Involvement of human plasma angiotensin I-converting enzyme in the degradation of the haemoregulatory peptide *N*-acetyl-seryl-aspartyl-lysyl-proline

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The degradation of *N*-Ac-Ser-Asp-Lys-Pro (AcSDKP), a negative regulator controlling the proliferation of the haematopoietic stem cell, by enzymes present in human plasma, has been investigated. Radiolabelled AcSD[4-^{3}H]KP ([^{3}H]AcSDKP, 1 mM) was completely metabolized in human plasma with a half-life of 80 min, leading exclusively to the formation of radiolabelled lysine. The cleavage of AcSDKP was insensitive to classical proteinase inhibitors including leupeptin, but sensitive to metalloprotease inhibitors. The degradation was completely blocked by specific inhibitors of angiotensin I-converting enzyme (ACE; kininase II; peptidyldipeptide hydrolase, EC 3.4.15.1), showing that the first step of the hydrolysis was indeed due to ACE. In dialysed plasma, the hydrolysis proceeded at only 17% of the maximal rate, whereas addition of 20 mM NaCl led to the recovery of the initial rate observed with normal plasma. Hydrolysis of AcSDKP by commercial rabbit lung ACE generated the C-terminal dipeptide Lys-Pro. Thus, ACE cleaves AcSDKP by a dipeptidyl carboxypeptidase activity. In fact the formation of Lys-Pro was observed when AcSDKP was incubated in human plasma in the presence of $HgCl_2$. These results suggest that ACE is involved in the first limiting step of AcSDKP degradation in human plasma. The second step seems to be under the control of a leupeptin- and E-64-insensitive, $HgCl_2$-sensitive plasmatic enzyme.

INTRODUCTION

The haematopoietic system is essentially under the control of growth-promoting and inhibitory regulators which maintain its homoeostasis. Such activities were detected in bone-marrow extracts [1,2]. The tetrapeptide Ac-Ser-Asp-Lys-Pro (AcSDKP) has been characterized recently [3] as one of the negative regulators secreted by bone marrow [4] which could be formed from thymosin β_4 [5]. It has been demonstrated that AcSDKP increases the survival of mice treated with cytotoxic drugs used in cancer chemotherapy without preventing the efficiency of these drugs on eradication of experimental grafted tumours [6]. This property has been attributed to a protective action of the peptide on the haematopoietic pluripotent stem cell (CFU-s), which suggested possible therapeutic applications for this molecule. It is therefore of interest to determine the stability of this peptide in biological fluids and to specify the enzymic systems involved in its degradation. For this purpose, we evaluated the behaviour of AcSDKP specifically labelled in the lysine side chain, referred to as [^{3}H]AcSDKP, in human plasma. Our strategy to determine the type of protease activity, responsible for the inactivation of AcSDKP *in vivo*, was to evaluate the effectiveness of different classes of protease inhibitors in blocking AcSDKP degradation. Our study demonstrated that in human plasma, AcSDKP metabolism is essentially under the control of angiotensin I-converting enzyme (ACE, EC 3.4.15.1). This enzyme hydrolysed in an initial and determinant step the peptide bond Asp^2-Lys^3 to release the C-terminal dipeptide Lys-Pro which is further degraded to radiolabelled Lys.

The present study suggests that *in vivo* ACE might be involved in the regulation of the local concentration of the haemoregulatory peptide AcSDKP.

MATERIALS AND METHODS

Chemicals

Purified rabbit lung ACE (lot 66F9610; specific activity 2–4 units/mg of protein) was obtained from Sigma (St. Louis, MO, U.S.A.). N^{α}-[(*S*)-1-Carboxy-3-phenylpropyl]-L-alanyl-L-proline (enalaprilat), N^{α}-[(*S*)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline (lisinopril) and L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)butane (E-64) were kindly provided by Dr. P. Corvol (Collège de France, Paris, France). Highly purified rabbit kidney neutral endopeptidase (NEP; membrane metallo-endopeptidase EC 3.4.24.11) and retrothiorphan were kindly provided by Dr. B. P. Roques (Faculté de Pharmacie, Paris, France). Bacterial *N*-aspartyl endoproteinase was purchased from Boehringer-Mannheim (Meylan, France). AcSDKP and SDKP were generously supplied by IPSEN–Beaufour (Les Ulis, France). Lysine was obtained from Pierce (Roissy, France). Ac-Ser-Asp-Lys, Ser-Asp-Lys, Asp-Lys-Pro, Asp-Lys and Lys-Pro analogues were synthesized in our laboratory using conventional methods of solution peptide synthesis. The radiolabelled tetrapeptide AcSD[4-^{3}H]KP ([^{3}H]AcSDKP) was prepared as previously described [7] and the specific radioactivity was found to be around 120 Ci/mmol (4329 GBq/mmol).

