Acute Angiotensin-converting Enzyme Inhibition Increases the Plasma Level of the Natural Stem Cell Regulator *N*-Acetyl-Seryl-Aspartyl-Lysyl-Proline

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Abstract

Angiotensin I–converting enzyme (ACE) has two homologous active NH_2- and COOH-terminal domains and displays activity toward a broad range of substrates. The tetrapeptide *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) has been shown to be hydrolyzed in vitro by ACE and to be a preferential substrate for its NH_2-terminal active site. This peptide is a regulatory factor of hematopoiesis which reversibly prevents the recruitment of pluripotent hematopoietic stem cells and normal early progenitors into S-phase. We found that a single oral dose of 50 mg of the ACE inhibitor, captopril, when administered to eight healthy subjects in a double-blind, crossover, placebo-controlled study, massively increased the plasma level of Ac-SDKP. ACE inhibition by captopril induced a 90–99% inhibition of in vitro [^{3}H]Ac-SDKP hydrolysis and a long-lasting 5.5-fold (range: 4–8.5-fold) increase in the plasma levels of Ac-SDKP.

These results demonstrate that Ac-SDKP is the first natural peptide hydrolyzed by the NH_2-terminal domain of ACE not only in vitro but also in vivo, confirming that both catalytic sites of ACE are physiologically active. Our data suggest that ACE may also be implicated in the process of hematopoietic stem cell regulation, by permanently degrading this natural circulating inhibitor of cell entry into S-phase. (*J. Clin. Invest.* 1996. 97:839–844.) Key words: oligopeptides • metabolism • peptidyl-dipeptidase A • angiotensin-converting enzyme inhibitor • hematopoiesis

Introduction

Angiotensin I–converting enzyme (ACE)[1] (kininase II, dipeptidyl carboxypeptidase I, EC 3.4.15.1), has two homologous active NH_2- and COOH-terminal domains and is involved in the metabolism of two major vasoactive peptides. It converts the inactive decapeptide angiotensin I (Ang I) into the active octapeptide angiotensin II (Ang II) and inactivates the vasodepressor peptide bradykinin, therefore playing a major role in blood pressure regulation and fluid and electrolyte homeostasis (1). The importance of the applications of oral ACE inhibitors in clinical medicine is now self evident, with many thousands of patients being treated with these drugs at the present time for either hypertension, congestive heart failure, postmyocardial infarction, or diabetic nephropathy. However, ACE inhibitors are nonspecific blockers of the renin-angiotensin system, since ACE displays activity toward a broad range of substrates, at least in vitro (1, 2).

ACE is involved in vitro in the hydrolysis of *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), a regulatory factor of hematopoiesis (3). By using selective mutants of the two homologous active domains of ACE expressed in Chinese hamster ovary cells, Rousseau et al. (4) have shown that the NH_2-terminal active site of ACE is preferentially involved in vitro in the catabolism of the hemoregulatory tetrapeptide Ac-SDKP. The hydrolysis of Ac-SDKP is blocked by ACE inhibitors such as captopril and lisinopril (3, 4). Ac-SDKP reversibly prevents the recruitment of pluripotent hematopoietic stem cells and normal early progenitors into S-phase of the cellular cycle by maintaining them in Go-phase (5, 6). It appears to exert this function indirectly by blocking the effect of an as yet unknown stem cell stimulator of proliferation (7). During phase-specific anticancer drug treatment, exogenous Ac-SDKP administration selectively maintains normal progenitors in the quiescent state (8). Additionally, when administered after two-thirds partial hepatectomy in mice, Ac-SDKP has been shown to reduce hepatocyte proliferation by up to 50%, as assessed by autoradiography and [^{3}H]thymidine incorporation (9), indicating that this peptide may also be involved in the homeostasis of cellular growth of different cell types. Ac-SDKP, whose precursor could be thymosin β4 (10), is known to be secreted by bone marrow (11), is normally present in human plasma and in circulating mononuclear cells (12), and is ubiquitously distributed in vivo (13). Interestingly, Ac-SDKP is localized in tissues where ACE is present.

