In Vivo Assessment of Captopril Selectivity of Angiotensin I-Converting Enzyme Inhibition: Differential Inhibition of Acetyl-Ser-Asp-Lys-Pro and Angiotensin I Hydrolysis

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ABSTRACT
Angiotensin I-converting enzyme (ACE) is a zinc metalloproteinase that plays a major role in blood pressure regulation. The demonstration that the hemoregulatory peptide acetyl-Ser-Asp-Lys-Pro (AcSDKP) is a natural and specific substrate of the N-active site of ACE suggests that this enzyme may have a new physiological role such as the modulation of hematopoietic stem cells. In vitro studies have shown that ACE inhibitors displayed various potencies in inhibiting the degradation of different natural or synthetic substrates of ACE, among which captopril inhibits AcSDKP hydrolysis more potently than angiotensin I hydrolysis. To look for this selectivity in vivo, we investigated the pharmacodynamic effect of increasing doses of captopril (0.01–10 mg/kg) during the 90 min after i.v. administration to spontaneously hypertensive rats. Plasma and urinary AcSDKP levels were measured. The renin-angiotensin system was evaluated by measurements of ACE activity in plasma samples, using the synthetic substrate Hip-His-Leu, by determinations of plasma renin concentrations and measurements of arterial blood pressure. The results showed that captopril (0.01–0.3 mg/kg) selectively inhibited AcSDKP hydrolysis, with limited effects on the renin-angiotensin system. AcSDKP levels in plasma and urine rose to a plateau 4 times the basal level for doses more than 0.3 mg/kg. All of the parameters reflecting the renin-angiotensin system were significantly affected at doses of 1 and 10 mg/kg. The present study therefore confirms that captopril can be used to protect hematopoietic stem cells during antitumor chemotherapy while having only a limited effect on cardiovascular homeostasis.

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) is a ubiquitous zinc metalloproteinase involved in cardiovascular homeostasis. Two isoforms are expressed in mammalian tissues (Corvol et al., 1995): a somatic isoform composed of two highly similar and active domains (N and C domains) and a smaller germlinal isoform composed of a single catalytic site identical with the C domain of somatic ACE. Besides its well known action in blood pressure control, ACE recently has been implicated in metabolism of acetyl-Ser-Asp-Lys-Pro (AcSDKP) (Rieger et al., 1993), a negative regulator of hematopoiesis that prevents the recruitment of pluripotent hematopoietic stem cells by maintaining them in the S phase (Lenfant et al., 1989; Robinson et al., 1992). AcSDKP has been used as a bone marrow protector during chemotherapy of cancer patients (Carde et al., 1992). However, this potential application of AcSDKP is limited by its pharmacokinetic properties such as a short half-life and a small volume of distribution (Ezan et al., 1994).

Recently, in vitro studies established that AcSDKP is a natural and specific substrate of the ACE N-terminal domain. Indeed, this tetrapeptide is hydrolyzed 50 times faster by this site than by the C site (Rousseau et al., 1995). In vivo, after single-dose administration to healthy volunteers, captopril, an ACE inhibitor, induced a 5- to 6-fold increase in plasma AcSDKP compared with levels in untreated healthy volunteers (Azizi et al., 1996). This was later confirmed with other ACE inhibitors in patients (Azizi et al., 1997). A consequence of this observation is that AcSDKP may be used as a reliable marker of ACE inhibition, which can help to verify compliance with ACE inhibitor treatment (Azizi et al., 1997). Another potential application involves administration of ACE inhibitors to cancer patients during antitumor chemotherapy with the aim of sustaining the bone marrow-protective effect of AcSDKP. It has been demonstrated recently that

ABBREVIATIONS: ACE, angiotensin I-converting enzyme; AcSDKP, acetyl-Ser-Asp-Lys-Pro; SHR, spontaneously hypertensive rat; PRC, plasma renin concentration; AUC, area under the curve; Cl R, renal clearance; E max, maximal effect.
the two domains of ACE interact differently with competitive inhibitors (Wei et al., 1992), raising the possibility of finding selective inhibitors of the N domain of ACE among previously synthesized and marketed ACE inhibitors. Such an inhibitor could increase AcSDKP levels with much less impact on the renin-angiotensin system. Interestingly, a recent in vitro study showed that captopril inhibits AcSDKP more potently than angiotensin I, with a 16-fold-lower $K_i$ with AcSDKP as substrate (Michaud et al., 1997).

