failure. These agents have also been shown to prevent or reverse some forms of cardiac hypertrophy and to prevent neointima formation in experimental animals. The extent to which inhibition of Ang I and BK-(1-9) metabolism contributes to these effects of ACE inhibitors remains to be defined. A large body of data supports the proposal that many of the effects of ACE inhibitors are due to the inhibition of Ang II formation. Moreover, accumulating evidence indicates that some of the antihypertensive and cardioprotective effects of ACE inhibitors may be due to reduced BK-(1-9) degradation, with resultant increased endogenous BK-(1-9) levels. Studies with the type 2 bradykinin receptor antagonist Hoe 140 also implicate BK-(1-9) in mediating the prevention of neointima formation by ACE inhibitors.

In the present study we investigated the mechanism of action of ACE inhibitors by studying the effect of a range of doses of two ACE inhibitors, perindopril and lisinopril, on circulating and tissue levels of angiotensin and bradykinin peptides. Using high-performance liquid chromatography (HPLC)-based radioimmunoassays we measured Ang-(1-7), Ang II, Ang I, BK-(1-7), and BK-(1-9) in plasma, blood, kidney, heart, lung, aorta, and brown adipose tissue. We monitored inhibition of Ang I conversion to Ang II and BK-(1-9) conversion to BK-(1-7) by determining the effects of each ACE inhibitor on the Ang II/Ang I and BK-(1-7)/BK-(1-9) ratios in each tissue. In addition to demonstrating the inhibition of both Ang I conversion to Ang II and BK-(1-9) conversion to BK-(1-7), these studies provide evidence suggesting that ACE inhibitors increase vascular BK-(1-9) levels by a mechanism different from inhibition of "classic" ACE.

Methods

Animals

Male Sprague-Dawley rats (250 to 300 g), maintained in a room with a 12-hour light/dark cycle (lights on 6 AM to 6 PM), were fed a diet of GR 2+ pellets (Clarke King & Co) and received tap water to drink. These studies were performed in accordance with the guidelines of the St Vincent's Hospital Animal Experimentation Ethics Committee.

In an initial dose-finding study, angiotensin and bradykinin peptides were measured in kidney, heart, lung, aorta, and brown adipose tissue of control rats and rats administered perindopril (1.4 and 4.2 mg/kg per day) in the drinking water for 7 days (6 rats per group). The results obtained for kidney...
have been published. Two further studies were performed whereby plasma renin, angiotensinogen, and ACE enzymatic activity, plasma angiotensin peptides, blood bradykinin peptides, and angiotensin and bradykinin peptides in kidney, heart, lung, aorta, and brown adipose tissue were measured in rats receiving either low-dose perindopril (0, 0.006, 0.017, 0.052, 0.156 mg/kg per day) or high-dose perindopril (0, 0.0467, 1.4, 4.2, 12.6 mg/kg per day) administered in the drinking water for 7 days. Both the low-dose and high-dose experiments were performed twice with 20 rats per experiment (4 rats per group), resulting in a total of 8 rats for each perindopril dose. There were separate control groups for the low-dose and high-dose experiments. In a separate experiment, plasma angiotensin peptides and aortic angiotensin and bradykinin peptides were measured in rats administered lisinopril (0, 0.006, 0.017, 0.052, 0.156 mg/kg per day) in the drinking water for 7 days. Drinking water containing ACE inhibitor was freshly prepared each evening. Water intake was approximately 30 mL per rat per 24 hours, and the concentrations of ACE inhibitor in drinking water were adjusted to provide the required dose. Perindopril and lisinopril were generous gifts from Servier Laboratories and ICI Pharmaceuticals, respectively. Rats were killed by decapitation between 10 AM and noon without prior anesthetic, unless specified otherwise.

**Extraction and Radioimmunoassay of Angiotensin Peptides From Plasma**

Trunk blood (2 to 3 mL) was rapidly collected in tubes containing 0.5 mL inhibitor solution (1 mmol/L SQ-30,697, 146 µmol/L pepstatin, 50 mmol/L 1,10-phenanthroline, 125 mmol/L EDTA, 2 g/L neomycin sulfate, 2% dimethyl sulfoxide, and 2% ethanol in water) at 4°C. The renin inhibitor SQ-30,697 was a generous gift from Bristol-Myers Squibb Pharmaceutical Research Institute. The final plasma concentration of SQ-30,697 (100 to 200 µmol/L) was sufficient to cause complete inhibition of rat renin (unpublished data from our laboratory). The blood was centrifuged and the plasma (1 to 2 mL) was immediately extracted with Sep-Pak C18 cartridges (Waters Chromatography Division). Angiotensin peptides were acetylated before HPLC, and assay of HPLC fractions was performed with N-terminal-directed radioimmunoassay.

