In Vitro Tissue Potencies of Converting Enzyme Inhibitors
Prodrug Activation by Kidney Esterase

Michèle Grima, Corinne Welsch, Bruno Michel, Mariette Barthelmebs, Jean-Louis Imbs

The inhibition of angiotensin converting enzyme by ramipril, ramiprilat, enalapril, enalaprilat, and captopril was studied in the plasma and various tissues (lung, heart, renal cortex, renal medulla) of normotensive rats and spontaneously hypertensive rats. Displacement curves for $[3H]$ramiprilat were established on each tissue with the converting enzyme inhibitors, and their potencies were expressed as the concentration that inhibited 50% of the specific $[3H]$ramiprilat binding. In the plasma, lung, and heart, the order of activities was: ramiprilat > enalaprilat > captopril > ramipril > enalapril. This order was different in the kidney (cortex and medulla): ramiprilat > enalaprilat > ramipril > captopril > enalapril. For ramiprilat, enalaprilat, and captopril, there were no differences in their respective potencies between tissues or between rat strains. However, the two prodrugs ramipril and enalapril were 10–30 times more active in the kidney than in the other tissues in both groups of rats. This was due to the deesterification of the prodrugs: in the presence of an esterase inhibitor (diethyl nitrophenyl phosphate, $10 \mu M$), the potencies of ramipril in the kidney were not different from that obtained in the lung, which was not affected by the presence of the esterase inhibitor. These results suggest that the variations in the tissue activities of an angiotensin converting enzyme inhibitor are probably not due to differences in tissue affinities of the angiotensin converting enzyme inhibitor but depend on the concentration of this angiotensin converting enzyme inhibitor in each tissue. (Hypertension 1991;17:492–496)

In recent years, several potent and specific inhibitors of angiotensin converting enzyme (ACE) have been proposed in the treatment of hypertension. Recently, a new concept has emerged, namely, that inhibition of the local renin-angiotensin systems, present in many tissues, was involved in the antihypertensive action of ACE inhibitors. Ex vivo studies on the tissue distribution of ACE inhibitors showed that the ACE in the aorta, heart, kidney, and brain was differently inhibited by these compounds. One might expect the effect of an ACE inhibitor to be modified by its tissue concentration, which depends on factors such as the physicochemical properties of the compound and the plasma half-life of the drug; we would like to add the ability of some tissues to transform the inactive prodrug into a potent inhibitor. It is also possible that the affinity for ACE may vary from one tissue to another because of differences in tissue ACE, as suggested by the studies of Johnston et al and Fabris et al.

In the present study, we compared the in vitro potencies of three ACE inhibitors (captopril, ramipril, ramiprilat, enalapril, enalaprilat) and their prodrug esters (ramipril, enalapril, ramiprilat, enalapril) in the plasma and various tissues (lung, heart, renal medulla, and renal cortex) of normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). The potencies of the ACE inhibitors were evaluated from displacement curves for specific $[3H]$ramiprilat binding. The binding technique with radiolabeled ACE inhibitor is a simple and rapid method, which was used to characterize ACE in the tissues of SHR and WKY rats or for potency measurement of ACE inhibitors in plasma and in tissues. In this study, we also demonstrated that the kidney was able to activate the prodrug esters ramipril and enalapril by deesterification.

**Methods**

Ramipril, ramiprilat, and $[3H]$ramiprilat (specific activity 55.5 Ci/mmol, radiochemical purity 96%) were obtained from Hoechst AG, Frankfurt-am-Main, FRG. Enalapril and enalaprilat were obtained from Merck Sharp & Dohme, Rahway, N.J., and captopril.
was obtained from Squibb, Paris la Defense, France. Diethyl 4'-nitrophenyl phosphate (DNP) was purchased from Aldrich-France, Strasbourg, France.

