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MODULATION OF PROTEOLYTIC ACTIVITY IN TISSUES FOLLOWING CHRONIC INHIBITION OF ANGIOTENSIN-CONVERTING ENZYME

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Abstract—The release of angiotensin-converting enzyme (ACE) (EC 3.4.15.1) from aortic rings and the modulation of the proteolytic balance of rat organs under chronic ACE inhibition were examined. ACE from rat organs had a higher apparent molecular mass than the circulating enzyme, but a similar behavior towards ACE inhibitors. Chronic treatment with ACE inhibitors (captopril or lisinopril) for 25 days, followed by 1 day without treatment, increased plasma ACE, but only slightly modified lung, aorta, heart and kidney specific ACE activity. In the lung the activities of aminopeptidases A and B, two angiotensin degrading enzymes, decreased, as did the activity of aminopeptidase A in the plasma. *In vitro*, the release of ACE from aortic rings was not suppressed by inhibitors of either serine proteases, metalloproteases, serine and thiol proteases, or aspartyl proteases. After chronic ACE inhibition, the release of ACE from aortic rings was not significantly modified by the presence of protease inhibitors. As shown by gel filtration experiments, ACE was converted from its tissue form into its circulating form only after release from the endothelium.

Angiotensin-converting enzyme (ACE†) (EC 3.4.15.1) is an exopeptidase of broad specificity located on the luminal side of various endothelia, epithelia and neuroepithelia, and anchored by the C-terminal part of its polypeptide chain to the endothelial membrane. It is released into the blood circulation by a mechanism, probably involving proteolysis [1, 2], but the pathway of conversion of the tissue form into the circulating form of ACE is not known.

The most widely studied activities of ACE are the cleavage of the dipeptide His-Leu from angiotensin I (Ang I) thus producing the bioactive hypertensive peptide Ang II, and cleavage of the dipeptide Phe-Arg from the hypotensive peptide bradykinin, which inactivates it. ACE is the target of widely used anti-hypertensive drugs and its inhibitors act either by decreasing the production of Ang II and/or degradation of bradykinin, or by modification of other peptides. However, the biological activity of these drugs does not always correlate with their effect on circulating ACE [3, 4]. The chronic intake of ACE inhibitors induces the immediate inhibition of enzymatic activity but also, after a period of latency, an increase in circulating and tissue enzymes [5–7]. *In vivo*, autoimmune diseases, sarcoidosis, AIDS or systemic sclerosis [8–10] modify the activity of circulating ACE. *In vitro*, dexamethasone, fibroblast growth factor (FGF), thyroid hormones and ACE inhibitors increase ACE expression in endothelial cell cultures [11–16].

It is not known whether the induction of ACE in endothelia by chronic ACE inhibition corresponds to an adaptive reaction to diminished metabolism of ACE substrates, to decreased metabolism of enzyme–inhibitor complexes and/or to increased secretion of this enzyme into the circulation; nor is the duration of these tissue effects known. Little information exists on the effect of ACE inhibition on the expression of other proteases [17–19]. To address these questions, the apparent molecular mass of ACE extracted from rat tissues and from plasma have been compared and the exchange rates with ACE inhibitors measured. In rats receiving ACE inhibitors chronically, followed by withdrawal from treatment for 1 day, and in non-treated animals as controls, the specific activities of ACE, aminopeptidases and carboxypeptidases (Ang and bradykinin degrading enzymes) γ-glutamyl-transpeptidase (gGTP) (an unrelated enzyme, but whose substrates may be inhibitors for ACE [20]) have been measured in plasma, aorta, heart, brain and kidney. The release of ACE from aortic rings in the presence of inhibitors of proteases has been evaluated.

MATERIALS AND METHODS

Animal handling. The drinking behavior of rats was determined. Captopril (Lopirin, Squibb) at a concentration of 200 mg/L or lisinopril (Merck, Sharp and Dohme) at a concentration of 22 mg/L, were given to adult rats in drinking water for 25 days. One day after discontinuing treatment, the animals were killed after ether anesthesia, and blood, aorta, heart, lung, kidneys and brain were removed in that order, cleaned of adhering tissue,

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† Abbreviations: ACE, angiotensin-converting enzyme; gGTP, γ-glutamyltranspeptidase; APA, aminopeptidase A; APB, aminopeptidase B; CPB, basic carboxypeptidase; Ang, angiotensin; SBTI, soy bean trypsin inhibitor; PMSF, phenylmethylsulfonylfluoride; PBS, phosphate-buffered saline.

washed in cold phosphate-buffered saline (PBS) and immediately frozen at −70°. Control rats without treatment were handled in the same manner.