To establish whether Ac-SDKP is a natural substrate of ACE in vivo, a single oral dose of the ACE inhibitor, captopril, was given to healthy volunteers to investigate whether ACE blockade inhibits Ac-SDKP hydrolysis in vivo and consequently increases the plasma levels of this hemoregulatory peptide.

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1. *Abbreviations used in this paper:* ACE, angiotensin I–converting enzyme; Ac-SDKP, *N*-acetyl-seryl-aspartyl-lysyl-proline; Ang, angiotensin; AUC_{0-8h}, area under the curve from 0 to 8 h; *I/D* polymorphism, insertion/deletion polymorphism.

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Methods

Study design. A single-dose, double-blind, two-way crossover study design was used. The two periods were separated by a washout interval of 1 wk. Treatment assignment was performed according to a random allocation schedule and each subject received a single oral dose of captopril 50 mg or a matching placebo on two separate occasions.

Subjects. Eight Caucasian, healthy, normotensive (supine blood pressure < 140/90 mmHg) male volunteers 20–35 yr old were recruited for the study. Volunteers gave their written and informed consent to participate in the study. The protocol was approved by the Comité Consultatif de Protection des Personnes se prêtant à des Recherches Biomédicales, Paris-Cochin.

Study protocol. For each phase, subjects were instructed to arrive at the Broussais Clinical Investigation Center at 7 a.m. on the study day. At 9 a.m., after a 1-h rest in a semirecumbent position to allow equilibration of hormones, volunteers received a single oral dose of the appropriate treatment (placebo or captopril 50 mg) with 50 ml water. They remained in the same position until 6 h after dose (3 p.m.). Blood was sampled before and 0.5, 1, 1.5, 2, 4, 6, and 8 h after dose for plasma ACE activity, Ac-SDKP, and active renin determinations. Blood samples for analyzing in vitro Ac-SDKP hydrolysis were drawn before and 0.5, 1, 2, 4, and 8 h after dose. Plasma Ang I and Ang II were determined before and 1, 2, and 8 h after drug intake.

Laboratory methods. Captopril (10^{-8} M) was immediately added to the blood samples for the measurement of plasma Ac-SDKP. These samples were then instantaneously centrifuged at 2,500 rpm at 4°C and stored at −20°C until assay. Blood samples for the analysis of in vitro Ac-SDKP hydrolysis were immediately centrifuged at 2,500 rpm at 4°C and stored at −80°C until assay. For plasma angiotensin radioimmunoassays, blood samples (10 ml) were rapidly collected (within 10 s) into prechilled EDTA-K_3 vacutainers and 0.5 ml of an inhibitor mixture of 62.5 mM EDTA, 100 μM of the renin inhibitor remikiren, and 100 μM enalaprilat was immediately added to prevent in vitro generation and degradation of angiotensins (14). Blood samples were immediately centrifuged at 3,500 rpm at 4°C and stored at −80°C until assay.

For plasma Ac-SDKP measurements, the supernatant of centrifuged methanol-treated plasma (3/1 ml) was collected, evaporated to dryness, and reconstituted in 0.1 M phosphate buffer (pH 7.4) containing 0.1% BSA, 0.001% sodium azide, 2.3% NaCl, and 0.2% EDTA. Plasma Ac-SDKP was determined by a competitive enzyme immunoassay (12) which has been used previously for pharmacokinetic studies in humans (15) and has a limit of quantification of 1 pmol/ml. Captopril at doses of up to 10^{-6} M does not interfere with this assay.

To evaluate the kinetics of Ac-SDKP hydrolysis, 15 μl of a solution of the radiolabeled peptide [^{3}H]Ac-SDKP (3 μCi, 100 nM), specifically tritiated on the lysyl residue, was added to 450 μl of plasma. After 1 h of incubation at 37°C, 30-μl samples were drawn, a 10-μl solution (2 mg/ml) of carriers (Ac-SDKP [Ipsen-Biotech, Paris, France]; Lys-Pro and Lys [ICSN-CNRS, Gif-sur-Yvette, France]) was immediately added, and the mixture was frozen on dry ice. The radioactivity specifically associated with [^{3}H]Ac-SDKP and its degradation products was then evaluated (3).

Plasma active renin was measured by immunoradiometric assay (16) using the monoclonal antibodies 3E8 and ^{125}I-4G1 in a commercially available kit (ERIA, Diagnostics Pasteur, Marnes-la-Coquette, France).

