Generation by the phosphoramidon-sensitive peptidases, endopeptidase-24.11 and thermolysin, of endothelin-1 and C-terminal fragment from big endothelin-1

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1 Phosphoramidon, a potent inhibitor of endopeptidase-24.11 (E-24.11) and thermolysin, has been shown to reduce the hypertensive effect of exogenous big endothelin-1 (big ET-1) in rats. To examine whether E-24.11 or thermolysin convert big ET-1 to endothelin-1 (ET-1) and C-terminal fragment (CTF), the effects on porcine and human big ET-1 of each of the purified enzymes were compared *in vitro*.

2 For E-24.11, the relative rates of hydrolysis were ET-1 > CTF >> big ET-1. The relative half-lives for hydrolysis of 3 nmol of each peptide by 200 ng enzyme were: big ET-1 > 24 h; ET-1, 37 min; CTF, 57 min. For comparison, the half-life for hydrolysis of substance P under similar conditions was 2.1 min.

3 For thermolysin the relative rates of hydrolysis were found to be big ET-1 > CTF > ET-1. The relative half-lives for hydrolysis of 3 nmol peptide by 50 ng enzyme were: big ET-1, 25 min; ET-1, 56 min; CTF, 47 min.

4 Because the low rate of conversion of big ET-1 to ET-1 by E-24.11 did not yield sufficient ET-1 for h.p.l.c. quantification a RIA specific for ET-1(16-21) was used to study further the hydrolysis of big ET-1 by E-24.11. Incubation of big ET-1 (0.2–2 nmol) with E-24.11 (4–400 ng) generated ET-1 levels of between 1.7 and 33 pmol measured by RIA. Incubation of big ET-1 (2 nmol) with E-24.11 (40 ng) for 8 h showed that steady state levels of ET-1 were achieved after 4 h indicating that the rate of ET-1 degradation was then equal to the formation of new ET-1. Characterization of the immunoreactivity by h.p.l.c. and RIA confirmed that authentic ET-1 had been produced, but the yield was insufficient for verification by mass spectrometry.

5 Both ET-1-like and CTF-like peaks were detected at 214 nm when the products of big ET-1 hydrolysis by thermolysin were resolved by h.p.l.c. RIA and mass spectrometry confirmed the production of ET-1 with amounts in the range 120–160 pmol.

6 The hydrolysis profile of ET-1 by E-24.11 and thermolysin shows that both enzymes have some common cleavage sites consistent with their similar specificities hydrolysing on the amino side of a hydrophobic residue.

7 Thermolysin, for which 3D structural information is available, may represent a better model for endothelin converting enzyme (ECE) action than E-24.11 and could be useful for the design of ECE inhibitors. Since E-24.11 can both synthesize and hydrolyse ET-1, the presence of E-24.11 in membrane fractions or in partially purified ECE preparations may produce misleading estimates of ECE activity.

Keywords: Endopeptidase-24.11; endothelin converting enzyme; thermolysin; phosphoramidon; thiorphan

Introduction

Endothelin-1, a 21 amino acid, potent vasoconstrictor peptide, is produced by cleavage of its precursor 'big endothelin' at the Trp²¹-Val²² bond by a putative endothelin converting enzyme (ECE) (Yanagisawa *et al.*, 1988). Several lines of study have recently implicated a phosphoramidon-sensitive metalloendopeptidase in the physiological conversion of big endothelin-1 to endothelin-1 and its C-terminal fragment (big ET-1(22-39)) (Ikegawa *et al.*, 1990; Matsumura *et al.*, 1990; 1991; McMahon *et al.*, 1991; Pollock & Opgenorth, 1991). For example, phosphoramidon was found to reduce the hypertensive effect of exogenous big ET-1, but not that of ET-1, in rats (McMahon *et al.*, 1991; Pollock & Opgenorth, 1991).