**Plasma and Tissue Sampling**

The main study was done on 8-week-old SHR of the Okamoto-Aoki strain and on normotensive WKY rats purchased from Iffa-Credo, L’Arbresle, France. Before the study began, the rats were kept in our laboratory for 10 days under identical conditions (tap water and food ad libitum). Plasma and tissue sampling were carried out under ether anesthesia. Six rats from each group were exsanguinated by puncture of the abdominal aorta; the blood, collected in a heparinized syringe, was rapidly centrifuged (2,000g for 10 minutes), and the plasma was preserved at -20°C. Organs were rapidly excised to obtain fragments of lung (right apex), heart (apex), and kidney (cortex and medulla). The tissue samples were rinsed in 0.9% ice-cold NaCl solution, frozen in liquid nitrogen, and then preserved at -20°C. After defrosting the tissue samples, 0.3% Triton X-100 was added (1 ml/100 mg tissue); the organs were then homogenized (Polytron, Kinematics, Kriens-Luzern, Switzerland, 20 seconds at maximum speed), and the suspension was centrifuged (11,800g for 20 minutes). Supernatants were diluted in 0.3% Triton for [3H]ramiprilat binding. An additional study was done on 8-week-old WKY rats to test the activation of the prodrugs ramipril and enalapril by kidney esterase. Lung and kidney sampling and tissue preparation were carried out as described before.

Protein concentration was assessed with the method of Lowry et al. using bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, Mo.) as standard. Triton X-100 was added to the standard at the same concentration as for the tissue samples.

**[3H]Ramiprilat Binding**

Fifty microliters of plasma or tissue supernatant were incubated with 50 μl buffer (Tris HCl 50 mM, pH 7.9, NaCl 100 mM), [3H]ramiprilat (10 nM), and increasing concentrations of ramipril, ramiprilat, enalapril, enalaprilat, and captopril according to Strittmatter and Snyder. In some experiments, an esterase inhibitor (DNP, 10 μM) was added to the incubation buffer. After 90 minutes incubation at room temperature, free and bound ligands were separated by filtration (cell harvester, Brandel, Gaithersburg, Md.) on Whatman GF/B filters treated with polyethyleneimine (0.3%) to allow the retention of soluble binding sites. Filters were washed three times with 5 ml ice-cold buffer (Tris HCl 10 mM, pH 7.4) and were transferred to counting vials containing 10 ml scintillation mixture (TM 299, Packard, Downers Grove, Ill.). Radioactivity was measured in a Packard counter with a 58% efficiency. Binding experiments were performed at a protein concentration that ensured linearity between binding and protein concentration and binding of less than 10% of added counts (0.2 mg/ml for lung, 1.0 mg/ml for plasma and other tissues). Displacement curves for [3H]ramiprilat were analyzed with the iterative LIGAND program for IC₅₀ determinations. No significant decrease in total [3H]ramiprilat was observed during the 6 months needed for this study, but to avoid any storage-dependent effect, the 300 plasma and tissue samples were randomized before [3H]ramiprilat binding measurements.

**Statistics**

Results are given as mean±SEM. Tissue IC₅₀ comparisons between SHR and WKY rats and between tissues were tested with two-way analysis of variance (ANOVA) with a dependent factor taking into account the fact that the tissues came from the same rat (BMDP 2V, BMDP Statistical Software Inc., Cork, Ireland). A paired t test was used to compare DNP-treated and nontreated tissues.

**Results**

**Relative In Vitro Potencies of Angiotensin Converting Enzyme Inhibitors in Plasma and Tissues of Spontaneously Hypertensive Rats and Wistar-Kyoto Rats**

[3H]Ramiprilat binds with high specificity (96% of specific binding) to plasma and tissue ACE. Total binding varied from one tissue to another; it was maximal in the lung (6,000 cpm) and minimal in the heart (400 cpm). Figure 1 gives an example of the displacement curves for [3H]ramiprilat-specific binding by each of the five drugs studied in the lung of a WKY rat. The results are expressed as the percentage of maximal specific binding measured in the absence of an inhibitor, which was defined as 100%. All five drugs inhibited, completely and dose-dependently, the binding of [3H]ramiprilat. The order of potencies in the lung was: ramiprilat > enalaprilat > ramipril > captopril > enalapril.