Organ extraction. Organs were extracted by homogenization at 4° for 2 × 15 sec in a Polytron homogenizer in 0.1 M phosphate buffer containing 0.3 M NaCl, pH 8.0. The respective volumes of extraction were: aorta, 1 mL; heart, 3 mL; lung, 4 mL; brain, 4 mL; kidney, 3 mL. After 30 min at 4° with occasional shaking, the suspensions were centrifuged for 30 min at 4000 rpm at 4°. Aliquots of tissue extracts were frozen at −70° for subsequent determinations.

ACE activity. Determination of ACE activity was performed by adapting the method described [21] for plasma enzyme determination. Briefly, aliquots of plasma or organ extracts, diluted if necessary, were added to 400 μL of phosphate–NaCl buffer and 40 μL of *Z*-Phe-His-Leu (Bachem) in 0.05 N NaOH, final concentration 1 mM. After incubation at 37°, 1 mL of 0.1 N NaOH, was added and the reaction was continued as described. Units of enzymatic activity were calculated from a standard solution of His-Leu.

The specificity of the reaction was controlled by preincubating samples with decreasing concentrations of ACE inhibitors or EDTA before adding the substrate.

gGTP activity. gGTP activity was determined for 30 min at 37° with 50 μL of organ extracts using L-*g*-Glu-*p*-nitroanilide (Bachem) as donor, final concentration 1 mM, and Gly-Gly as acceptor, final concentration 20 mM [22]. The reaction was stopped by adding 2 vol. of 2 M acetic acid and absorbance was measured at 405 nm. Units of enzymatic activities were calculated using an ε value of 8800 M^{-1} cm^{-1}.

Aminopeptidase A (APA) and B (APB) activities. APA activity was evaluated with L-*a*-Glu-*p*-nitroanilide and APB activity with L-Arg-*p*-nitroanilide (Bachem) as substrates (final concentration 1 mM) in 0.1 M Tris buffer pH 7.6 for 30 min at 37°. The reaction was stopped with 2 vol. of 2 M acetic acid, absorbance was measured at 405 nm and units of enzymatic activities were calculated as for gGTP.

Basic carboxypeptidase (CPB) activity. This activity was assessed with a new sensitive fluorescent method based on the estimation of lysine [23] released from hippuryl-L-Lys. Carboxypeptidase was activated for 15 min at 37° in 0.1 M phosphate: 0.1 mM $CoCl_2$ pH 7.5. Hippuryl-L-Lys (Bachem) (final concentration 1 mM) was added, aliquots were removed at 2 and 12 min, and the reaction was stopped with 1 vol. of 1 M HCl. The lysine content was estimated by adding 0.1 M borate pH 10, *o*-diacetylbenzene (Fluka) (final concentration 0.5 mg/mL) and 2-mercaptoethanol (final concentration 0.25 μg/mL). After 10 min at room temperature, fluorescence was measured at 355/455 nm and units of enzymatic activity calculated by comparing with a Lys standard solution.

Protein estimation. Protein concentration was determined with the BCA kit (Pierce) using bovine serum albumin as standard.

Gel filtration chromatography. Organ extracts (0.5–1 mL) were filtered on a calibrated Sephadex G-200 (Pharmacia) (1.6 × 90 cm) column in 0.05 M phosphate buffer: 0.15 M NaCl pH 8.0. Eluted

Table 1. Effects of protease inhibitors on ACE secretion by rat aortic rings

	Inhibitor concentration (mM)	% ACE secretion over control
Ser-proteases		
Benzamidine	10.0	80
SBTI	0.05	76
Bowman–Burk trypsin inhibitor	0.05	63
Kunitz trypsin inhibitor	0.05	105
Chymostatin	1.2	76
Aprotinin	0.3	90
PMSF	9.0	85
Thiol and Ser-proteases		
Leupeptin	1.0	83
Antipain	1.6	89
Iodoacetamide	2.7	120
Metallo-proteases		
Phosphoramidon	1.8	94
EDTA	1.3	105
Asp-proteases		
Pepstatin A	0.7	52