N-(α-L-Rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan (phosphoramidon), trypsin/chymotrypsin inhibitor

Abbreviations used: ACE, angiotensin I-converting enzyme; FA-FGG, *N*-[3-(2-furylacryloyl)]-L-phenylalanylglycylglycine; AcSDKP, *N*-Ac-Ser-Asp-Lys-Pro; NEP, neutral endopeptidase; PCMB, *p*-chloromercuribenzoic acid; TFA, trifluoroacetic acid; PMSF, phenylmethanesulphonyl fluoride; FAB, fast atom bombardment; DMSO, dimethyl sulphoxide.

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from soybean, aprotinin, Ac-Leu-Leu-Arg (leupeptin), isovaleryl-Val-Val-Sta-Ala-Sta (pepstatin), [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine (bestatin), [(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl]-Val-Val-Asp (amastatin), phenylmethanesulphonyl fluoride (PMSF), 1,10-phenanthroline, DL-3-mercapto-2-benzylpropanoylglycine (thiorphan), *p*-chloromercuriobenzoic acid (PCMB), L-benzylsuccinic acid, mercaptoethanol, succinyl-L-proline (ACE-inhibitor), 1-[(2*S*)-3-mercapto-2-methylpropionyl]-L-proline (captopril), EDTA and trifluoroacetic acid (TFA) were purchased from Sigma (l'Isle d'Abeau, France). $HgCl_2$ and $MgCl_2$ were obtained from Merck (Nogent sur Marne, France) and $CuSO_4$ from Prolabo (Paris, France), and *N*-[3-(2-furylacryloyl)]-L-phenylalanylglycylglycine (FA-FGG) was purchased from Bachem (Bubendorf, Switzerland). Acetonitrile 'h.p.l.c. grade' was purchased from Carlo Erba (Milano, Italy). Water for h.p.l.c. analysis was of 'Milli-Q grade'.

H.p.l.c. analysis

Aliquots (100 μl) of each reaction mixture were analysed in a Waters automated gradient h.p.l.c. system. Two types of reverse-phase columns were used: 7.8 mm × 300 mm semi-preparative column (Waters, Delta-Pak C_{18}), and 4.6 mm × 250 mm Hypersil ODS C_{18} column (Alltech, France). Elution was performed using a ternary gradient consisting of 0.1 % TFA in water (solvent A), 0.1 % TFA in 4.5 % acetonitrile (solvent B) and 0.1 % TFA in acetonitrile (solvent C). The elution profile incorporated an isocratic elution with 100 % solvent A for 25 min at a flow rate of 0.5 ml/min, followed by isocratic elution with 100 % solvent B for 30 min at a flow rate of 1 ml/min and a linear gradient from 100 % solvent B to 100 % solvent C over 15 min at a flow rate of 1 ml/min. Peptides were detected by measuring u.v. absorbance at 215 nm.

Fractions were collected, lyophilized and the associated radioactivity was evaluated using a LKB liquid-scintillation counter (Model 1214 Rackbeta). Each peptide was identified by comparison with synthetic standards.

Hydrolysis of FA-FGG by rabbit lung ACE was monitored by h.p.l.c. as previously described [8].

Paper electrophoresis and t.l.c.

High-voltage paper electrophoresis was carried out according to the method of Offord [9]. Analysis was performed on Whatman 3 MM paper for 1 h (800 V), using pyridine/acetic acid/water (25:1:225, by vol., pH 6.5) as the solvent system. Radioactivity associated with each centimetre-long paper strip was evaluated by counting.

T.l.c. was carried out on DC-Alufolien Kieselgel 60F plates (Merck, Darmstadt, Germany) with TFA/water/acetonitrile (50:10:200, by vol.) as the solvent system. Detection was performed with 0.1 % ninhydrin in acetone. AcSDKP, Ac-Ser-Asp-Lys, Ser-Asp-Lys-Pro, Ser-Asp-Lys, Asp-Lys-Pro, Asp-Lys, Lys-Pro and Lys were characterized by their R_F values (respectively: 0.3, 0.4, 0.43, 0.48, 0.6, 0.68, 0.65 and 0.73). Radioactivity associated with each spot was measured using an automatic t.l.c. analyser (Berthold, Tracemaster 20).