**Extraction and Radioimmunoassay of Angiotensin and Bradykinin Peptides From Tissues**

Kidney, heart (cardiac ventricles), lung, aorta, and periaortie brown adipose tissue were rapidly removed, weighed, and immediately homogenized in 4 mol/L guanidine thiocyanate (GTC) and 1% (vol/vol) trifluoroacetic acid (TFA) in water and then processed as described previously before acetylation, HPLC, and measurement of angiotensin and bradykinin peptides by N-terminal-directed radioimmunoassay. The time delay between decapitation and homogenization of the kidney was 60 seconds; heart and lung were homogenized within 90 seconds, and aorta and brown adipose tissue were homogenized within 150 seconds.

**Extraction and Radioimmunoassay of Bradykinin Peptides From Blood**

The effects of perindopril on blood levels of BK-(1-7) and BK-(1-9) were studied in separate groups of rats. After receiving perindopril in the drinking water for 7 days, rats were anesthetized with ether, and 2 mL blood was withdrawn from the inferior vena cava directly into a syringe containing 10 mL GTC/TFA. The blood and GTC/TFA were then mixed, homogenized by a Polytron, and processed as described previously. By collecting 2 mL blood into 10 mL GTC/TFA, we achieved lower minimum detectable levels for blood kinins [BK-(1-7): 0.7 fmol/mL; BK-(1-9): 0.4 fmol/mL] than for our earlier study in which 2 mL blood was collected into 20 mL GTC/TFA and only half the sample extracted. Low-dose and high-dose perindopril were studied in separate experiments with 40 rats per experiment (8 rats per group).

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

The plasma concentrations of ACE enzymatic activity, active renin, and angiotensinogen were measured as described previously. ACE enzymatic activity was measured using 3-(2-furylacryloyl)-L-phenylalanine-glycyl-glycine (FAPGG) as substrate.

**Statistical Analysis**

Data are presented as mean ± SEM. When more than half of the samples comprising a mean had values below the minimum detectable level, the sample mean is shown as less than the minimum detectable. For each experiment, data were calculated as the ratio to the mean of the respective control group. Data from the initial dose-finding study (0, 1.4, 4.2 mg/kg per day perindopril) were pooled with the data from the high-dose perindopril study. Data from the five rat groups (including control) were analyzed by ANOVA for the low-dose and high-dose studies separately. Comparisons with control were examined by Dunnett's two-tailed test. When values were below the minimum detectable, they were set at half the minimum detectable for statistical calculations. Logarithmic transformation of data was performed when appropriate to obtain similar variances among groups. Analyses were performed using SuperANOVA (Abacus Concepts).

**Results**

Circulating and tissue levels of angiotensin and bradykinin peptides for the pooled control rat groups are given in the Table. The blood levels of BK-(1-7) and BK-(1-9) in control rats were lower than previously reported from this laboratory; this may be due to the sampling of blood from the inferior vena cava in this study, whereas in previous studies arterial blood was collected. Blood levels of BK-(1-7) and BK-(1-9) in control rats were less than the minimum detectable for 6 of 15 samples and 12 of 16 samples, respectively. Control plasma levels of ACE, renin, and angiotensinogen were 241 ± 14 U/L, 11 ± 2 pmol/mL per hour, and 484 ± 23 pmol/mL, respectively (mean ± SEM, n = 16).

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

The dose-related effects of perindopril on plasma ACE enzymatic activity, renin, and angiotensinogen are shown in Fig 1. Plasma ACE activity showed a progressive inhibition throughout the range of perindopril doses studied, with a threshold dose of 0.017 mg/kg per day; ACE activity was still measurable at 12.6 mg/kg per day, being approximately 14% of control. The threshold dose of perindopril for an increase in plasma renin levels was also 0.017 mg/kg per day, with a maximal 100-fold increase in response to 4.2 to 12.6 mg/kg per day. Plasma angiotensinogen levels showed a 40% decrease in response to 0.156 mg/kg per day perindopril, corresponding to an eightfold increase in plasma renin levels. With higher doses of perindopril, plasma angiotensinogen levels fell to approximately 9% of control (approximately 40 pmol/mL).