Rat aorta, cleaned of adventitia, were cut into equivalent rings and incubated in PBS in the absence or presence of protease inhibitors for various times at 37°. Aortic rings of each animal were used as controls without inhibitor. Aliquots of supernatants were assayed for ACE activity. Control values were between 21 and 60 mU ACE/mL. hr.

fractions were assayed for protein by measuring absorbance at 280 nm and for ACE activity as described. The specificity of the reaction was controlled by preincubating the fractions with lisinopril (final concentration 0.05 mM).

Inhibitor exchange rate. One milliliter of plasma, lung or kidney extracts in phosphate–NaCl buffer was incubated with 500 μL of ^{125}I-MK 521 (kindly prepared by Dr Fenner, Merck, Sharp and Dohme, and provided by Dr Nussberger, Lausanne), 2.10^4 cpm/mL, for 90 min at 37°, then 50 μL of enalaprilat (Merck, Sharp and Dohme), final concentration 0.02 mM, were added. The mixture was maintained at 37°, and 150 μL aliquots were removed at various times, added to 1 mL ethanol, centrifuged for 15 min at 4000 rpm, washed with 1 mL ethanol under the same conditions and radioactivity was measured in the supernatant and the pellet. Controls were treated under the same conditions without addition of enalaprilat.

ACE release from aortic rings. Rat aorta, cleaned of adventitia and washed in cold PBS were cut into eight equivalent rings which were either incubated in 1 mL PBS at 37° for 0, 2 or 7 hr. Incubation supernatants or extracts of homogenized rings were filtrated on Sephadex G-200 as described. ACE activity was determined as described. Specificity of the reaction was controlled with lisinopril.

Alternatively, two aortic rings were incubated in 200 μL of PBS at 37° containing one of the protease inhibitors at the concentration indicated in Table 1 (with the exception of EDTA: 0.3 mM), and aliquots of the mixture were assayed for ACE activity at various times. Inhibitors were from Serva (soy bean trypsin inhibitor, SBTI); Fluka (iodoacetamide, phenylmethylsulfonylfluoride PMSF, aprotinin, phosphoramidon and EDTA); Bachem (pepstatin A, chymostatin, leupeptin and antipain); the Sigma Chemical Co. (benzamidine). Two aortic rings of each animal were included in each determination.

At the end of the incubation period, supernatants were removed and aortic rings were homogenized. Protein content and ACE activity were measured in supernatants and ring extracts.

For assaying ACE activity when EDTA was used as an inhibitor, $ZnSO_4$ (final concentration 0.3 mM) was added to the activity determination medium prior to substrate.

Secretion of ACE by endothelial cells. An endothelial cell line of rat brain origin (line EC219) [11] was grown to confluence in six well plates in Dulbecco's modified Eagle's medium (DMEM) (Seromed) containing 10% fetal calf serum (Seromed) as described. One milliliter of DMEM without fetal calf serum was then added to each well and the cells were maintained at 37° for various periods of time. Supernatants were removed and ACE activity determined in supernatants and cells, and after gel filtration on Sephadex G-200.

Statistics. Statistical significance was evaluated by non-paired Student's *t*-test, N − 1 degrees of freedom.

RESULTS

ACE release from aortic rings

In order to determine the biochemical characteristics of tissue and circulating ACE, ACE was extracted from various rat organs and compared to circulating ACE. The spontaneous release of ACE from aortic rings and endothelial cells in culture was used as a model to analyse the mechanisms involved in the processing of ACE and to determine the cells involved.