Plasma ACE activity was quantified by a spectrophotometric method (using Hip-His-Leu as substrate) according to Cushman and Cheung (17). To minimize dissociation of captopril from plasma ACE, blood samples were immediately centrifuged at 4°C and stored at −80°C, and plasma ACE activity was determined within 48 h (18).

Detection of the insertion-deletion (I/D) polymorphism of the ACE gene by enzymatic amplification. ACE gene *I/D* polymorphism was detected by PCR using oligonucleotide primers flanking the ALU insertion (19). Three subjects were homozygous for the D allele (*DD*) and five were heterozygous (*I/D*).

Statistical methods. Areas under the curve from 0 to 8 h (AUC_{0-8h}) were calculated according to the trapezoidal rule for plasma Ac-SDKP. The time to peak effect and the maximum value at peak for each of the pre-cited variables were graphically determined for each subject.

All parameters were analyzed by two-sample *t* tests taking into account period, carry-over, and treatment effects according to the method for repeated measures in a two-period crossover design proposed by Armitage (20). The relationship between two continuous variables was studied with Spearman's rank correlation test. Natural logarithmic transformation was applied where appropriate. Comparisons between the baseline level of all parameters and the 8-h post–captopril dose level were made by a Wilcoxon paired test. Calculations were done with the STATVIEW II and SUPERANOVA statistical software programs (Apple Macintosh Abacus Concepts Inc., Berkeley, CA). Data are expressed as mean±1 SD in text and mean±1 SEM in the graphs. The times to peak effect are expressed as median (range). A probability value < 0.05 was considered as significant.

Results

The stability of all parameters over the 8 h of investigation was consistently obtained after placebo intake (Fig. 1) and no period or carry-over effect was present. Therefore, only treatment effect is reported.

Effects of captopril on plasma Ac-SDKP (Fig. 1). Plasma Ac-SDKP levels steeply increased from 3.3±0.7 to 16.0±5.7 pmol/ml 4 h after captopril administration, decreasing slowly thereafter. The time to maximum concentration of plasma Ac-SDKP was observed after a median (range) time interval of 4 h (2–6 h) which corresponds with a maximum value for plasma Ac-SDKP of 18.1±6.1 pmol/ml. At peak, the mean difference (95% confidence interval) between captopril and placebo for plasma Ac-SDKP levels was 14.2 (9.7–18.7) pmol/ml. 8 h after dose, plasma Ac-SDKP levels remained significantly higher (11.9±1.7 pmol/ml) than their pretreatment values (3.3±0.7 pmol/ml, $P < 0.001$). The AUC_{0-8h} of plasma Ac-SDKP levels after captopril administration was highly and significantly different from that of placebo (100±18 vs. 27±7 pmol·h·ml^{-1}, $P < 0.001$). The increase in plasma Ac-SDKP after captopril administration was consistently observed for all eight subjects.

Mean plasma Ac-SDKP levels (average of all values during the placebo phase for each subject) were not significantly correlated with either mean plasma ACE activity measured by Cushman's assay or mean plasma Ang II/Ang I ratio (not shown). The ACE *I/D* polymorphism did not influence basal plasma Ac-SDKP levels (*DD*, range: 2.4–3.6 pmol/ml and *ID*, range: 2.4–4.4 pmol/ml).

Effect of captopril on in vitro [^{3}H]Ac-SDKP hydrolysis. During the placebo phase and before captopril administration, human plasma hydrolyzed the radiolabeled peptide [^{3}H]Ac-SDKP in vitro, and the residual radioactivity specifically associated with [^{3}H]Ac-SDKP was 55±6%.

Captopril (50 mg) almost completely inhibited [^{3}H]Ac-SDKP hydrolysis: 1 h (range: 0.5–2 h) after dose, the maximum residual radioactivity associated with [^{3}H]Ac-SDKP was 99±1% after 1 h of in vitro incubation. A significant inhibition of [^{3}H]Ac-SDKP hydrolysis persisted throughout the investigation, as shown by the presence of 90±5% of residual radioactivity associated with [^{3}H]Ac-SDKP 8 h after captopril intake.