**Effect of Perindopril on Angiotensin Peptides**

The effects of perindopril on circulating and tissue levels of angiotensin peptides varied considerably, al-
Angiotensin and Bradykinin Peptide Levels in Plasma, Blood, and Tissues of Control Rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ang-(1-7), fmol/mL or fmol/g</th>
<th>Ang II, fmol/mL or fmol/g</th>
<th>Ang I, fmol/mL or fmol/g</th>
<th>Ang-(1-7)/ Ang I Ratio</th>
<th>Ang II/ Ang I Ratio</th>
<th>BK-(1-7), fmol/mL or fmol/g</th>
<th>BK-(1-9), fmol/mL or fmol/g</th>
<th>BK-(1-7)/ BK-(1-9) Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1.7±0.3</td>
<td>25±2</td>
<td>15±2</td>
<td>0.15±0.03</td>
<td>1.9±0.1</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Blood</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Kidney</td>
<td>28±4</td>
<td>107±10</td>
<td>38±3</td>
<td>0.8±0.1</td>
<td>3.0±0.2</td>
<td>35±7</td>
<td>57±6</td>
<td>0.55±0.06</td>
</tr>
<tr>
<td>Heart</td>
<td>&lt;4</td>
<td>5.6±0.7</td>
<td>2.7±0.4</td>
<td>...</td>
<td>2.7±0.4</td>
<td>7.6±1.2</td>
<td>13±2</td>
<td>0.59±0.05</td>
</tr>
<tr>
<td>Lung</td>
<td>4.0±0.4</td>
<td>55±5</td>
<td>3.3±0.5</td>
<td>1.7±0.3</td>
<td>25±5</td>
<td>14±2</td>
<td>28±3</td>
<td>0.58±0.06</td>
</tr>
<tr>
<td>Aorta</td>
<td>&lt;20</td>
<td>106±14</td>
<td>10±2</td>
<td>...</td>
<td>17±3</td>
<td>84±26</td>
<td>121±30</td>
<td>0.72±0.07</td>
</tr>
<tr>
<td>Brown adipose tissue</td>
<td>&lt;8</td>
<td>38±4</td>
<td>5±1</td>
<td>...</td>
<td>13±2</td>
<td>82±26</td>
<td>71±20</td>
<td>1.1±0.1</td>
</tr>
</tbody>
</table>

Ang indicates angiotensin; BK, bradykinin. Data are mean±SEM, n=15-28.

Angiotensin and Bradykinin Peptide Levels in Plasma, Blood, and Tissues of Control Rats

though both plasma and tissues showed a decrease in the Ang II/Ang I ratio, indicating effective inhibition of Ang I conversion. The Ang II/Ang I ratio for plasma showed a 30% decrease at 0.006 mg/kg per day perindopril and was a more sensitive index of ACE inhibition than plasma ACE enzymatic activity, with the ratio falling to 2% of control at 1.4 mg/kg per day (Fig 2). For tissues (Figs 3 through 7), the Ang II/Ang I ratio showed a significant decrease for perindopril doses of 0.467 mg/kg per day or less, falling to less than 10% of control for the higher doses of perindopril.

For plasma, both Ang-(1-7) and Ang I showed marked increases, reaching a maximal increase of approximately 25-fold at 0.467 mg/kg per day perindopril (Fig 2). By contrast, plasma Ang II levels showed no significant change until plasma Ang I levels reached a plateau, when Ang II levels fell to 30% to 40% of control at perindopril doses of 1.4 to 12.6 mg/kg per day. At these perindopril doses, plasma Ang II levels were approximately 10 fmol/mL (minimum detectable, approximately 0.9 fmol/mL), with no tendency to decrease further with higher doses of perindopril. The plasma Ang-(1-7)/Ang I ratio showed no change with perindopril administration.

For kidney, the effects of perindopril were quite different from those seen for plasma (Fig 3). Perindopril had little effect on Ang-(1-7) and Ang I levels, but Ang II levels showed a 40% decrease at 0.017 mg/kg per day perindopril, which was the threshold dose for an increase in plasma renin levels. With higher doses of perindopril, renal Ang II levels fell to 7% of control (approximately 7 fmol/g; minimum detectable, approximately 3 fmol/g). The renal Ang-(1-7)/Ang I ratio showed no change with perindopril administration.