Metastable ion kinetic energy (MIKE spectroscopy) analysis

The plasma constituent isolated by h.p.l.c. at the elution time of the dipeptide Asp-Lys was purified by semi-preparative h.p.l.c. This fraction and the authentic dipeptide Asp-Lys were separately treated at a final concentration of 150 ng/μl with a 30 % (w/v) solution of acetyl chloride in hexanol at 0 °C for 30 min. Fast atom bombardment (FAB)-m.s. analysis was performed with a reverse geometry ZAB-HS spectrometer (VG Analytical, Manchester). The acceleration voltage of the xenon ions was 8 keV. Similarly, the FAB gun was also operated at +8 keV. The matrix was a 1/1 mixture of glycerol and thioglycerol. The spectra of plasma fraction and authentic Asp-Lys derivatives exhibit a similar molecular ion m/z 430 [MH^+].

Enzyme inhibitor assays

Peripheral normal human venous blood was collected in the presence of heparin (Roche, Neuilly-sur-Seine, France; 50 units/ml of blood). Plasma was removed by centrifugation at 400 *g* for 10 min at 4 °C. In one case plasma was dialysed for 24 h at 4 °C against tridistilled water and supplemented with NaCl (20, 100, 300 or 800 mM) in the presence or absence of $ZnCl_2$ (10^{-4} M) before incubation with the substrate. Plasma was incubated for different periods of time at 37 °C with [^{3}H]AcSDKP (20 μCi, 0.16 μM) alone or in the presence of 1 mM AcSDKP. Aliquots (200 μl) were periodically withdrawn and reactions were stopped with 800 μl of ice-cold methanol and centrifuged at 1500 *g* for 10 min. Supernatants were concentrated in a Speed-Vac Concentrator (Savant, France), analysed by h.p.l.c., and radioactivity was evaluated. The extent of AcSDKP degradation was determined by the decrease of the radioactivity in the peak corresponding to the radiolabelled tetrapeptide eluted between 48 and 52 min.

Protease inhibitors were solubilized in PBS (150 mM, pH 7.2) except for pepstatin and PMSF which were solubilized in methanol or PCMB and benzylsuccinic acid dissolved in dimethyl sulphoxide (DMSO).

AcSDKP (1 mM) and [^{3}H]AcSDKP (20 μCi) were incubated with 1 ml of plasma for 2 h at 37 °C in the presence or absence of chosen inhibitors. Samples were then treated as described previously. Inhibition was evaluated relative to the control values.

For the determination of IC_{50} values of captopril, enalaprilat and lisinopril in human plasma, inhibitors (10^{-11} M to 10^{-8} M) were preincubated with 1 ml of plasma for 15 min before substrate {AcSDKP (1 mM), [^{3}H]AcSDKP (20 μCi)} addition. The inhibitory efficiency was evaluated after 1 h of incubation by comparison with the control values.

Identification of AcSDKP metabolites

[^{3}H]AcSDKP (20 μCi) was incubated with plasma for 4 h at 37 °C and aliquots (50 μl) were analysed successively by h.p.l.c. and paper electrophoresis using Lys and the dipeptides Asp-Lys and Lys-Pro as standards.

Enzyme assays

Hydrolysis of substrates by purified rabbit kidney NEP, bacterial *N*-aspartyl endoproteinase and commercial rabbit lung ACE was measured by either h.p.l.c. or t.l.c. AcSDKP (0.1 mM) and [^{3}H]-AcSDKP (0.1 μCi), when spiked, in 0.1 ml Tris/HCl (100 mM, pH 7.5) containing 300 mM NaCl, were incubated at 37 °C for various periods of time with ACE (0.005 unit). Captopril, when added, was preincubated with the enzyme for 15 min at 37 °C. NEP (2.2 μg/100 μl) was incubated with [^{3}H]AcSDKP (0.1 μCi) in 0.5 ml Tris/HCl (50 mM, pH 7.4) at 25 °C for various periods of time. [^{3}H]AcSDKP (0.1 μCi) was dissolved in 450 μl of phosphate buffer (50 mM, pH 8), then 2 μg of *N*-aspartyl endoproteinase in 50 μl of water was added. The digestion was carried out at room temperature for 18 h. Reactions were stopped by freezing in liquid nitrogen.

Inhibitory activity of the dipeptide Lys-Pro on rabbit lung ACE was measured with FA-FGG in Tris/HCl (100 mM, pH 7.5) containing 300 mM NaCl.

RESULTS

Kinetics of AcSDKP hydrolysis by normal human plasma

Incubations of AcSDKP spiked with [^{3}H]AcSDKP in normal human plasma were analysed by h.p.l.c. and the radioactivity associated with AcSDKP fractions was determined. The profile of AcSDKP (1 mM) degradation exhibited an apparent half-life of 80 min and total degradation had occurred within 4 h (Figure 1a).