The aim of the present work was to show that captopril may induce selective inhibition of AcSDKP hydrolysis by ACE in rats. After administration of i.v. boluses of increasing doses of captopril to spontaneously hypertensive rats, we investigated plasma and urinary AcSDKP levels. The plasma renin concentration was selected as a marker of the in vivo inhibition of angiotensin II formation. The N and C domain selectivity of ACE inhibition in vivo was evaluated by studying the hydrolysis of the synthetic substrate Hip-His-Leu, which has been shown to be a selective C domain substrate at high chloride concentrations (Rousseau et al., 1995; Michaud et al., 1997).

Materials and Methods

Peptides and Reagents. AcSDKP was synthesized by Neosystem (Strasbourg, France). Hip-His-Leu was obtained from Bachem (Bubendorf, Switzerland), angiotensin I was obtained from Calbiochem (La Jolla, CA), and captopril was obtained from Sigma (St. Louis, MO).

Animals. Male spontaneously hypertensive rats (SHR) 8 to 9 weeks old and weighing about 250 g were used (Charles River, Saint-Aubin-Les-Elbeuf, France). All studies on animals comply with the Décret sur l’Expérimentation Animale (French law on rules for animal experimentation, Decree 87-848, October 19, 1987).

Surgical Procedures. Inactin-anesthetized (10 mg for 100-g weight) SHR were cannulated in the femoral artery and in the right and left jugular veins. An additional vesicle catheter was implanted for the collection of urinary fractions. At $T_0$, i.v. boluses of captopril at doses of 0, 0.01, 0.03, 0.1, 0.3, 1, and 10 mg/kg (12 rats/group) were administered in the right jugular vein. An i.v. infusion of furosemide at doses of 0, 0.01, 0.03, 0.1, 0.3, 1, and 10 mg/kg (12 rats/group) were administered in the right jugular vein.

AcSDKP Measurements. AcSDKP was quantified in plasma and urine by means of a competitive enzyme immunoassay described elsewhere (Pradelles et al., 1990). Polyclonal antibodies were obtained after immunization of AcSDKP conjugated to BSA. The tracer was AcSDKP bound to Electrophorus electricus acetylcholinesterase (EC 3.1.1.7). Before assay, plasma samples were treated with methanol. After centrifugation, the supernatants were collected and evaporated to dryness, and reconstituted in enzymeimmunoassay (EIA) buffer. Urinary samples were directly diluted in EIA buffer. Sample concentrations were calculated from a standard curve linearized with a cubic spline fitting. All measurements of standards and samples were performed in duplicate. Assay repeatability and reproducibility were in the 10 to 20% range, and the limit of quantification was 0.2 nM in plasma and 1 nM in urine.

Plasma Renin Concentration (PRC) Measurement. PRCs were evaluated by the ability of renin in plasma samples to hydrolyze an excess of angiotensinogen present in the plasma of binephrectomized rats. Angiotensin I formation then was measured by means of a radioimmunoassay (RIA) (Menard and Catt, 1972). PRC was expressed in nanograms of formed angiotensin I per milliliter of plasma and per hour of incubation (ng angiotensin I/ml/h).

Hip-His-Leu Hydrolysis by SHR Plasma. The enzymatic assays were performed in duplicate with 10 or 20 µl of each plasma, using Hip-His-Leu as substrate under the following conditions: 5 mM substrate, 300 mM NaCl, 10 µM ZnSO$_4$, 1 mg/ml BSA at 37°C, pH 8.3. The hippuric acid released from the substrate was resolved by isocratic reversed-phase HPLC on a 10-µm Nucleosil C$_18$ column in acetonitrile/10 mM potassium phosphate, pH 3.0 (23:78, v/v), at a flow rate of 1 ml/min with UV detection at 228 nm. The interassay coefficient of variation was determined by using an internal standard plasma and was 10%. To minimize dissociation of captopril from plasma ACE, the activity was determined within 48 h after the sampling. Results were expressed in nmol/ml/min of generated hippuric acid.

Statistical and Pharmacokinetic Analysis. At each sampling time, plasma AcSDKP, PRC levels, Hip-His-Leu hydrolysis, and variation in arterial blood pressure were analyzed by one-way ANOVA for the dose effect. The mean values of each group then were compared with the control group by Dunnett’s test. The same procedure was applied to compare amounts of AcSDKP excreted in urine, plasma AcSDKP area under the curve ($AUC_{0–90}$), and renal clearances of AcSDKP obtained at each captopril dose to values in the control group. Normality was checked before running the ANOVAs. When distributions were skewed, a Kruskal-Wallis nonparametric ANOVA followed by Dunnett’s test was used. Values of $p < .05$ were considered significant. Calculations were done by using SIGMA-STAT statistical software (Systat Corporation, San Rafael, CA).