For heart, aorta, and brown adipose tissue (Figs 4 through 6), the effects of perindopril were similar to those described for plasma. Ang I levels in these tissues reached a maximal twofold to fourfold increase at perindopril doses above 0.467 mg/kg per day, and a decrease in Ang II levels was not seen until perindopril doses of 0.467 mg/kg per day or higher. At 12.6 mg/kg per day perindopril, Ang II levels were below the minimum detectable for more than half the samples of each of these three tissues. The decrease in Ang II levels shown in Figs 4 through 6 may be underestimated because values below the minimum detectable were set at half this value for calculation of the ratio to mean control and for statistical calculations. The minimum detectable levels of Ang II were 2 fmol/g for heart, 12 fmol/g for aorta, and 3 fmol/g for brown adipose tissue. Ang-(1-7) levels in these tissues were less than the

![Graphs showing dose-response effects of perindopril on plasma levels of angiotensin-converting enzyme (A), renin (B), and angiotensinogen (C). Low-dose perindopril (0.006, 0.017, 0.052, 0.156 mg/kg per day) and high-dose perindopril (0.467, 1.4, 4.2, 12.6 mg/kg per day) were studied in separate experiments, each with its own control group, and data are expressed as the ratio to the mean of the respective control group, where each point represents mean±SEM, n=8. *P<.05, **P<.01 compared with control.](http://hyper.ahajournals.org/)

![Graph showing ratio to mean control for Ang-(1-7) Ang I ratio with perindopril administration.](http://hyper.ahajournals.org/)

![Graph showing ratio to mean control for Ang II Ang I ratio with perindopril administration.](http://hyper.ahajournals.org/)
Effect of Perindopril on Bradykinin Peptides

Perindopril had markedly different effects on circulating and tissue levels of BK-(1-7) and BK-(1-9). For blood, perindopril caused an eightfold increase in BK-(1-9) levels and a twofold increase in BK-(1-7) levels (Fig 2). Given the fact that the blood levels of BK-(1-7) and BK-(1-9) in control rats were less than the minimum detectable for 6 of 15 samples and 12 of 16 samples, respectively, the calculated increases and changes in the BK-(1-7)/BK-(1-9) ratio are only approximate. The threshold dose of perindopril for an increase in BK-(1-9) was 0.052 mg/kg per day. However, a significant decrease in the BK-(1-7)/BK-(1-9) ratio was not seen until 0.467 mg/kg per day perindopril, with a maximal decrease to 30% of control at higher perindopril doses.

For kidney (Fig 3), perindopril caused an approximate twofold increase in BK-(1-9) levels, although this achieved statistical significance only for 0.052 and 4.2 mg/kg per day perindopril. Renal BK-(1-7) levels showed no significant change, and the BK-(1-7)/BK-(1-9) ratio showed a significant decrease to approximately 70% of control at 0.052 and 1.4 to 4.2 mg/kg per day perindopril.

For heart (Fig 4), perindopril caused a twofold to threefold increase in BK-(1-9) levels, with no change in BK-(1-7) levels, and the BK-(1-7)/BK-(1-9) ratio fell to 50% of control at 12.6 mg/kg per day perindopril. Similar results were obtained for lung, in which BK-
(1-9) levels increased twofold to threefold. However, lung also showed a twofold increase in BK-(1-7) levels, and the BK-(1-7)/BK-(1-9) ratio fell to 50% to 60% of control (Fig 7).

For aorta and brown adipose tissue, all doses of perindopril increased both BK-(1-7) and BK-(1-9) levels; thus, the threshold dose of perindopril was less than 0.006 mg/kg per day. As shown in Figs 5 and 6, the extent of the increase in BK-(1-7) and BK-(1-9) levels varied between 3- and 17-fold for aorta and between 3- and 24-fold for brown adipose tissue. The bimodal shape of the dose-response curves for BK-(1-7) and BK-(1-9) levels in aorta and brown adipose tissue may be a consequence of the study of low-dose (0.006, 0.017, 0.052, 0.156 mg/kg per day) and high-dose perindopril (0.467, 1.4, 4.2, 12.6 mg/kg per day) perindopril in separate experiments. Aorta showed a significant 40% decrease in the BK-(1-7)/BK-(1-9) ratio for 0.467 to 12.6 mg/kg per day perindopril, but there was no significant decrease in the BK-(1-7)/BK-(1-9) ratio of brown adipose tissue.