IC₅₀ determined for the five drugs in plasma and tissues, is presented in Table 1 for WKY rats and in

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Line graph showing displacement curves for [3H]ramiprilat-specific binding in lung of Wistar-Kyoto (WKY) rats by ramiprilat (○), ramipril (△), enalaprilat (□), enalapril (○), and captopril (△). These curves are representative of six experiments.
TABLE 1. Relative Potencies for Angiotensin Converting Enzyme Inhibitors in Wistar-Kyoto Rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>ACE inhibitor</th>
<th>Plasma</th>
<th>Heart</th>
<th>Lung</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ramiprilat (\times 10^{-7}\text{M})</td>
<td>5.83±1.18</td>
<td>8.35±0.94</td>
<td>3.91±0.29</td>
<td>7.45±1.22</td>
</tr>
<tr>
<td></td>
<td>Enalaprilat (\times 10^{-8}\text{M})</td>
<td>10.67±3.30</td>
<td>3.02±0.32</td>
<td>6.95±2.72</td>
<td>4.88±1.29</td>
</tr>
<tr>
<td></td>
<td>Captopril (\times 10^{-8}\text{M})</td>
<td>3.47±0.83</td>
<td>3.54±1.38</td>
<td>2.86±0.22</td>
<td>10.85±2.96</td>
</tr>
<tr>
<td></td>
<td>Ramipril (\times 10^{-6}\text{M})</td>
<td>2.83±1.66</td>
<td>2.51±0.99</td>
<td>2.26±1.01</td>
<td>0.16±0.07*</td>
</tr>
<tr>
<td></td>
<td>Enalapril (\times 10^{-5}\text{M})</td>
<td>6.30±0.67</td>
<td>5.07±0.74</td>
<td>4.16±0.66</td>
<td>0.12±0.04†</td>
</tr>
</tbody>
</table>

Potencies are expressed as the concentration needed to produce 50% inhibition of \[^{3}H\]ramiprilat binding in the plasma and tissues of Wistar-Kyoto rats (mean±SEM, \(n=6\)). ACE, angiotensin converting enzyme.

\*p<0.001, significantly different from plasma, heart, and lung.
†p<0.05, significantly different from plasma, heart, and lung.

Table 2 for SHR. Two-way ANOVA showed that there was no statistical difference between SHR and WKY rats, whatever the compound or the tissue.

Comparison between tissues showed that for ramiprilat, enalaprilat, and captopril, their respective IC\(_{50}\) did not vary significantly from one tissue to another. On the contrary, the IC\(_{50}\) in the kidney (cortex and medulla) for the two prodrugs enalapril and ramipril was 10 to 30 times lower than in the other tissues.

Inhibition of Esterase Activity in the Kidney

The lower IC\(_{50}\) obtained in the kidney with the prodrugs ramipril and enalapril might be linked to the deesterification of the compounds. To test this hypothesis, lung and renal cortex samples were taken from three WKY rats. Displacement curves for \[^{3}H\]ramiprilat binding by ramiprilat and ramipril were determined in the absence or in the presence of a specific esterase inhibitor DNP (10 \(\mu\text{M}\)). Results are given in Figures 2 and 3 and in Table 3. In the lung, DNP treatment did not modify the displacement curves for \[^{3}H\]ramiprilat binding by ramiprilat and ramipril. In the renal cortex, the displacement curve for \[^{3}H\]ramiprilat binding by ramiprilat was unchanged in the presence of DNP, but the ramipril curve was displaced to the right. In the lung, the IC\(_{50}\) for ramiprilat and ramipril were unchanged in the presence of DNP (Table 3). In the kidney cortex, the IC\(_{50}\) for ramipril was also unaffected by DNP treatment, whereas the IC\(_{50}\) for ramipril increased significantly in the presence of DNP and then did not differ significantly from the IC\(_{50}\) measured in the lung.