Each pharmacokinetic parameter first was determined for individual animals and then averaged. The plasma AcSDKP $AUC_{0–90}$ was determined by the trapezoidal rule. Renal clearances (CLR) for AcSDKP were calculated by the amount of urinary AcSDKP excreted over 90 min divided by $AUC_{0–90}$.

Results

Time-dependent changes in plasma AcSDKP after captopril i.v. bolus administration are given in Table 1. In the absence of captopril, AcSDKP remained stable, with mean values ranging from 1.7 to 2.3 nM. Captopril at doses between 0.01 and 10 mg/kg induced an increase in plasma AcSDKP levels that was statistically significant at all doses and at all times, except for 0.01 mg/kg captopril at $T_{90}$ (Table 1). The maximum AcSDKP concentration was 8.4 nM and was obtained at the dose of 10 mg/kg, 90 min after captopril administration. Between doses of 0.01 and 0.3 mg/kg, the increase was dose-dependent and reached a plateau for doses more than 0.3 mg/kg. This was confirmed by the AcSDKP $AUC_{0–90}$ analysis reported in Table 2. To confirm the plasma data, urinary levels of AcSDKP were assessed, and Table 2 shows that the amount of peptide excreted in urine was increased significantly from the lowest captopril dose upward. As a consequence of the parallel increases in plasma and urinary AcSDKP, renal clearance remained unchanged for all doses of captopril (Table 2).

The time course of PRC is presented in Table 3. Except for the 10-mg/kg dose, the maximum value was observed 30 min after captopril injections for all doses. Although PRC was increased significantly at 30 and 60 min, only the 0.3-, 1-, and 10-mg/kg doses significantly increased PRC 90 min after captopril administration (Table 3). At 10 mg/kg captopril, PRC increased steeply, with values 15 and 20 times those in the controls at 30 and 90 min, respectively.
TABLE 1
Plasma AcSDKP levels

<table>
<thead>
<tr>
<th>Captopril Dose</th>
<th>AUC_{0-90} (nM)</th>
<th>Urinary Levels (pmol/90 min)</th>
<th>CL_{ul} (ml/min)</th>
<th>Plasma AcSDKP Levels*</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/kg</td>
<td>30 min</td>
<td>0 min</td>
<td>30 min</td>
<td>60 min</td>
</tr>
<tr>
<td>0</td>
<td>165 ± 31</td>
<td>157 ± 33</td>
<td>1.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>193 ± 40</td>
<td>233 ± 43a</td>
<td>1.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>0.03</td>
<td>327 ± 41a</td>
<td>368 ± 101a</td>
<td>1.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>443 ± 64a</td>
<td>525 ± 102a</td>
<td>1.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>520 ± 58a</td>
<td>576 ± 193a</td>
<td>1.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>573 ± 151a</td>
<td>681 ± 293a</td>
<td>1.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>565 ± 73a</td>
<td>567 ± 263a</td>
<td>1.1 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± S.D. (n = 12). **Statistically significant difference from control group (p < .05).

Values are means ± S.D. (n = 12). **Statistically significant difference from control group (p < .05).

For captopril-induced changes in arterial blood pressure (data not shown), a slight decrease in blood pressure with time was observed in the absence of captopril, probably because of inactin anesthesia. Compared with the control group, only the doses of 1 and 10 mg/kg captopril significantly lowered the blood pressure at all the sampling times.

In vitro plasma ACE activity was estimated by using the synthetic substrate Hip-His-Leu. The values corresponding to the time course of Hip-His-Leu hydrolysis are given in Table 4. Results corresponding to 0.01 mg/kg captopril have not been included because they were erratic and not interpretable. In the absence of captopril, ACE activity remained stable, with mean values of hippuric acid ranging from 17.7 to 22.4 nmol/ml/min. Inhibition of Hip-His-Leu degradation by captopril was dose-dependent, with maximal inhibition (hippuric acid: 3.4 ± 2.1 nmol/ml/min) at 10 mg/kg captopril 30 min after drug administration. However, except for 1 mg/kg captopril at T_{90} and 10 mg/kg captopril, ACE activity evaluated using Hip-His-Leu as substrate did not significantly differ from that observed with the control group.

Dose-response curves of captopril for plasma AcSDKP AUC_{0-90}, PRC AUC_{0-90}, and inhibition of Hip-His-Leu hydrolysis AUC_{0-90} are presented in Fig. 1. Although maximal effects (E_{max}) on PRC and Hip-His-Leu were not reached, inhibition profiles were modeled according to an E_{max} model. Maximal effects for PRC and Hip-His-Leu were arbitrarily fixed at 10 mg/kg captopril. E_{50} (median effective dose) for AcSDKP (0.02 mg/kg) was 40 times lower than that of Hip-His-Leu (0.8 mg/kg) and 100 times lower than that of PRC (2 mg/kg).