Effect of Lisinopril on Plasma Angiotensin Peptides and Aortic Angiotensin and Bradykinin Peptides

Lisinopril had approximately one tenth the potency of perindopril with respect to its effects on plasma angiotensin peptide levels (Fig 8), with a threshold for increased Ang I levels and a decreased Ang II/Ang I ratio at 0.052 mg/kg per day. No change in plasma Ang II levels was seen for the lisinopril doses studied. At the doses studied, lisinopril had little effect on aortic Ang II levels, and Ang-(1-7) and Ang I levels remained below the minimum detectable. By contrast, lisinopril increased aortic levels of BK-(1-7) and BK-(1-9) by fourfold to fivefold, with a threshold dose of 0.017 mg/kg per day.

Discussion

The present study is the first detailed examination of the dose-related effects of ACE inhibition on circulating and tissue levels of angiotensin and bradykinin peptides and the first to demonstrate that ACE inhibitors in-
FIG 6. Line graphs show dose-response effects of perindopril on brown adipose tissue levels of angiotensin I (Ang I) (A); Ang II and the Ang II/Ang I ratio (B); and bradykinin-(1-7) [BK-(1-7)], BK-(1-9), and the BK-(1-7)/BK-(1-9) ratio (C). Low-dose perindopril (0.006, 0.017, 0.052, 0.156 mg/kg per day) and high-dose perindopril (0.467, 1.4, 4.2, 12.6 mg/kg per day) were studied in separate experiments, each with its own control group, and data are expressed as the ratio to the mean of the respective control group, where each point represents mean±SEM, n=7-14. *P<.05, **P<.01 compared with control.

For blood and tissues other than kidney, perindopril increased BK-(1-9) levels at doses much lower than those required to reduce Ang II levels, and it is of interest to consider to what extent the measured changes in Ang II and BK-(1-9) levels correspond to other effects of ACE inhibitors. The present data were obtained in normal male Sprague-Dawley rats, and one must be careful in extrapolating these data to other experimental models. Moreover, no previous study has examined such a broad range of ACE inhibitor doses. Perindopril doses as low as 0.1 mg/kg per day reduced blood pressure of stroke-prone spontaneously hypertensive rats,18 and 0.4 mg/kg per day perindopril reduced vascular hypertrophy in spontaneously hypertensive rats.19 Moreover, 0.5 mg/kg per day perindopril normalized blood pressure, left ventricular weight, and left ventricular isomyosin profile in rats with two-kidney, one clip hypertension.20 This perindopril dose also decreased the lesions of hypertensive microangiopathy in the unclipped kidney.21 Gohlke et al22 have reported that 0.01 mg/kg per day perindopril increased aortic levels of cyclic GMP in spontaneously hypertensive rats. Taken together with the present data, these studies suggest that perindopril has effects on blood pressure and cardiac and renal function at doses below the threshold for reduction in circulating and tissue (other than kidney) levels of Ang II and raise the possibility that the effects of perindopril are mediated by effects on renal Ang II levels and/or effects on tissue BK-(1-9) levels.

With respect to the renin-angiotensin system, the dose-related effects of perindopril demonstrate a complex interaction between the various components of the system and emphasize the fact that the effects of ACE inhibition on plasma and tissue levels of Ang II are dependent on the concomitant changes in Ang I levels, which themselves are dependent on the associated changes in renin and angiotensinogen.14,23,24 The threshold dose of perindopril for inhibition of ACE activity...
was at or below 0.006 mg/kg per day, as indicated by the plasma Ang II/Ang I ratio. At this dose, an approximate 20% decrease in plasma ACE enzymatic activity and approximate 30% increase in plasma renin occurred, but changes in these components did not achieve statistical significance until 0.017 mg/kg per day perindopril. Plasma Ang I levels did not increase to the same extent as renin and reached a plateau at 0.467 mg/kg per day, probably as a consequence of the fall in plasma angiotensinogen levels that occurred. For perindopril doses up to 0.467 mg/kg per day, the increase in plasma Ang I was sufficient to maintain plasma Ang II levels despite ACE inhibition. However, when Ang I levels were unable to increase further, higher perindopril doses caused plasma Ang II levels to fall.