After extraction in the buffer system, ACE eluted in all organs studied as a high molecular mass component, while in plasma, it eluted with an apparent molecular mass of 140 kDa. A mixture of the circulating (plasma) and the tissue form (aorta homogenized in 1 mL of rat plasma instead of buffer) were separated completely under the conditions of chromatography, indicating that the plasma form of the enzyme was not adsorbed on a tissue carrier (results not shown). Tissue and circulating forms of ACE were inhibited to the same extent by ACE inhibitors (enalaprilat, lisinopril, captopril and EDTA) in a concentration dependent fashion. The initial exchange rate of ACE inhibitors (lisinopril–

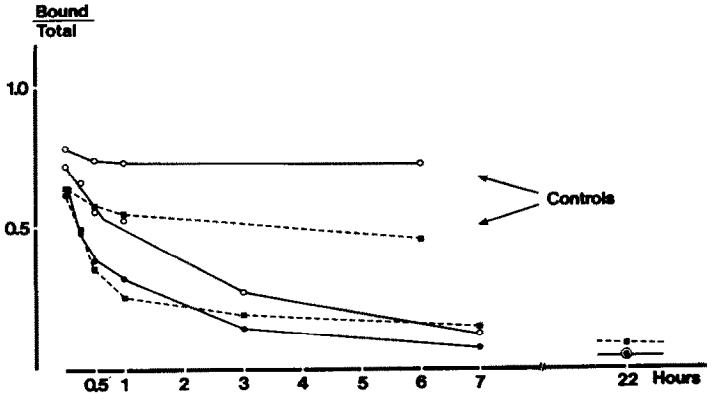

Fig. 1. Exchange rate of ACE inhibitors in plasma and organs. One milliliter of plasma (■), lung (○) or kidney (●) extracts was incubated with labeled MK 521 then the label was displaced with non-labeled MK 422.

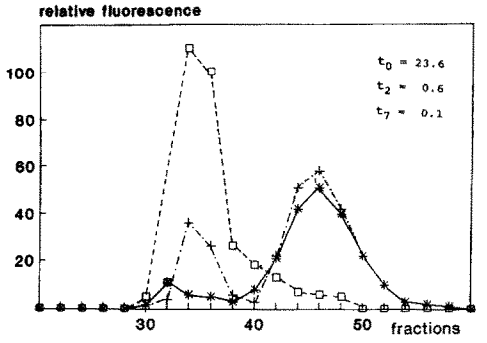

Fig. 2. Gel filtration through Sephadex G-200 of ACE released from aortic rings. Incubation time: (□) 0 hr; (+) 2 hr; (*) 7 hr. The proportion between the high and the low molecular mass forms of the enzyme at various incubation times is indicated in the panel at the right of the diagram.

enalaprilat) was similar whatever the tissue or origin of the enzyme (Fig. 1), showing that under the experimental conditions, ACE activity was in fact measured. These results show that there exists two forms of ACE, a tissue and a circulating form, with comparable kinetic properties.

ACE was spontaneously released from aortic rings by a mechanism which was not inhibited by any single protease inhibitor at the concentration used (Table 1). Preliminary experiments showed that the experimental procedure for preparation of aortic rings gave comparable levels of ACE activity in each aortic ring of the same animal, but that there was great variability between aorta from different animals. Thus, aortic rings of the same animal had to be taken as their own controls in all experiments. The effective concentrations of inhibitors leupeptin, antipain, iodoacetamide, pepstatin A, chymostatin and EDTA were evaluated with specific synthetic substrates in extracts of organs. The concentrations used for the other inhibitors were obtained from previous experience with these substances. The inhibitory effect of EDTA on ACE was totally reversed by concentrations of EDTA and $ZnCl_2$, as used on plasma (results not shown).

As shown by gel filtration experiments (Fig. 2), the form of ACE initially released from cell membrane was the high molecular mass "tissue form". The appearance of the low molecular mass "circulating form" of ACE increased with increasing incubation time. The enzyme finally obtained was indistinguishable from plasma ACE as ascertained by gel filtration of rat plasma. However, only the high molecular mass form could be detected in extracts of aortic rings, even after incubation for 7 hr at 37° or 24 hr at 4°. Preincubation of the eluted fractions with lisinopril abolished enzymatic activity in all experiments.

Endothelial cells in culture released ACE spontaneously in a time-dependent fashion without the addition of external proteases (Fig. 3A). The same two-peak pattern of elution was obtained with endothelial cell supernatants as with aortic ring supernatants (Fig. 3B), indicating that the presence of non-endothelial cells was not required in the processing of ACE by aortic rings.