The fungal metabolite, phosphoramidon, was first recognised as an inhibitor of certain bacterial neutral metalloproteases such as thermolysin (EC 3.4.24.4) (K_i = 28 nM; Suda *et al.*, 1973). Subsequently, phosphoramidon was shown to be a highly selective and potent (K_i = 2 nM) inhibitor of mammalian endopeptidase-24.11 (EC 3.4.24.11; E-24.11) (Kenny, 1977; Turner, 1987). Endopeptidase-24.11 is a membrane

glycoprotein existing as an ectoenzyme (M_r approx 90 kDa). It exhibits a broad tissue distribution and substrate specificity, hydrolysing a wide range of biologically active peptides by cleaving on the amino side of a hydrophobic residue (Turner *et al.*, 1987; Roques *et al.*, 1993). This would suggest the enzyme to be a possible candidate for cleavage of the Trp²¹-Val²² bond of big ET-1. However E-24.11 also hydrolyses ET-1 *in vitro* (Vijayaraghavan *et al.*, 1990; Fagny *et al.*, 1991) and has been implicated in the physiological inactivation of endothelin (Abassi *et al.*, 1992). For this reason E-24.11 seems unlikely to serve as the principal ECE activity in cells. Furthermore, Abassi *et al.* (1993) have recently suggested that recombinant E-24.11 is unable to generate ET-1 and C-terminal fragment from big ET-1.

The substrate specificity of E-24.11 is similar to that of thermolysin. Thus, thermolysin also hydrolyses peptide bonds involving the amino groups of hydrophobic amino acid residues with bulky side chains (Monzingo & Matthews, 1984). The active site of E-24.11 has been explored by chemical modification (Beaumont & Roques, 1986; Jackson & Hersh, 1986), site-directed mutagenesis (Bateman & Hersh, 1987; Devault *et al.*, 1988) and by hydrophobic cluster analysis of the primary sequence (Benchetrit *et al.*, 1988).

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These studies have revealed that the Zn^{2+} -binding and catalytic residues present in thermolysin are conserved in E-24.11. Although E-24.11 and thermolysin are sensitive to phosphoramidon in the nanomolar range, they differ substantially in their sensitivity to thiorphan (Fulcher *et al.*, 1982). In this respect thermolysin more closely resembles ECE which is relatively insensitive to thiorphan (Ohnaka *et al.*, 1993).

As yet, no data have been reported comparing the actions of these two enzymes on big ET-1. We have re-evaluated the potential of E-24.11 to function as an endothelin converting enzyme and have studied its ability to degrade ET-1 and CTF. These actions have been compared with those of thermolysin to determine if the bacterial phosphoramidon-sensitive metalloprotease may represent a more useful model for metallopeptidase ECE, especially since the 3D structure of thermolysin has been solved both in the absence and presence of inhibitors (Weaver *et al.*, 1977; Monzingo & Matthews, 1982; 1984).

Methods

Hydrolysis of peptides by E-24.11 and thermolysin

Time courses of hydrolysis of ET-1, big ET-1 (1-39), pCTF and substance-P by E-24.11 Endopeptidase-24.11 (200 ng protein) in 0.1 M Tris/HCl at pH 7.4 was incubated with ET-1, big ET-1 (1-39), pCTF or substance P (3 nmol of each) at 37°C (final volume 100 µl) for up to 180 min. Reactions were stopped either by acidification with 50 µl 9% v/v trifluoroacetic acid (TFA) or by addition of 50 µl of phosphoramidon (150 µM). The hydrolysis of each of the peptides was monitored by reverse phase high performance liquid chromatography (r.p.-h.p.l.c.) analysis as described below. The above procedure was also repeated for big ET-1 but using 4 µg E-24.11 protein instead of 200 ng.

Time courses of hydrolysis of ET-1, big ET-1 and pCTF by thermolysin Thermolysin (50 ng) in 0.1 M Tris/HCl, 3 mM CaCl_2 at pH 7.4 was incubated with either ET-1, big ET-1 (1-39) or pCTF (3 nmol of each) at 37°C (final volume 100 µl) for up to 80 min. All reactions were stopped by acidification with 50 µl 30% v/v acetic acid. The hydrolysis of each of the peptides was monitored by r.p.-h.p.l.c. analysis as described below. Hydrolysis was completely inhibited by 10 µM phosphoramidon.