Plasma Ang II levels remained at 30% to 40% of control levels despite an increase in perindopril dosage from 1.4 to 12.6 mg/kg per day. This persistence of plasma Ang II levels despite maximal ACE inhibition may represent Ang II formation by an alternate pathway of conversion of Ang I to Ang II. Several studies have provided evidence for Ang I conversion to Ang II by serine protease activity and Ang II formation by such an alternate pathway may have been amplified by the 20- to 30-fold increase in plasma Ang I levels. Nevertheless, the plasma Ang II/Ang I ratio fell to 1% of control at 12.6 mg/kg per day perindopril, indicating that for control rats ACE was responsible for 99% of plasma Ang II formation. Perindopril decreased the Ang II/Ang I ratio to less than 10% of control in all tissues, indicating effective inhibition of tissue ACE. This result is in agreement with studies of the inhibition of tissue ACE by perindopril using in vitro autoradiography. For heart, aorta, and brown adipose tissue, the changes in tissue levels of angiotensin peptides were similar to those for plasma, although the threefold to fourfold increases in Ang I levels were much less than for plasma. For each of these three tissues, perindopril caused Ang II to fall to levels below the minimum detectable. Lung differed from the other tissues in that the 30- to 50-fold increase in Ang I levels was similar to that of plasma. Moreover, pulmonary Ang II levels remained above the minimum detectable. This may represent Ang II formation by an alternate pathway, as suggested for plasma. The failure of pulmonary Ang-(1-7) levels to increase in parallel with Ang I levels may represent a limitation of this pathway of Ang I metabolism in lung. A similar limitation may also apply to heart, aorta, and brown adipose tissue, in which Ang-(1-7) levels did not increase with perindopril administration.

In contrast to the results obtained for plasma and the other tissues, perindopril did not increase renal Ang I levels, and renal Ang II levels were reduced by much lower perindopril doses. The threshold dose of perindopril for reduction of renal Ang II levels (0.017 mg/kg per day) was the same as that observed for an increase in plasma renin and Ang I levels, consistent with a major role for local renal Ang II levels in regulating renin release. These data suggest an important role for reduced renal Ang II levels in mediating the effects of ACE inhibitors. We have previously presented data in support of the argument that the failure of renal Ang I levels to increase during ACE inhibition is due to local exhaustion of angiotensinogen. The possible role of BK-(1-9) in mediating the effects of ACE inhibitors has been the subject of many inves-
tigations. Several studies have shown that ACE inhibitors increase circulating kinin levels\textsuperscript{29-37} and urinary kinin excretion,\textsuperscript{29,38,39} although other studies have reported that ACE inhibitors do not increase circulating kinin levels.\textsuperscript{33,38-41} For most of these studies, however, the very high levels reported for circulating immunoreactive kinins raise questions concerning the validity of their estimation.\textsuperscript{12} Indirect evidence for a role of BK-(1-9) in mediating some of the effects of ACE inhibitors has come from the use of BK-(1-9) antagonists, kinin antibodies, and aprotinin, which partially reverse the hypotensive effect of ACE inhibitors\textsuperscript{7,9,42-48} and also antibodies, and aprotinin, which partially reverse the effects of ACE inhibitors on renal papillary blood flow, urine flow, and sodium excretion\textsuperscript{49,50} on cardiac hypertrophy\textsuperscript{11} and on neointima formation.\textsuperscript{13}

In the present study perindopril administration increased BK-(1-9) levels in blood and tissues. We have performed an extensive validation of the methods used to measure bradykinin peptides in blood and tissues.\textsuperscript{12} As we have previously discussed,\textsuperscript{12} the measurement of tissue levels of bradykinin peptides presents methodological problems, the main one being the difficulty of preventing artifactual generation of bradykinin peptides due to activation of prekallikrein during dissection of the tissue before homogenization. The relatively large SEM for BK-(1-7) and BK-(1-9) in aorta and brown adipose tissue of control rats (Table) is a consequence of a large variation between experiments in the levels of these peptides in control rats, probably in part due to our failure to prevent artifactual generation of bradykinin peptides during collection of tissue. Artifactual generation of bradykinin peptides will contribute to the variance between animals for tissue bradykinin peptide levels and make it difficult to demonstrate statistical significance for increases of twofold or less. Another consequence of this increased variance may be the apparently bimodal dose-response curves we obtained when aortic and brown adipose tissue bradykinin peptide responses to low-dose and high-dose perindopril were measured in separate experiments (Figs 5 and 6). It is of note that the effects of low-dose perindopril on aortic levels of BK-(1-7) and BK-(1-9) were reproduced in our separate study of the effects of similar doses of lisinopril (Fig 8).