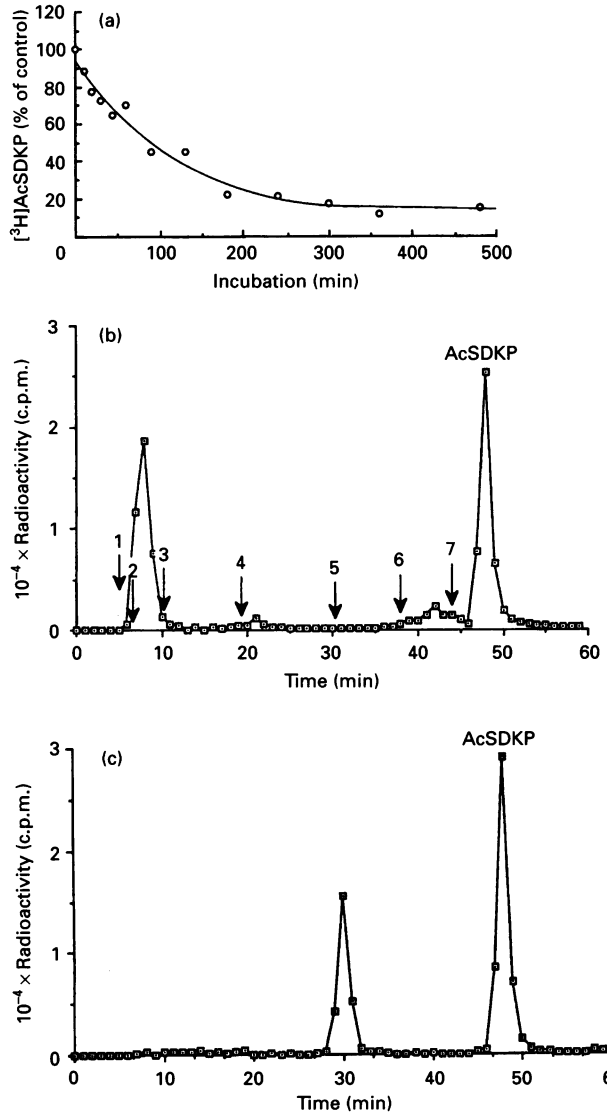

Figure 1 Degradation of AcSDKP in normal human plasma

(**a**) Kinetic profile of AcSDKP degradation. AcSDKP (1 mM) spiked with [^{3}H]AcSDKP (20 μCi) was incubated at 37 °C for different periods of time. After methanol precipitation supernatants were separated by h.p.l.c. as described. The extent of degradation was determined by the decrease of the radioactivity in the peak corresponding to the radiolabelled tetrapeptide eluted between 48–52 min. (**b**) Profile of radioactivity eluted by h.p.l.c. after a 2 h incubation period. ↓ elution time of standards: 1, Lys; 2, Asp-Lys; 3, Ser-Asp-Lys; 4, *N*-Ac-Ser-Asp-Lys; 5, Lys-Pro; 6, Asp-Lys-Pro; 7, Ser-Asp-Lys-Pro. (**c**) Effect of $HgCl_2$ (1 mM) on AcSDKP hydrolysis. Radioactivity profile eluted by h.p.l.c. after incubation for 2 h.

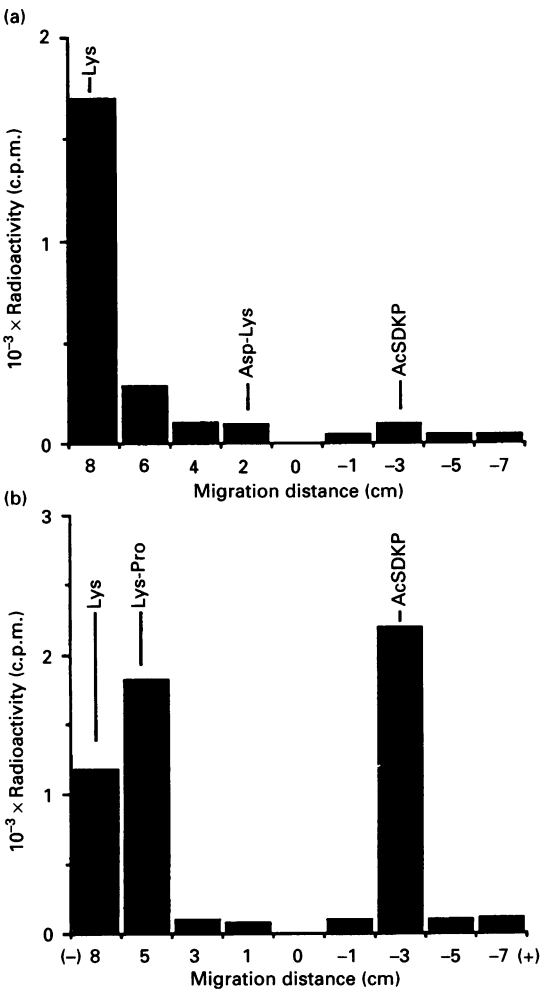

Figure 2 Paper-electrophoresis analysis of the radiolabelled metabolite formed from [^{3}H]AcSDKP