Discussion

In the present study, we evaluated the relative in vitro potencies of three ACE inhibitors and their prodrugs in the plasma and tissues of SHR and WKY rats. The potencies of the ACE inhibitors were determined from the displacement curves for the binding of \[^{3}H\]ramiprilat to ACE in the plasma or in tissue extracts. Because tissue ACE is a membrane-bound enzyme, all the tissues were treated with Triton X-100 for total extraction and solubilization of ACE, which allowed us to obtain high binding.6 This in vitro determination of ACE inhibition on tissue extracts ruled out the influence of the bioavailability and the physicochemical properties of the ACE inhibitors.

TABLE 2. Relative Potencies for Angiotensin Converting Enzyme Inhibitors in Spontaneously Hypertensive Rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>ACE inhibitor</th>
<th>Plasma</th>
<th>Heart</th>
<th>Lung</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ramiprilat (\times 10^{-6}\text{M})</td>
<td>4.52±1.06</td>
<td>6.67±0.93</td>
<td>5.45±0.60</td>
<td>6.27±1.50</td>
</tr>
<tr>
<td></td>
<td>Enalaprilat (\times 10^{-4}\text{M})</td>
<td>4.80±0.76</td>
<td>2.90±0.28</td>
<td>7.68±2.58</td>
<td>4.40±0.40</td>
</tr>
<tr>
<td></td>
<td>Captopril (\times 10^{-7}\text{M})</td>
<td>8.73±2.88</td>
<td>4.18±1.14</td>
<td>7.05±2.30</td>
<td>3.72±0.95</td>
</tr>
<tr>
<td></td>
<td>Ramipril (\times 10^{-6}\text{M})</td>
<td>2.78±0.53</td>
<td>5.05±2.97</td>
<td>2.49±0.92</td>
<td>0.19±0.04*</td>
</tr>
<tr>
<td></td>
<td>Enalapril (\times 10^{-5}\text{M})</td>
<td>3.28±0.25</td>
<td>5.54±0.78</td>
<td>3.24±0.52</td>
<td>0.12±0.03†</td>
</tr>
</tbody>
</table>

Potencies are expressed as the concentration needed to produce 50% inhibition of \[^{3}H\]ramiprilat binding in the plasma and tissues of spontaneously hypertensive rats (mean±SEM, \(n=6\)). ACE, angiotensin converting enzyme.

\*p<0.001, significantly different from plasma, heart, and lung.
†p<0.05, significantly different from plasma, heart, and lung.
No difference was detected between hypertensive and normotensive rats. This agrees with other data for 3-week-old SHR and WKY rats,13 showing that differences in ACE activities between the two strains resulted from differences in ACE concentration and not in enzyme affinity. The potency of ramiprilat was nearly identical in each of the five tissues studied, as were the relative potencies for enalaprilat and captopril. These results are in accordance with those of Cushman et al3 who compared by means of an enzymatic assay several ACE inhibitors, among them ramiprilat, enalaprilat, and captopril, in six tissues from SHR. All these data support the notion that ACE arises from a single gene.14 Different results were obtained by Johnston et al4 and Fabris et al5 who, using a radioassay with I25I-MK351A as ligand, found significant differences for in vitro ACE inhibitor potencies in rat tissues, in particular in the heart. Nevertheless, these differences were slight and do not indicate that the characteristics of ACE differ markedly from one tissue to another. The order of potency for the three ACE inhibitors studied here was similar to those obtained by other authors,3,7,15 although IC50 values differed from one study to another. This is probably due to the different methods used to measure ACE inhibition.16 In the plasma, lung, and heart, the two prodrugs ramipril and enalapril were 300-400 times less potent than ramiprilat and enalaprilat but were not completely inactive. The activity of the esterified form might be linked to the binding with auxiliary sites,15 but it cannot be ruled out that ramipril or enalapril may be contaminated by very small amounts of their diacid forms. Contamination of ramipril by 0.25% ramiprilat could explain its inhibitory effect.