Given that artifactual generation of bradykinin peptides may have occurred during collection of tissues, it can be argued that the effects of such artifactual bradykinin generation on the measured levels of bradykinin peptides in tissues may have been amplified by ACE inhibition. We offer two responses to this argument. First, artifactual bradykinin generation will increase the measured bradykinin levels for tissues of control rats and thus make any effect of ACE inhibition on these levels more difficult to detect. Second, any effect of ACE inhibition on those bradykinin levels consequent to artifactual generation will be similar to the effects of ACE inhibition on bradykinin levels in vivo. Although the extent of artifactual bradykinin generation during collection of tissues cannot be estimated, it is likely that the relative effects of ACE inhibition on the measured levels of bradykinin peptides are a reliable indication of the relative effects of ACE inhibition on endogenous bradykinin levels in vivo. In support of this contention, the present results for the effects of perindopril on bradykinin peptide levels in tissues are largely in agreement with the results obtained for blood, in which the potential for artifactual generation of bradykinin peptides was minimized by collection of blood directly into GTC/TFA.

The failure of the BK-(1-7)/BK-(1-9) ratio to fall to the same extent as the Ang II/Ang I ratio during ACE inhibition reflects the operation of alternate pathways of conversion of BK-(1-9) to BK-(1-7).\textsuperscript{12} Moreover, the changes in BK-(1-7) and BK-(1-9) levels during perindopril administration suggest that perindopril had actions on bradykinin peptides additional to inhibition of “classic” ACE. This is most clearly evident for aorta and brown adipose tissue, in which BK-(1-7) and BK-(1-9) levels increased in parallel at perindopril doses at the threshold for inhibition of Ang I conversion. There are two possible explanations for this result: (1) Perindopril inhibited an alternative pathway of BK-(1-7) and BK-(1-9) metabolism, whereby these peptides were cleaved between residues 1 and 7, such that BK-(1-7) and BK-(1-9) increased in parallel when this pathway was inhibited; and (2) perindopril increased BK-(1-9) production.

With respect to the first proposal, some ACE inhibitors have been shown to inhibit aminopeptidase P,\textsuperscript{51} which metabolizes both BK-(1-7) and BK-(1-9). Whether perindopril inhibits aminopeptidase P has not been reported, but lisinopril does not inhibit this enzyme.\textsuperscript{51} Our demonstration that lisinopril also increased aortic levels of BK-(1-7) and BK-(1-9) at doses below the threshold for ACE inhibition shows that this action of ACE inhibitors was independent of inhibition of both "classic" ACE and aminopeptidase P. Other possible alternate pathways of BK-(1-7) and BK-(1-9) metabolism that may be inhibited by ACE inhibitors include enzymes that are related to or are isomers of ACE\textsuperscript{52,53} or that may be related to endopeptidase 24.15,\textsuperscript{54} which cleaves BK-(1-9) between residues 5 and 6.\textsuperscript{55} If such an alternate pathway exists, the present data suggest that it makes an important contribution to BK-(1-7) and BK-(1-9) metabolism in the vasculature and that vascular BK-(1-9) may play an important role in mediating some of the effects of ACE inhibitors. The increase in vascular levels of BK-(1-7) may have been responsible for the small increases in the levels of this peptide in blood and lung during perindopril administration.

With respect to the second proposal, further studies will be required to determine whether ACE inhibitors increase BK-(1-9) production. Possible mechanisms by which BK-(1-9) production may increase include increased local levels of kininogen and kallikrein activity, which may result from increased local synthesis. Increased kallikrein activity may also result from increased activation of prekallikrein or reduced inhibition of tissue kallikrein by kallikrein-binding protein.