Effect of chronic inhibition of ACE

The effect of chronic treatment with ACE inhibitors was studied to evaluate the consequences of prolonged modulation of proteolysis on the functions of the endothelium, as ascertained by the release of ACE from aortic rings.

With the exception of plasma, where ACE activity was elevated 24 hr after withdrawal of ACE inhibitors (captopril or lisinopril) from drinking water, ACE activities in all organs were not statistically different between treated and control animals (Table 2). Plasma carboxypeptidase N activity was not modified. This enzymatic activity was not readily extracted under our experimental conditions from the various organs. The activity of gGTP was used as a control and was not modified in any of the extracts. The amount of protein extracted in organs of treated animals was comparable to that of non-treated animals. However, a compensatory mechanism to the decrease of ACE activity seemed to exist in the lung, where a statistically significant decrease of two angiotensin-degrading enzymes, APA and APB activities, was demonstrated in this organ. The decrease of APA activity was further reflected in plasma levels of this enzyme (Table 2).

Since ACE levels remained elevated in plasma after discontinuation of treatment with ACE inhibitors, the spontaneous release of ACE from aortic rings was used to assess the contribution of this release to the maintenance of that induction in plasma and the possible implication in this phenomenon of proteolytic enzymes, after chronic inhibition. In Fig. 4, the release of ACE from aortic rings in the presence of protease inhibitors is compared between rats which had received captopril for 25 days and in control rats. The secretion of ACE by aortic rings after chronic *in vivo* inhibition of this enzyme with captopril was not significantly modified by the presence of inhibitors or by captopril treatment. These results show that the functions of vascular cells, as determined by the release of ACE, were not modified after chronic inhibition of ACE.

DISCUSSION

Peptides are important mediators of biological systems and their metabolism is under the control of various peptidases. Modification of proteolytic enzymes will therefore affect regulation of peptide metabolism.

It is known that chronic intake of an ACE inhibitor produces inhibition of plasma and tissue enzyme activities, and an increase in the amount of both forms of this enzyme [5–7]. How long this effect lasts in these various physiological compartments and the mechanism associated with this increase in concentration are not known. It has been shown that 1 day after discontinuation of either acute ACE or chronic ACE inhibition, plasma, but not tissue ACE activity was fully restored [24, 25]. Since ACE has a broad specificity [26], this effect is not necessarily related to the regulation of Ang

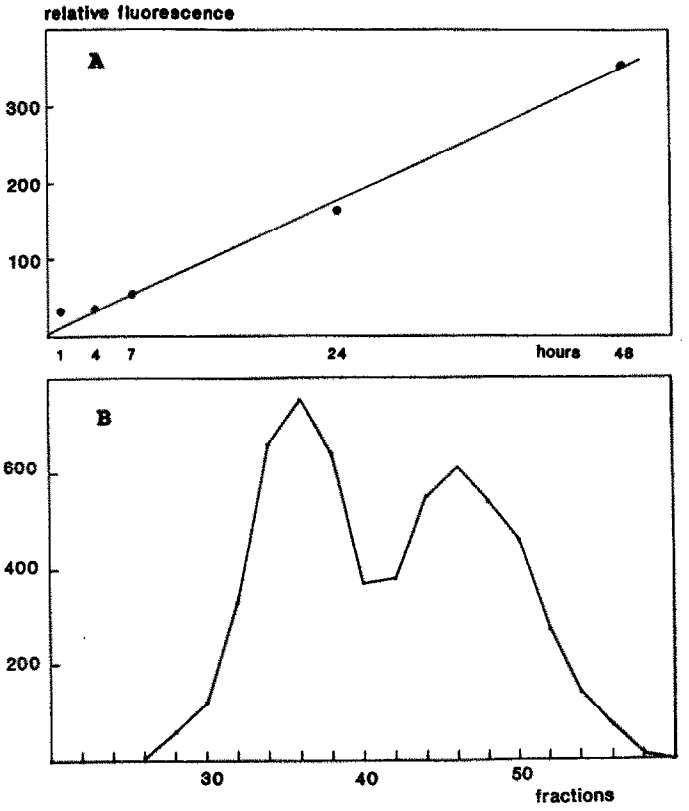

Fig. 3. Release of ACE by endothelial cells in culture. (A) Kinetics of appearance of ACE in cell supernatant. (B) Gel filtration through Sephadex G-200 of supernatant after 72 hr of incubation.