Effects of captopril on plasma ACE activity (Cushman's assay) and on the renin-angiotensin system. In vitro plasma ACE

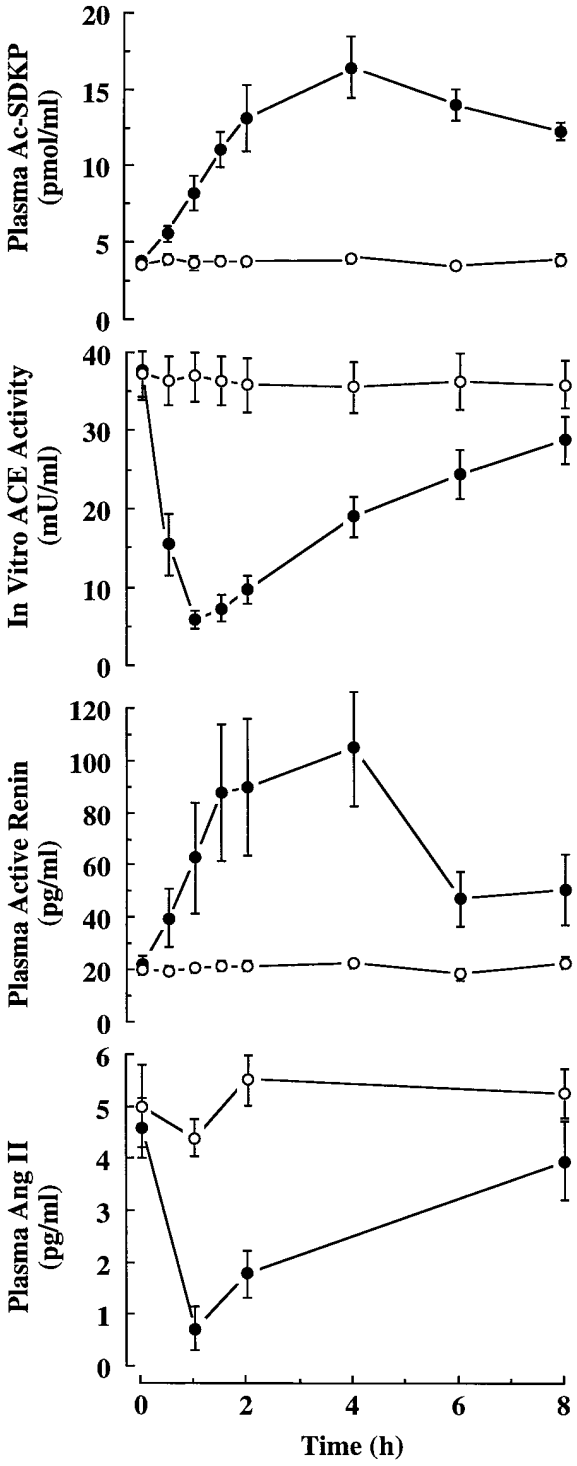

Figure 1. (From top to bottom): Plots of the time course evolution of plasma Ac-SDKP levels, in vitro ACE activity (Cushman's assay), plasma active renin, and Ang II levels after captopril 50 mg (*filled circles*) and placebo (*open circles*) intake in eight healthy subjects (mean±SEM).

activity measured by Cushman's assay decreased rapidly toward a nadir (13±7% of its baseline value) 1 h (range: 0.5–1.5 h) after captopril intake and began to increase from the second hour after dose onwards, reaching 75±6% of its baseline value 8 h after dose (Figs. 1 and 2). Plasma Ang II fell to very low levels 1 h after dose (0.6±1.2 pg/ml), this fall being associated with a parallel increase in plasma active renin which reached its maximum value (115±77 pg/ml) after a median time interval of 4 h (range: 2–8 h) after dose (Fig. 1). Whereas, 8 h after dose, plasma Ang II levels returned to their pretreatment values (3.8±2.2 vs. 4.4±1.6 pg/ml at baseline, NS), plasma active renin levels remained significantly higher than their baseline values (48±38 vs. 20±8 pg/ml, respectively, $P < 0.05$), despite their gradual decrease from the fourth hour after dose onwards (Fig. 1). The plasma Ang I profile after captopril intake was parallel to that of plasma active renin (not shown). The plasma Ang II/Ang I ratio, an index of in vivo ACE activity (21), decreased from 0.83±0.14 to 0.01±0.03 1 h after captopril intake and remained at a low value of 0.24±0.10 8 h after dose.