Human plasma was incubated for 4 h at 37 °C with AcSDKP (1 mM) spiked with [^{3}H]AcSDKP (20 μCi) and analysed by h.p.l.c. (**a**) In the absence of $HgCl_2$. The fraction eluted between 8 and 11 min was collected and submitted to paper electrophoresis in the presence of Lys and the dipeptide Asp-Lys (both at 10^{-7} M) as standards. Associated radioactivity was evaluated. (**b**) In the presence of $HgCl_2$. Radioactivity was associated with dipeptide Lys-Pro (10^{-7} M) as standard.

The radioactivity profiles obtained after various periods of incubation time of substrate with plasma indicated in all cases the formation of a radiolabelled metabolite(s) which eluted between 8 and 11 min (Figure 1b). Comparison with the retention times of synthetic peptides used as standards indicated that the radiolabelled degradation product formed from [^{3}H]AcSDKP might correspond either to the dipeptide [^{3}H]Asp-Lys or [^{3}H]Lys, or to a mixture of both molecules. Paper electrophoretic analysis of this fraction established that Lys was the only radiolabelled metabolite generated from [^{3}H]AcSDKP (Figure 2a). However, the presence of the dipeptide Asp-Lys in human plasma was demonstrated in the corresponding h.p.l.c. fraction (8–11 min) by comparison with the synthetic dipeptide using MIKE-spectrometry analysis. After formation of the corresponding

hexyl ester derivatives, both samples exhibited an identical molecular ion m/z 430 [MH^+].

Effect of proteinase inhibitors on AcSDKP-metabolizing enzymes

The effect of various proteinase inhibitors on the hydrolysis of AcSDKP by human plasma was evaluated after 2 h incubation at 37 °C. After h.p.l.c. analysis of plasma samples the associated radioactivity was determined. The degree of inhibition was calculated relative to controls which led to 50 % hydrolysis (here referred to as 100 % of enzyme activity). As summarized (Table 1), serine enzyme inhibitors (soybean inhibitor, PMSF and aprotinin) have no effect on AcSDKP degradation. Aspartic protease inhibitor (pepstatin) and cysteine protease inhibitors (leupeptin and E-64) did not block AcSDKP degradation in normal human plasma. In all cases the pattern of hydrolysis was identical with that observed when the tetrapeptide was incubated

Table 1 Inhibition of [^{3}H]AcSDKP hydrolysis by human plasma enzymes

[^{3}H]AcSDKP was incubated for 2 h at 37 °C with normal human plasma in the presence or absence of inhibitors. After methanol precipitation, supernatants were analysed by h.p.l.c. The percentage of activity was evaluated by counting the radioactivity associated with [^{3}H]AcSDKP relative to the control. The metabolite formed was [^{3}H]Lys. *The metabolite formed was [^{3}H]Lys-Pro.

Inhibitor	Molarity (M)	Activity (%)
None	–	100
Aprotinin	1×10^{-3}	100
PMSF	1×10^{-3}	100
Soybean inhibitor	1×10^{-3}	100
Pepstatin	1×10^{-3}	100
Leupeptin	1×10^{-3}	100
E-64	1×10^{-5}	100
Bestatin	1×10^{-3}	85
Amastatin	1×10^{-3}	100
Phosphoramidon	1×10^{-3}	100
L-Benzylsuccinic acid	1×10^{-3}	27
Succinyl-L-proline	1×10^{-4}	0
Captopril	1×10^{-6}	0
	1×10^{-7}	5
	1×10^{-8}	12
Enalaprilat	1×10^{-8}	15
Lisinopril	1×10^{-8}	20
Thiorphan	5×10^{-5}	30
Retrothiorphan	3×10^{-3}	0
	5×10^{-7}	87
1,10-Phenanthroline	1×10^{-3}	58
Mercaptoethanol	1×10^{-3}	17
EDTA	1×10^{-3}	58
	1×10^{-2}	0
PCMB	1×10^{-3}	31*
$HgCl_2$	1×10^{-3}	85*
$CuSO_4$	1×10^{-3}	93
$MgCl_2$	1×10^{-3}	100

Table 2 Effect of Cl^- ions on AcSDKP hydrolysis by human plasma

Plasma or plasma dialysed against tridistilled water for 24 h at 4 °C were incubated with AcSDKP (1 mM) spiked with [^{3}H]AcSDKP (20 μCi) in the presence of different NaCl concentrations.