R.p.-h.p.l.c. methods for time course studies

(a) **Endothelin peptides** – big ET-1 (1-39), ET-1 and pCTF were resolved by r.p.-h.p.l.c. on a µBondapak C18 column (3.9 × 300 mm, Millipore Corporation, Milford, MA, U.S.A.) using a Waters h.p.l.c. system. Elution was performed by using 0.02% TFA in water (solvent A) and 0.02% TFA in acetonitrile (solvent B). The gradient was linear from 10 to 36.5 %v/v solvent B in 15 min, followed by a further gradient up to 43% v/v solvent B over the next 20 min. The flow rate was 1 ml min⁻¹ and detection was at 214 nm. pCTF, big ET-1 (1-39) and ET-1 typically eluted with retention times of 17.2 min, 23.5 min and 25.3 min respectively. Samples from reactions were centrifuged and a known volume of the supernatant analysed by r.p.-h.p.l.c.

(b) **Substance P** – substance P was resolved using the same column as above but under different conditions. Solvent A was 0.08% H_3PO_4 while solvent B consisted of 45% acetonitrile/0.08% H_3PO_4 . The gradient was linear from 9% to 100% v/v solvent B in 20 min followed by 5 min at final conditions. Substance P typically eluted with a retention time of 16.9 min.

Characterization by radioimmunoassay, h.p.l.c. and mass spectrometry of the products of human big ET-1 incubation with E-24.11 or thermolysin

The studies described above used porcine big ET-1. However, to facilitate the identification of ET-1 and CTF by radioimmunoassay, human big ET-1 was employed because a specific RIA for human big ET-1(22-38) (hCTF) was available (Corder *et al.*, 1993).

Incubations with E-24.11 and thermolysin: E-24.11 To define the most appropriate enzyme to substrate ratio for E-24.11, either 1 µM (0.2 nmol) or 10 µM (2 nmol) human big ET-1 was incubated with a range of concentrations of E-24.11 (20 ng ml⁻¹–2 µg ml⁻¹) for 2 h at 37°C in a total volume of 200 µl. The reaction was terminated by 10 min of heat treatment at 80°C. Samples were centrifuged at 10,000 g and, after appropriate dilutions in RIA buffer, the ET-1 generated in each sample was assayed by RIA. The time course for ET-1 formation by E-24.11 was investigated by incubating 2 nmol human big ET-1 with 40 ng E-24.11 in a total volume of 200 µl, and samples were taken for RIA at 1, 2, 4 and 8 h. All incubations were performed in triplicate.

Thermolysin 10 µM (2 nmol) big ET-1 was incubated with thermolysin at 40 or 400 ng for 2 h at 37°C in a total volume of 200 µl. At the end of the incubation the samples were processed as described above and subjected to RIA for ET-1 immunoreactivity.

RIA of ET-1 and hCTF ET-1 was measured using a RIA specific for the C-terminal ET-1(16-21) sequence (crossreactivity with big ET-1 <0.015%) (Corder *et al.*, 1993). This antibody was raised by injecting rabbits with His-Leu-Asp-Ile-Ile-Trp conjugated to bovine thyroglobulin with glutaraldehyde. [¹²⁵I]-ET-1 was used as tracer and dilutions of ET-1 were used as standard. Samples from incubations with big ET-1 or h.p.l.c. fractions were measured for ET-1(16-21)-immunoreactivity after appropriate dilution. Identification of hCTF in h.p.l.c. fractions was performed using a RIA specific for the C-terminal 22-38 sequence of human big ET-1. This antibody was raised by injecting rabbits with hCTF (Val-Asn-Thr-Pro-Glu-His-Val-Val-Pro-Tyr-Gly-Leu-Gly-Ser-Pro-Arg-Ser) conjugated to bovine serum albumin with glutaraldehyde (Corder *et al.*, 1993). [¹²⁵I]-human big ET-1 was used as tracer and dilutions of human big ET-1 were used as standard.