Comparison of the in vitro and in vivo effects of captopril on Ac-SDKP and the renin-angiotensin system. The relative changes of the residual radioactivity associated with [^{3}H]Ac-SDKP after 1 h of incubation, which represent in vitro plasma ACE activity using [^{3}H]AcSDKP as substrate (expressed as a percentage), can be compared with the relative changes in plasma ACE activity measured either by Cushman's assay (using Hip-His-Leu as substrate) or by the relative changes in plasma Ang II/Ang I ratio, which in fact represent in vivo endothelial and plasma ACE activity. The time course of ACE activity inhibition measured by these three methods is shown in Fig 2.

1 h after captopril administration (i.e., at peak), ACE activity was reduced 98±3, 92±5, and 87±8% from its baseline level when using the relative changes in plasma Ang II/Ang I ratio, [^{3}H]AcSDKP hydrolysis, and Hip-His-Leu hydrolysis, respectively (Fig. 2). From 1 h after dose onwards, the time course evolution of both the relative changes in plasma Ang II/Ang I ratio and the residual radioactivity associated with [^{3}H]AcSDKP were superimposed, leading to residual ACE inhibitions of 76±13 and 74±4%, respectively, 8 h after captopril administration (Fig. 2). The time course evolution of the relative changes in plasma ACE activity using Hip-His-Leu as substrate was clearly dissociated from the two precedent curves.

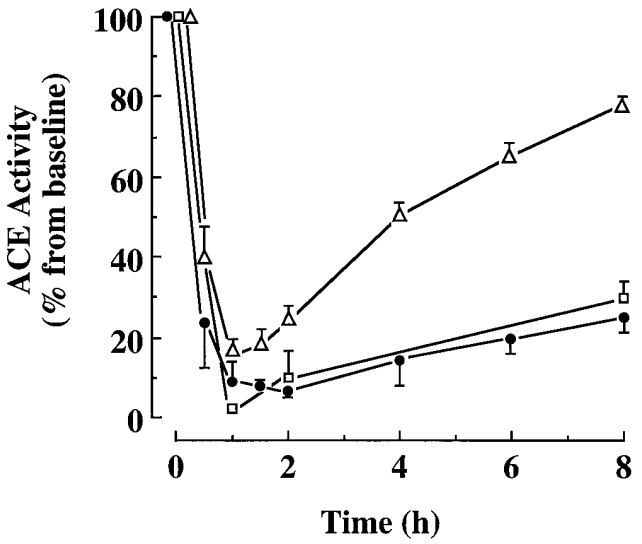

Figure 2. Comparison of the time course evolution of ACE inhibition measured in vitro by two methods using Hip-His-Leu (*open triangles*) and [^{3}H]Ac-SDKP (*filled circles*) as substrates of ACE and estimated in vivo using the Ang II/Ang I ratio (*open boxes*) (mean±SEM).

8 h after drug intake, only an apparent 25±6% residual inhibition was detected when using Hip-His-Leu as substrate (Fig. 2).

Discussion

This study shows that acute in vivo ACE inhibition induced by a single oral 50-mg dose of captopril and quantified by its usual parameters (plasma ACE activity assessed by Cushman's assay, plasma Ang II, and plasma Ang II/Ang I ratio) resulted in a 5.5-fold (range: 4–8.5-fold) increase in the natural stem cell regulator Ac-SDKP plasma level when compared with placebo. Captopril was selected because (*a*) among the ACE inhibitors studied in vitro, it has the lowest IC_{50} (10^{-9} M) for inhibition of Ac-SDKP hydrolysis by ACE (3, 4); (*b*) it binds preferentially to the NH_2-terminal domain rather than to the COOH-terminal domain of ACE in vitro (22), being therefore a potent inhibitor of the NH_2-terminal domain, which is involved in the catabolism of Ac-SDKP (4); and (*c*) it is not a prodrug, it is rapidly absorbed by the intestinal tract, and its pharmacokinetics is well known.