Discussion

ACE is involved in the hydrolysis of a variety of biologically active peptides in vivo and is a major component of the cardiovascular system through the degradation of angiotensin I and Bradykinin. The demonstration that ACE is also involved in the metabolism of AcSDKP, a negative regulatory factor of hematopoiesis, suggests that the enzyme may also have a new physiological role such as the modulation of hematopoietic stem cell regulation. This new function of ACE has been considered recently in vivo in mice (Rousseau-Plass et al., 1998) and in humans (Comte et al., 1997).

Although the N and C domains, the two active sites of ACE, are of equal potency in their ability to hydrolyze the two vasoactive peptides (Michaud et al., 1997), the N domain is 50-fold more active than the C domain in the hydrolysis of AcSDKP (Rousseau-Plass et al., 1995; Michaud et al., 1997). Recent studies have demonstrated the diversity of various ACE inhibitors in their relative binding to both ACE domains (Wei et al., 1992) and in their potency in inhibiting synthetic or natural substrates (Michaud et al., 1997). The major finding was that captopril and fosinoprilat were more potent inhibitors of AcSDKP than of angiotensin I hydrolysis (Michaud et al., 1997). Although 4-fold less potent than fosinoprilat, captopril displays the best selectivity for the N-terminal domain. For wild-type ACE, the K_{i} values of captopril for AcSDKP and angiotensin I as substrate were 0.24 and 3.98 nM, respectively. These values were 0.08 and 13.25 nM, respectively, with the ACE mutant bearing only the N-terminal domain.

The purpose of the present study was to look for an in vivo dissociation of captopril-inhibiting properties with the aim of using an ACE inhibitor to protect hematopoietic stem cells via an increase in AcSDKP without affecting the renin-angiotensin system and its hemodynamic effects. Therefore, we investigated the pharmacodynamic effect of captopril in spontaneously hypertensive rats that received captopril i.v. at doses ranging from 0.01 to 10 mg/kg. This was achieved by measuring plasma renin concentrations, which are known to increase through a feedback mechanism resulting from the inhibition of angiotensin II formation and AcSDKP. A captopril dose of 10 mg/kg was necessary to increase the renin plasma concentration substantially. This is confirmed by the measurement of the in vitro capacity of rat plasma samples taken after captopril administration to inhibit the degradation of Hip-His-Leu, a synthetic substrate of ACE that mimics the last two amino acids of angiotensin I and for which captopril has a similar K_{i} to that of angiotensin I (Michaud et al., 1997). At doses ranging from 0.01 to 0.1 mg/kg and 90 min after captopril administration, the plasma renin concentration and blood pressure were not statistically different from control values, whereas AcSDKP metabolism was still
inhibited. This absence of parallelism of captopril action indicates that this inhibitor selectively inhibits the metabolism of two natural substrates of ACE, AcSDKP and angiotensin I.

The experimental model that has been developed uses i.v. administered furosemide at a dose that does not induce sodium depletion. Its helps to maintain a urinary flow sufficient to measure AcSDKP in urines, whereas the animals are infused with saline. This model provides plasma renin concentrations that are much more variable than plasma AcSDKP levels from one rat to another. This variability depends on the multiplicity of factors involved in renin release regulation (blood pressure level, blood volume, angiotensin II feedback), whereas plasma AcSDKP is influenced mainly by its metabolism. However, the differences in the dose-response curves on the selected end points have such a magnitude that they can be identified despite experimental results variability.

So far, the existence of two separate, functional catalytic sites (N- and C-terminal, as in humans) has not been demonstrated rigorously for rat ACE. However, using radioligand-binding techniques on rat lung ACE, two distinct binding sites that displayed different affinities for ACE inhibitors have been found (Perich et al., 1992). The degradation of bradykinin was attributed to the high-affinity site, suggesting that these two active sites may be selective for different natural substrates. Furthermore, using rat testis and rat lung ACE (Bevilaqua et al., 1996), it has been demonstrated that captopril was N-selective, as in humans (Michaud et al., 1997). Lastly, it has been demonstrated that rat and human ACE genes are highly conserved (Koike et al., 1994). These arguments may support that rat ACE possesses two selective catalytic active sites that display a similar reactivity to that observed for human ACE.