Table 2. Effect of chronic ACE inhibition on enzymatic activities of APA, APB and gGTP

	gGTP	APA	APB	CPB	ACE
Plasma					
Control	0	1.25 ± 0.12	0.97 ± 0.09	17.9 ± 2.4	2.59 ± 0.23
Treated	0	0.90 ± 0.26*	1.04 ± 0.20	18.3 ± 2.4	5.17 ± 0.84†
P value		2.427			5.849
Lung					
Control	56.9 ± 5.1	41.3 ± 2.7	56.6 ± 6.4	0	57.8 ± 5.6
Treated	56.1 ± 9.4	24.6 ± 4.5†	36.8 ± 9.9*	0	42.4 ± 9.1
P value		6.314	3.172		
Kidney					
Control	8055 ± 641	90.8 ± 10.5	183.7 ± 18.9	0	7.88 ± 1.52
Treated	7970 ± 597	90.7 ± 21.6	204.7 ± 17.5	0	8.36 ± 1.18
Brain					
Control	11.2 ± 1.5	0	0	0	12.54 ± 2.52
Treated	12.4 ± 2.8	0	0	0	14.10 ± 2.44

Lisinopril was given to adult rats (N = 5 in each group) in drinking water for 25 days. After 1 day without treatment, animals were killed, blood and organs immediately removed, extracted in phosphate–NaCl buffer and enzymatic activities were determined. Control animals were treated in the same manner, without the inhibitor.

Activities: U/mg protein (ACE: mU/mg); means ± SD, N = 5.

Statistical significance was assessed with a non-paired Student's *t*-test, df = 9.

0, no measurable activity.

* $P < 0.05$, treated versus control animals.

† $P < 0.001$, treated versus control animals.

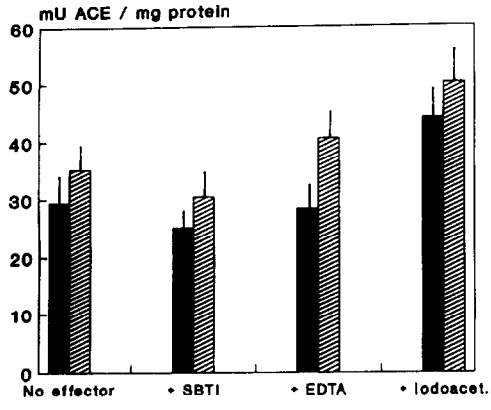

Fig. 4. Effect of chronic inhibition of ACE on ACE release by aortic rings. Captopril was given to rats for 25 days (N = 5 in each group), then aorta were removed, cut into eight equivalent pieces and ACE release was monitored for up to 60 min in the absence or presence of protease inhibitors (EDTA, SBTI or iodoacetamide). Aorta of control rats were treated in the same manner. After completion of the release experiments, aortic rings were homogenized and the protein content was measured.

processing. Ang II, one of the products of the catalytic activity of ACE, can also act as an angiogenic factor, as an activating factor for oncogenes or cause phosphorylation of proteins (for a review see Refs 27–29).

Plasma ACE is thought to originate mainly from pulmonary endothelium. It is released into the blood stream by a non-defined proteolytic event [1, 2]. The accumulation of ACE in the blood during chronic ACE inhibition may result from diminished clearance of the inhibitor–enzyme complex, increased release from the membrane of this complex, increased synthesis of the enzyme, a direct effect of the inhibitor on endothelium or a combination of several of these mechanisms. In this study, an attempt has been made to elucidate aspects of this process. To induce ACE, rats were given ACE inhibitors for 25 days, then 1 day after a washout period, the animals were killed and ACE, as well as other enzymatic activities, were measured in several organs and compared to non-treated animals. The release of ACE from aortic rings and endothelial cells in culture was also evaluated.