Both the plasma levels of Ac-SDKP attained after captopril intake and the AUC_{0-8h} of its time course evolution were of a similar magnitude to those results obtained during a 12-h continuous infusion of 62.5 μg/kg of the synthetic peptide Ac-SDKP (15). Lower dosages of exogenous Ac-SDKP (from 2.5 and 12.5 μg/kg) have been shown to be active doses in preventing the myelotoxic effects of anticancer chemotherapy (cytarabin and ifosfamide) in cancer patients (23). The increase in plasma Ac-SDKP was due to a prolonged and profound inhibition of its hydrolysis after captopril intake. Whereas 45% hydrolysis of the radiolabeled peptide was measured in vitro before drug intake and during the placebo phase, 90–99% of the intact [^{3}H]Ac-SDKP persisted after captopril intake for the entire procedure. Since captopril inhibits both plasma and endothelial ACE, it is therefore highly conceivable that it increased plasma Ac-SDKP by inhibiting its in vivo hydrolysis. That both the plasma and endothelial enzyme are probably involved in the catabolism of Ac-SDKP is suggested by the comparison of the very short half-life (4.5 min) of the peptide when infused exogenously (15) to that obtained after its incubation with plasma in vitro (50 min) (3, 4). Taking into account the very short in vivo half-life of the tetrapeptide, the combination of the prevention of Ac-SDKP hydrolysis by captopril in vitro with the persistence of high plasma levels for more than 8 h after its intake suggests that Ac-SDKP is either not degraded by peptidases other than ACE or that it is rapidly removed in vivo. In agreement with the in vitro study (3), our results confirm that ACE is the principal enzyme implicated in Ac-SDKP catabolism in vivo and that it is also likely to determine the rate-limiting step of Ac-SDKP hydrolysis.

Another interesting result emerging from this study concerns Ac-SDKP metabolism. This peptide probably undergoes an intense permanent process of production and degradation. Plasma Ac-SDKP levels during the placebo period were remarkably stable at ∼ 2–4 pmol/ml as a consequence of a balance between the production and the degradation rates of the peptide, with no detectable influence of the ACE gene *I/D* polymorphism on its plasma levels. When complete inhibition of Ac-SDKP degradation by captopril was obtained (99% inhibition at peak), plasma levels of the peptide increased toward a maximum value of 18.1±6.1 pmol/ml, indicating that a permanent production process exists and that ACE is a physiological regulator of Ac-SDKP clearance. Maintaining physiologically low plasma levels of Ac-SDKP of ∼ 2–4 pmol/ml necessitates a permanent degradation, which seems mainly dependent on ACE activity.

This study also demonstrates that the natural hemoregulatory tetrapeptide Ac-SDKP is a newly discovered physiological substrate for ACE in vivo and the first natural peptide hydrolyzed by the ACE's NH_2-terminal domain not only in vitro but also in vivo. Rousseau et al. (4) have shown that if Ac-SDKP can be bound by both the COOH- and NH_2-terminal domains of ACE with similar affinity (K_m), only the NH_2-terminal domain is likely to physiologically hydrolyze this peptide, since its catalytic efficiency (K_{cat}/K_m) is 50 times greater than that of the COOH-terminal domain. Since luteinizing hormone–releasing hormone, the other known substrate for the NH_2-terminal domain in vitro (1), is 43-fold less efficiently cleaved than Ac-SDKP (24), this hemoregulatory peptide is the first natural NH_2-terminal domain–specific substrate actually discovered for ACE. In addition, the increased plasma Ac-SDKP level after ACE inhibition demonstrates that the ACE NH_2-terminal domain is functional in vivo. Ang I, which is cleaved by both domains in vitro (25), is probably converted in plasma and at tissue level by the NH_2-terminal domain in conjunction with the COOH-terminal domain. Physiologically, ACE is therefore a bifunctional zinc metallopeptidase and both active sites of ACE are inhibited in vivo by the ACE inhibitor, captopril.

The apparent association rates between captopril and both active sites of ACE were similar. This is suggested by the identical 1-h delay necessary to reach peak ACE inhibition assessed either by the in vitro hydrolysis of Hip-His-Leu (a preferential substrate for the COOH-terminal domain) (25) or that of Ac-SDKP (a preferential substrate for the NH_2-terminal domain) and by the Ang II/Ang I ratio in vivo.