Plasma	NaCl (mM)	AcSDKP hydrolysis (%)
Not dialysed	–	100
Dialysed	–	17
Dialysed	20	97
Dialysed	100	125
Dialysed	100	118
Dialysed	300	134
Dialysed	800	125

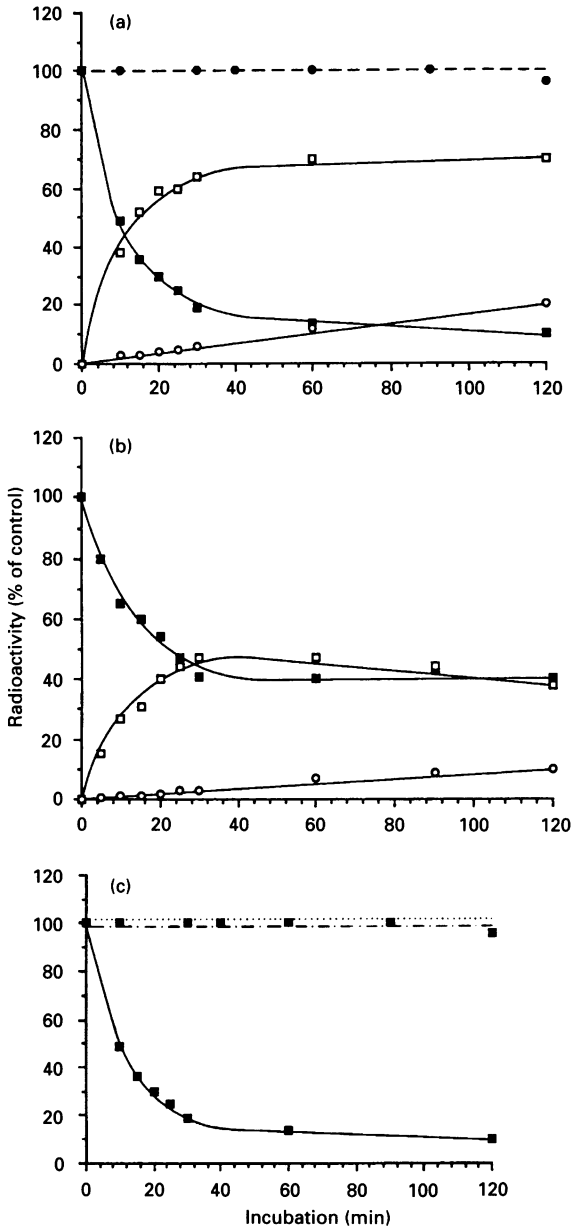

Figure 3 Hydrolysis of AcSDKP by rabbit lung ACE, rabbit kidney NEP and bacterial *N*-aspartyl endoproteinase

(**a**) [^{3}H]AcSDKP (0.1 μM) was incubated with ACE (0.005 unit) at 37 °C for different periods of time and analysed as described in the Materials and methods section (——). The enzyme was preincubated with captopril (1 μM) before [^{3}H]AcSDKP addition (– –). (**b**) [^{3}H]AcSDKP (100 μM) was incubated with ACE (0.005 unit) at 37 °C for different periods of time and analysed as mentioned above (——). (**c**) [^{3}H]AcSDKP (0.1 μM) was incubated with either ACE (0.005 unit) (——), or NEP (2.2 μg/100 μl) (· · · ·), or *N*-aspartyl endoproteinase (2 μg/500 μl) (–·–). Analyses were carried out as described in the Materials and methods section. Detected radiolabelled molecules: ■, [^{3}H]AcSDKP; □, [^{3}H]Lys-Pro; ○, [^{3}H]Lys. Results are from an experiment representative of two.

in the absence of any inhibitor. Aminopeptidase inhibitor, bestatin, weakly inhibited AcSDKP degradation (15%) whereas amastatin was ineffective. Both inhibitors led to the formation of radiolabelled Lys. Whereas $CuSO_4$ and $MgCl_2$ (both at concentrations of 1 mM) were ineffective, addition of $HgCl_2$ (1 mM) led to a unique radiolabelled intermediate product (Figure 1c) which was identified by paper electrophoresis as the dipeptide Lys-Pro (Figure 2b). Formation of the radiolabelled dipeptide Lys-Pro was also observed when AcSDKP was incubated in the presence of PCMB (1 mM). Conversely mercaptoethanol and several metalloproteinase inhibitors (1,10-phenanthroline, L-benzylsuccinic acid, succinyl-L-proline, thiorphan, retrothiorphan and EDTA) inhibited the degradation of AcSDKP to various extents (42–100%) and led exclusively to the formation of radiolabelled Lys. Compared with retrothiorphan (IC_{50} 2×10^{-6} M), captopril (IC_{50} 1×10^{-9} M), enalaprilat (IC_{50} 5.4×10^{-9} M) and lisinopril (IC_{50} 5.5×10^{-9} M) were shown to be significantly more potent.