In support of our finding that low doses of ACE inhibitor increase vascular levels of BK-(1-9), Gohlke et al\textsuperscript{52} reported that low-dose (0.01 mg/kg per day) ramipril and perindopril increased aortic levels of cyclic GMP in the rat. Moreover, Linz and Scholkkens\textsuperscript{11} reported that a low, nonhypotensive dose of ramipril (0.01 mg/kg per day) prevented or reversed cardiac hypertrophy in rats with aortic coarctation, an action that was reversed by Hoe 14011; however, this antihypertrophic
effect of low-dose ramipril was not confirmed by Rhaleb et al.56

Several lines of evidence support the proposal that ACE inhibitors increase BK-(1-9) levels by a mechanism independent of inhibition of "classic" ACE. For tight-binding ACE inhibitors such as perindopril and lisinopril, for which nanomolar concentrations cause 50% inhibition of ACE, one might predict that a given dose of inhibitor would have similar effects on the metabolism of Ang I (Km, 16 to 90 nM/L),57,58) and BK-(1-9) (Km, 0.18 to 1.0 nM/L).57,58) For example, captopril has a similar K (1.3 to 1.7 nmol/L) for inhibition of recombinant human ACE when either BK-(1-9) or hippuryl-His-Leu (Km, approximately 2 mmol/L) is used as substrate.58,59) Moreover, the administration of anti-ACE antisera causes similar effects on the dose-response curves for the pressor and depressor effects of Ang I and BK-(1-9), respectively.60,61) Thus, it is somewhat paradoxical that much lower doses of ACE inhibitor are required to potentiate the depressor effects of BK-(1-9) than are required to inhibit the pressor effects of Ang I.52,63) In addition, Bendhack et al54) have reported the potentiation by enalaprilat of the vasodilator effects of BK-(1-9) in isolated perfused rat mesenteric artery without any detectable effect on the vasoconstrictor actions of Ang I. Further evidence in support of the proposal that ACE inhibitors increase BK-(1-9) levels by a mechanism independent of inhibition of "classic" ACE is provided by Ondetti et al,65) who found a wide variation between different ACE inhibitors in the relative doses required for 50% inhibition of Ang I constrictor effects and for 50% of maximal augmentation of BK-(1-9) constrictor effects on guinea pig ileum. Given the fact that the doses of ACE inhibitor required to augment BK-(1-9)-mediated responses were lower than those required to prevent Ang I-mediated responses, these ACE inhibitor-specific differences in the relative inhibition of Ang I and augmentation of BK-(1-9) effects suggest that the metabolism of BK-(1-9) in guinea pig ileum is due to enzymes additional to ACE, with varying susceptibility to inhibition by different ACE inhibitors. In support of this proposition, Greene et al56) found that the ACE inhibitor pyroglytanyl-Lys-Trp-Ala-Pro potentiated the ileum-constrictor effects of bradykinin analogues resistant to cleavage by ACE. Based on their studies of Ang I and BK-(1-9) metabolism in isolated rabbit aortas, Gohlke et al57) have also suggested that ACE inhibitors prevent vascular BK-(1-9) degradation by inhibiting BK-(1-9)-degrading enzymes other than ACE.

Figs 1 and 2 reveal a discrepancy between the dose-related effects of perindopril on plasma ACE enzymatic activity, measured using FAPGG as substrate, and the plasma Ang II/Ang I ratio. Thus, for 0.156 mg/kg per day perindopril, plasma ACE enzymatic activity was 50% of control, whereas the plasma Ang II/Ang I ratio had fallen to 10% of control. Similar discrepancies between the effects of ACE inhibitors on plasma ACE enzymatic activity and plasma Ang II/Ang I ratio have been reported in previous studies.67,68) One possible explanation for this discrepancy is that FAPGG may be cleaved by enzymes other than "classic" ACE that are less susceptible to inhibition by perindopril. If this is the case, it is evident that higher doses of perindopril are able to inhibit these alternate FAPGG-cleaving enzymes.

In conclusion, our study of the dose-related effects of ACE inhibitors on circulating and tissue levels of angiotensin and bradykinin peptides has provided evidence for a role for both peptide systems in mediating the effects of ACE inhibitors. Renal Ang II levels and vascular BK-(1-9) levels responded to low doses of ACE inhibitor and may be important mediators of the effects of these compounds. The paradoxical increases in BK-(1-9) and BK-(1-7) levels in aorta and brown adipose tissue, at doses of inhibitor below the threshold for inhibition of Ang I conversion, may result from a mechanism different from inhibition of "classic" ACE.

Acknowledgments

This study was funded by grants from the National Health and Medical Research Council of Australia and from Servier Laboratories. We are grateful to Thaddeus P. Gorski for performing the assays for plasma ACE.

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Hypertension. 1994;23:439-449
doi: 10.1161/01.HYP.23.4.439

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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