After peak inhibition, time course evolution of the in vitro ACE inhibition measured by both substrates diverged, as shown by a 76% residual inhibition of ACE when using Ac-SDKP as substrate compared with a 25% residual inhibition when using Hip-His-Leu as substrate, 8 h after drug intake. One explanation of this divergence is that captopril may dissociate more rapidly from the COOH- than from the NH_2-terminal domain, since this ACE inhibitor binds to the NH_2-terminal domain with a much better affinity at both low and high chloride concentration (22). Another explanation for the discrepancy between Cushman's and the Ac-SDKP method resides in a methodological issue. Ac-SDKP, like Ang I, and contrary to Hip-His-Leu, is a physiological substrate for ACE in vivo, having a low K_m (41 μM), close to that of Ang I (16 μM), in comparison with that of Hip-His-Leu (1,540 μM) (4, 25). The ACE inhibition measured in vitro depends both on the substrate's K_m and concentration and on the inhibitor's K_i and concentration. The divergence between the two curves may thus only reflect the fact that Ac-SDKP is a much better substrate than Hip-His-Leu for measuring ACE activity. Interestingly, the profile of ACE inhibition as assessed by the hydrolysis of Ac-SDKP in vitro was very close to that of both endothelial and plasma ACE inhibition as assessed by the time course evolution of the in vivo Ang II/Ang I ratio, thus more closely reflecting the in vivo ACE inhibition than Cushman's method. The measurement of Ac-SDKP in plasma and of its hydrolysis in vitro may thus provide a valuable tool in screening specific inhibitors of the NH_2-terminal domain of ACE and

in monitoring ACE inhibition during chronic treatment in place of using either Cushman's assay, which certainly underestimates the magnitude of the inhibition, or the Ang II/Ang I ratio, which requires time-consuming radioimmunoassays.

Historically, Ang I and bradykinin were the two substrates which permitted the discovery of ACE and the subsequent development of ACE inhibitors based on physiopathological concepts implicating both peptides. The discovery that the endogenous levels of circulating Ac-SDKP are so dependent on ACE activity orientates research on this enzyme toward a possible new physiological role related to the permanent catabolism of this natural inhibitor of hematopoietic cell entry into S-phase. Depending on the importance of this peptide in normal and pathological conditions, ACE inhibition may still have unknown or underinvestigated effects. Considering that this peptide accumulates in plasma during chronic ACE inhibition, an area which remains to be investigated, a potential deleterious effect of the accumulation of Ac-SDKP in plasma during chronic ACE inhibition could be anemia. Anemia has been reported in some particular situations such as renal failure or after renal transplantation (26, 27), an adverse event which has not been observed with the new Ang II type 1 receptor antagonist, losartan (28). On the contrary, a potential beneficial role for the accumulation of this peptide after ACE inhibition could be considered in at least two situations. The antiproliferative effects of ACE inhibitors have been attributed to the fall in Ang II, considered as a trophic factor (29). The increase in the plasma level of Ac-SDKP during ACE inhibitor therapy could also play a role in this phenomenon. Considering that Ac-SDKP activity is not restricted to the hematopoietic system, as suggested by its inhibitory effects on the entry of hepatocytes into S-phase, a role for Ac-SDKP in inhibiting pathological hyperplastic processes such as neointima proliferation after desendothelialization should be interesting to evaluate.

Bearing in mind the hematopoietic effects of Ac-SDKP, the administration of an ACE inhibitor preferentially specific for the NH_2-terminal active site to patients undergoing cancer chemotherapy, alone or in combination with exogenous Ac-SDKP, could greatly enhance the endogenous level of the peptide and increase its ability to block the effects of cytotoxic drug-induced stimulators of hematopoietic stem cell proliferation (7). Although unexplained, a potential role for ACE inhibitors is actually suggested by the observation that captopril inhibits radiation-induced pulmonary fibrosis in rats (30), most likely through a direct inhibition of fibroblast proliferation (31), and protects the jejunal mucosa in mice from acute radiation damage (32).

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