Effect of NaCl on AcSDKP hydrolysis

The rate of hydrolysis of AcSDKP in dialysed plasma (chloride ion-free) was slowed down to 17% of the maximal rate (Table 2). When NaCl was added at a concentration of 20 mM, the rate increased to the control value. The optimal rate was reached in 100 mM NaCl and no further modification of the hydrolysis rate was observed by increasing NaCl concentration up to 800 mM.

Hydrolysis of AcSDKP by ACE

The degradation of AcSDKP at a concentration of 0.1 μM by rabbit lung ACE was carried out in the presence of 300 mM NaCl at 37 °C. Under these conditions AcSDKP exhibited an apparent half-life of 10 min and total degradation was obtained after incubation for 30 min (Figure 3a). Hydrolysis led to the formation essentially of the dipeptide Lys-Pro and to a small amount of Lys attributed to contamination of the ACE preparation with aminopeptidase activity. Complete inhibition of AcSDKP degradation was observed in the presence of captopril (10^{-6} M) (Figure 3a). If AcSDKP (0.1 mM) was treated under the same conditions the half-life was found to be 25 min and after 2 h incubation only 60% of the substrate was degraded (Figure 3b). Purified rabbit kidney NEP and bacterial *N*-aspartyl endoproteinase appeared ineffective for AcSDKP degradation (Figure 3c).

DISCUSSION

We have proposed several lines of evidence to indicate the involvement of human plasma ACE in the degradation of the tetrapeptide AcSDKP, a negative regulator controlling the proliferation of the haematopoietic stem cell (CFU-S) [3], which is actually under clinical trials. This peptide was shown to ameliorate or prevent the aplasia associated with the destruction of haematopoietic stem cells during cancer chemotherapy [6]. The stability of AcSDKP in human plasma was investigated and the enzymic systems involved in its degradation were characterized.

When the specifically radiolabelled bioactive peptide [^{3}H]AcSDKP was incubated at 1 mM with normal human plasma the half-life was found to be 80 min (Figure 1a). This value was reduced to 50 min for a 10 nM concentration of AcSDKP (which corresponds to the physiological concentration of this peptide) (result not shown). This remarkable resistance to proteolytic cleavage can be attributed to the double protection presented by this molecule: acetylation of the N-terminus and the presence of a C-terminal proline. These naturally occurring protective groups considerably diminish the possibility of a C- or N-terminal attack by usual plasma proteases.

Whatever the length of incubation with human plasma or the concentration of added peptide, the degradation of AcSDKP led exclusively to the formation of a unique radiolabelled metabolite (Figure 1b), identified as [^{3}H]Lys (Figure 2a).

In order to characterize the plasma enzymes responsible for such degradation the effect of various enzyme inhibitors was investigated (Table 1). Whereas cysteinyl, aspartyl and seryl endoproteinase inhibitors were ineffective in protecting AcSDKP, a significant inhibition of the peptide hydrolysis was observed with: (i) non-specific inhibitors of metalloproteases i.e. EDTA (10^{-2} M), 1,10-phenanthroline (10^{-3} M), (ii) inhibitors of carboxypeptidase i.e. L-benzylsuccinic acid (10^{-3} M), succinyl-L-proline (10^{-4} M); (iii) inhibitors of NEP i.e. thiorphan (5×10^{-5} M) and retrothiorphan ($IC_{50} = 2 \times 10^{-6}$ M); (iv) inhibitors of ACE i.e. captopril ($IC_{50} = 10^{-9}$ M), enalaprilat ($IC_{50} = 5.4 \times 10^{-9}$ M) and lisinopril ($IC_{50} = 5.5 \times 10^{-9}$ M). This suggests an involvement of metalloproteases, and in particular of ACE, in the degradation of AcSDKP.

Indeed, AcSDKP degradation by plasma enzymes was considerably reduced when carried out with plasma which had been extensively dialysed. The residual degradation observed in the absence of chloride ions suggested that besides ACE, which seemed to be implicated in the major degradative pathway, another enzyme in human plasma, such as carboxypeptidase, might participate in the catabolism of AcSDKP. It is worthwhile to note that the reconstitution of dialysed plasma with Cl^- ion, an ion indispensible for ACE activity, at a concentration as low as 20 mM is sufficient to recover the initial proteolytic rate. An acceleration of the catabolism of AcSDKP in the presence of 100 mM NaCl was observed (Table 2). ACE has been reported to present two homologous catalytic domains bearing identical active sites but exhibiting different sensitivity to chloride activation [11]. This suggests that AcSDKP might be degraded by the low-Cl^- ion-dependent enzymic site which was shown to be implicated in the N-terminal degradation of luteinizing hormone-releasing hormone [12].