In the renal cortex and medulla, the respective IC50 for ramipril and enalapril was 10-30 times lower than in the other tissues. This effect was completely reversed in the presence of DNP, which is a specific inhibitor of the B-esterase described in the kidney.12 This demonstrates that, in vitro, ramipril and enalapril were transformed into their active diacid form by kidney extracts. Under our experimental conditions, 1−2% of ramipril or enalapril were deesterified. This is low compared with the 6−10% metabolism measured by Lannoy et al17 with enalapril in the isolated rat kidney. This is also lower than the 20% metabolism after 1 hour of incubation reported by Cushman et al.3 This discrepancy may be because of the different methods used to prepare the tissues. We worked on a partially Triton-extracted enzyme preparation, whereas Cushman et al3 used a crude homogenate, and it is possible that esterases were not completely extracted by Triton in our preparation.

Esterases such as arylesterase, which is not inhibited by DNP,12 have also been described in human plasma. A DNP-insensitive esterase activity might exist in rat plasma. Nevertheless, our results showed no shift in the displacement curves with ramipril or enalapril. This differs from other data, in particular those of Cushman et al.7 The main discrepancies between our experimental conditions and those of

### Table 3. Inhibition of Kidney Esterase

<table>
<thead>
<tr>
<th>Tissue ACE inhibitor</th>
<th>Lung</th>
<th>Renal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (mean±SEM, n=3)</td>
<td>DNP-treated (mean±SEM, n=3)</td>
</tr>
<tr>
<td></td>
<td>Control (mean±SEM, n=3)</td>
<td>DNP-treated (mean±SEM, n=3)</td>
</tr>
<tr>
<td>Ramiprilat</td>
<td>5.10±0.86x10^-9</td>
<td>5.23±1.86x10^-9</td>
</tr>
<tr>
<td></td>
<td>4.26±0.53x10^-9</td>
<td>4.25±0.28x10^-9</td>
</tr>
<tr>
<td>Ramipril</td>
<td>1.16±0.06x10^-6</td>
<td>1.07±0.18x10^-6</td>
</tr>
<tr>
<td></td>
<td>9.33±3.76x10^-8</td>
<td>1.53±0.37x10^-8</td>
</tr>
</tbody>
</table>

Effects of 10 μM diethyl 4-nitrophenyl phosphate (DNP) treatment on potencies of ramiprilat and ramipril in the lung and renal cortex of Wistar-Kyoto rats. The potencies are expressed as the concentration (M) needed to produce 50% inhibition of [3H]ramiprilat binding. *p<0.001, significantly different from control.
Cushman were the use of Triton for the dilution of plasma and the incubation at room temperature, which could lower the activity of plasma esterase. The in vivo significance of ramipril activation in the kidney is difficult to assess because, in the rat, ramipril may be principally deesterified in the liver, but this activation phenomenon may become significant if the prodrug accumulates in the tissue after multiple doses of an ACE inhibitor. An accumulation of the prodrug might also lead to misinterpretation of ex vivo measurements of ACE inhibition after long-term treatment since the prodrug might be activated during the experimental procedure for measuring ACE inhibition.

In conclusion, our results show that there are no differences in ACE inhibitor potencies between tissues and between the two strains of rats studied here. This suggests that the characteristics of the ACE binding site, which is probably the active site, do not vary significantly either from one tissue to another or between SHR and WKY rats. So, variations in tissue ACE inhibition after the administration of an inhibitor probably result merely from differential tissue distribution due to the physicochemical properties of the compound and perhaps also to variations in the ability of some tissues to activate a prodrug.

Acknowledgments

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References


Key Words • angiotensin converting enzyme inhibitors • Wistar-Kyoto rats • spontaneously hypertensive rats
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