The tissue form of this enzyme could be extracted under these experimental conditions. It was almost exclusively represented by an enzyme with high apparent molecular mass, however, kinetic characteristics of the tissue and circulating forms of the enzyme were comparable as shown by the exchange rate of two ACE inhibitors, confirming results of other groups [12, 30, 31].

There was evidence from gel filtration experiments that complete conversion from the tissue to circulating form of ACE *in vitro* occurred after release into the incubation medium. However, this may reflect the limitations of the experimental *in vitro* model. Endothelial cells in culture behaved like aortic rings, showing that the mechanisms of conversion of the enzyme are dependent on endothelium only. We are presently investigating the characteristics of ACE released by endothelial cells of various origins in culture.

In the experimental model represented by the aortic ring, inhibitors of several classes of proteases did not suppress ACE release. These results in aorta did not confirm those obtained by Hooper *et al.* [1] on the transformation of the membranous into the circulating form of ACE in the kidney, where EDTA was an inhibitor for the unidentified processing enzyme(s). These discrepancies might be due to methodological problems since in Hooper's experiments, the inhibitory effect of EDTA was eliminated by dialysis while in the experiments described here, Zn^{2+} was added. There may also be differences between kidney and aorta, but experiments performed on both organs eliminated the involvement of a Ser-protease in the processing of ACE. The use of a single inhibitor or a single concentration of inhibitor in the incubation medium cannot exclude the possibility that more than one protease may be implicated in this process, in comparison with the postulated processing of other proteins from cell membrane, such as tumor necrosis factor-α [32]. This possibility is presently under investigation using cells in culture.

A persistent tissue inhibition has been observed after chronic inhibition of ACE [25]. Estimation of the residual inhibition 1 day after discontinuation of treatment was not a goal of this study. The discrepancies observed between the two ACE inhibitors might be explained either by differences between the induction mechanisms, since the induction of plasma ACE was higher with captopril which is a radical scavenger [33], than with lisinopril, or by differences between the dissociation of enzyme–inhibitor complexes, since lisinopril has a higher affinity for ACE.

The content of ACE in the brain was increased slightly, while that of gGTP, a specific marker of cerebral endothelium [34], but whose substrates may inhibit ACE [20], was not. The infusion of acid or neutral aminopeptidases into the brain decreased the blood pressure of spontaneously hypertensive rats [35]. However, 1 day after discontinuation of chronic treatment, a compensatory mechanism to the decreased activity of ACE persisted only in the lung, and partly in the blood, since aminopeptidase activities were diminished. An increase of neutral, but not basic, carboxypeptidases and a decrease of neutral endopeptidase 24.11 and of an amastatin-sensitive basic aminopeptidase has been described in the kidney after chronic inhibition with the ACE inhibitor, enalapril [17]. In the experiments described here, neutral endopeptidase was not tested, but no differences were observed in the level of aminopeptidase activity in the kidney. Differences in the extraction pH, or the washout period could explain this discrepancy. Chronic inhibition of ACE has been shown to modulate the activity of prolyl-hydroxylase [18].

The differences between organs may be explained by the variability of endothelia, or by differences in the localization of the different proteases. We have found that endothelial cell lines from the brain, but

not from the lung, express APA activity, and that dexamethasone differentially regulates these various proteolytic activities [11].

In conclusion, 24 hr after stopping the chronic intake of an ACE inhibitor, the secretion of ACE by aortic rings and the level of that enzyme in rat organs were only slightly increased, while it was still significantly elevated in blood. The results obtained are compatible with the hypothesis that increased plasma ACE is due to a decrease in the elimination of the enzyme–inhibitor complex. In humans, the elevated levels of circulating ACE lasted for more than 2 weeks (Ref. 3 and L. Juillerat, unpublished results). There exists in the lung a long-lasting compensatory mechanism to the chronic inhibition of ACE, and the effects of ACE inhibition observed on aminopeptidase activity, by modifying the metabolism of biologically active peptides, may explain some of the side-effects of ACE inhibitors. The *in vitro* release of ACE from aortic rings could not be inhibited by a single protease inhibitor, and chronic inhibition did not change this pattern, providing evidence that biochemical modification of the ACE molecule might be effected by various endothelial or circulating proteases, either before or after the release of this enzyme from the endothelium.

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