Furthermore, the degradation observed after incubation for 2 h was inhibited from 88 to 80% by captopril, enalaprilat or lisinopril at a concentration of 10^{-8} M. The efficiency of ACE was confirmed using commercial purified rabbit lung ACE; this enzyme degraded 10^{-7} and 10^{-4} M solutions of [^{3}H]AcSDKP with respective half-lives of 10 and 25 min and led to the formation of the dipeptide [^{3}H]Lys-Pro. The appearance in the digestion media of a small quantity of [^{3}H]Lys was attributed to contamination of the ACE preparation with aminopeptidase activity. Under the conditions used the degradation was completely inhibited by the addition of captopril (Figure 3a). Purified rabbit kidney NEP and bacterial *N*-aspartyl endoproteinase appeared ineffective at degrading AcSDKP (Figure 3c). When a large ratio of AcSDKP to ACE was used, the peptide degradation was limited by an accumulation in the incubation media of the dipeptide Lys-Pro (Figure 3b). In fact this dipeptide appeared to be able to inhibit the degradation by the enzyme of FA-FGG, an ACE-specific substrate (results not shown). It is worthwhile noting the structural similarity between the dipeptide Lys-Pro and lisinopril, a specific inhibitor of ACE which presents the Lys-Pro C-terminal sequence.

The role of soluble plasma ACE as the key enzyme involved in the first step of the degradation of AcSDKP leading to the

formation of the dipeptide Lys-Pro, which was previously recognized as devoid of biological activity [13], was sustained by further experiments which showed that $HgCl_2$ (1 mM) and PCMB (1 mM) prevented the formation of [^{3}H]Lys, leading instead exclusively to the formation of [^{3}H]Lys-Pro (Figure 2b). This dipeptide was protected under these conditions from further degradation. As mercuric compounds are known to inhibit acyl amino-acid-releasing enzymes, the participation of such enzymes in a degradation pathway leading from AcSDKP to Lys-Pro, which might be further degraded into Pro and Lys, can be excluded. This conclusion is reinforced by the fact that there is no protective effect of Cu^{2+} ions on AcSDKP degradation. Carboxypeptidase Y, which is sensitive to mercuric ions, appeared unable to degrade [^{3}H]AcSDKP (results not shown). Furthermore the identification by MIKE m.s. of the dipeptide Asp-Lys as a natural constituent of human plasma, and the fact that this radiolabelled dipeptide is not formed during [^{3}H]AcSDKP degradation, excluded its formation as an intermediate during the degradative process. On the other hand the lack of sensitivity to amastatine, an inhibitor of aminopeptidase A, which was recently identified to be the BP-1/6C3 pre-B lymphocyte antigen [14], excludes its involvement in AcSDKP degradation process in human plasma. However, the participation of such an enzyme in the metabolization of the tetrapeptide in haematopoietic tissue cannot be ruled out. An involvement of classical aminopeptidases seems improbable as such enzymes are not sensitive to Hg^{2+} ions. However, further degradation of the dipeptide Lys-Pro into its constitutive amino acids might be carried out by a $HgCl_2$- and PCMB-sensitive, E-64-insensitive plasmatic enzyme. Until now dicarboxydipeptidase activity has been established for ACE using various peptidic models. In particular its role in the conversion of angiotensin I into the active angiotensin II and the degradation of bradykinin makes it an important enzyme with a key physiological role. In those cases ACE cleaves hydrophobic peptidic bonds Phe^8–His^9 and Pro^7–Phe^8 respectively. It is worth pointing out that in the present case ACE exerts its proteolytic activity on a highly hydrophilic peptide cleaving the Asp^2–Lys^3 bond. Besides such activities ACE has been shown to release N- and C-terminal tripeptides from the luteinizing-hormone-releasing hormone [15] and to hydrolyse cholecystokinin and gastrin analogues [16].

ACE can be postulated to be one of the enzymes involved in the control of the concentration of circulating AcSDKP, an inhibitor of the haematopoietic system. However, the participation to a low extent of acetylases and diaminopeptidases in AcSDKP degradation in human plasma cannot be excluded. The fact that the conversion of angiotensin I into angiotensin II occurred mainly to a level of 60–80% by lung endothelial ACE suggests that the lung might play an important role *in vivo* in the catabolism of AcSDKP. Besides this enzymic proteolysis, clearance of the circulating tetrapeptide by the kidney might constitute another eliminating pathway for the active molecule *in vivo*. The large concentration of ACE associated with lung macrophages in sarcoidosis [17] and with B cells in non-T acute lymphoid leukaemia [18] suggests that this enzyme might contribute to a deregulation of haematopoietic system homoeostasis in these diseases.

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