As in humans (Rieger et al., 1993; Azizi et al., 1996), the present study shows that ACE is also involved in AcSDKP metabolism in rats. Between doses of 0.01 and 10 mg/kg, a dose-response curve revealed that the maximum effect occurred at the dose of 0.3 mg/kg, indicating that full inhibition of AcSDKP hydrolysis by ACE is achieved at this dose and above. Because the 0.03 mg/kg-dose corresponds to half of the

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**TABLE 3**

<table>
<thead>
<tr>
<th>Captopril Dose (mg/kg)</th>
<th>Plasma Renin Concentration* (ng/ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-30 min</td>
</tr>
<tr>
<td>0</td>
<td>35 ± 10</td>
</tr>
<tr>
<td>0.01</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>0.03</td>
<td>26 ± 4</td>
</tr>
<tr>
<td>0.1</td>
<td>46 ± 30</td>
</tr>
<tr>
<td>0.3</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>1</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>10</td>
<td>29 ± 7</td>
</tr>
</tbody>
</table>

* Mean ± S.E.M. (n = 12).

**TABLE 4**

<table>
<thead>
<tr>
<th>Captopril Dose (mg/kg)</th>
<th>ACE Hip-His-Leu Activity (hippuric acid nmoles/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-30 min</td>
</tr>
<tr>
<td>0</td>
<td>22.4 ± 2.7</td>
</tr>
<tr>
<td>0.03b</td>
<td>17.7 ± 3.9</td>
</tr>
<tr>
<td>0.1c</td>
<td>18.4 ± 3.5</td>
</tr>
<tr>
<td>0.3c</td>
<td>17.5 ± 5.4</td>
</tr>
<tr>
<td>1d</td>
<td>16.6 ± 5.6</td>
</tr>
<tr>
<td>10d</td>
<td>16.3 ± 6.9</td>
</tr>
</tbody>
</table>

* Mean ± S.D. (n = 4).

**Fig. 1.** Dose-response curves of captopril for plasma AcSDKP AUC₀−₉₀ (●), inhibition of Hip-His-Leu hydrolysis AUC₀−₉₀ (■), and plasma renin concentration AUC₀−₉₀ (▲). Values are expressed as percentages of maximal AUC₀−₉₀.
maximal inhibition it may be supposed that plasma or tissue captopril levels at this dose are of the same order as the $K_i$ of captopril for AcSDKP metabolism. As shown in the data of pharmacokinetic studies in rats (Endoh et al., 1989), the plasma captopril concentrations at this dose are in the range 10 to 20 nM between 30 and 60 min after administration. This corresponds to 50 and 200 times the $K_i$ of captopril observed in vitro for the inhibition of AcSDKP degradation by wild-type ACE or ACE N domain, respectively (Michaud et al., 1997).

This discrepancy between in vitro binding characteristics and in vivo data may indicate that AcSDKP metabolism is governed by captopril concentrations in an effect compartment rather than in the vascular compartment. Such a kinetic-dynamic relation has been demonstrated for the action of captopril on the hemodynamic response in pigs (Pereira et al., 1996). Indeed, in ACE-rich tissues such as the lung, captopril pharmacodynamics are faster than in plasma, possibly because of the tissular pharmacokinetic properties of the inhibitor (Cushman et al., 1989). The hypothesis that AcSDKP plasma concentrations are the resultant of tissue rather than plasma metabolism is in agreement with previous data showing that AcSDKP administered i.v. to humans has a half-life of 5 min (Ezan et al., 1994), which is much faster than the 45 min determined in vitro in human plasma (Rieger et al., 1993).

In this study, renal clearance was measured to confirm the plasma results and to study the pharmacodynamics of AcSDKP excretion in the kidney at different captopril doses. The increase in plasma AcSDKP concentrations induced a parallel increase in the amount of the peptide recovered in urine. In the absence of captopril, the renal clearance was 1 ml/min, which is half of the glomerular filtration rate in rats (Lin, 1995). This suggests that the peptide is filtered and partially degraded by ACE present in the kidney tubules. Although it has been demonstrated that captopril is excreted in urine (Cushman et al., 1989), we found no significant increase in renal clearance compared with basal levels after captopril administration. It is therefore likely that AcSDKP is reabsorbed or degraded by other enzymes in the kidney.

In conclusion, our results demonstrate that the in vivo potency of an ACE inhibitor is substrate-dependent, extending the in vitro demonstrations reported earlier (Michaud et al., 1997). Thus captopril, the first ACE inhibitor, is itself a good candidate for the selective inhibition of AcSDKP compared with inhibition of angiotensin I. This finding and the fact that ACE inhibition affects human blood hematopoietic progenitors (Comte et al., 1997) are of clinical importance because captopril may be used to protect the hematopoietic system of cancer patients receiving chemotherapy with limited effect on the cardiovascular system.

References


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