Both RIAs were performed as follows: standards or unknowns (200 µl) in RIA buffer (50 mM sodium phosphate buffer, pH 7.4, containing 0.15% bovine serum albumin, 0.005% Triton X-100 and 0.01% sodium azide) were incubated overnight at 4°C with 50 µl antibody and 50 µl tracer (7500 c.p.m.). The following day, 100 µl sheep anti-(rabbit IgG Fc region) serum and 100 µl normal rabbit serum were added to each tube and left to incubate for 3 h. At the end of the incubation period, 500 µl of 4% polyethylene glycol was added to each tube and bound ligand was separated from free by centrifugation at 2000 g for 30 min.

Identification by h.p.l.c. of ET-1, big ET-1 and big ET-1(22-38) This was performed using a column of 5 µm TSK gel ODS-120T, (4.6 × 250 mm; TOSOH Corporation, Japan from Anachem, Luton, Beds). Samples were first acidified and extracted using TechElut SPE C₁₈ columns, eluted with 1 ml 80% acetonitrile containing 0.1% TFA, concentrated to 0.2 ml under a stream of N_2 , and injected onto the h.p.l.c. system. The h.p.l.c. column was eluted at a flow rate of 1 ml min⁻¹ with a gradient of acetonitrile in 0.1% TFA using a Pharmacia LKB model 2249 gradient pump and 0.5 min fractions were collected. The acetonitrile gradient used was 0–20% over 3 min, 20–32% over 12 min, 32–40% over 15 min, 40–48% over 8 min. The column effluent was monitored at 280 nm using a Pharmacia LKB model 2141

variable wavelength monitor. Using this system, elution times for standard peptides were hCTF 15.1 min, big ET-1 27.5 min and ET-1 30.1 min.

Mass spectrometry To verify the identity of the immunoreactivity measured in h.p.l.c. fractions, samples were subjected to continuous flow FAB mass spectrometry using a VG-analytical 70 VSEQ mass spectrometer (Fisons Instruments, Manchester).

Materials

Porcine big ET-1 (1-39), human big ET-1 (1-38) and ET-1 were purchased from the Peptide Institute Inc., Osaka, Japan. Porcine big ET-1 (22-39) (porcine C-terminal fragment; pCTF) and phosphoramidon were obtained from Peninsula Laboratories Europe Ltd., St. Helens, Lancs. ET-1(19-21) Ile-Ile-Trp (IIW) was from Zinsser Analytic (UK) Ltd, Maidenhead, Berks. Human big ET-1 (22-38) (human C-terminal fragment; hCTF) was synthesized by Prof. Ramage and Dr A. Stewart, Department of Chemistry, University of Edinburgh. Substance P and recrystallized thermolysin (type X protease) were from the Sigma Chemical Company, Poole, Dorset. Porcine endopeptidase-24.11 was isolated by immunoaffinity chromatography as described by Gee *et al.* (1983), except that the monoclonal antibody used was GK4A9 (Gee & Kenny, 1985). The purified enzyme was homogeneous as assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate. [125 I]-ET-1 and [125 I]-big ET-1 were from Amersham International plc, Amersham, Bucks.

Figure 1 Hydrolysis of big endothelin-1 (big ET-1), ET-1, C-terminal fragment and substance P by purified porcine E-24.11. Peptide (3 nmol) was incubated at 37°C, pH 7.4 with E-24.11 (200 ng protein) for up to 180 min. Products were separated and analysed by reverse phase h.p.l.c. and depletion of substrate was quantified by A_{214} . The symbols correspond to: (□) ET-1; (○) substance P; (■) big ET-1; (●) C-terminal fragment. The experiment was repeated twice, with similar results.

Results

ET-1 and pCTF were efficiently hydrolysed by E-24.11. The respective half-lives for hydrolysis of 3 nmol of ET-1 and CTF by 200 ng E-24.11 were 37 and 57 min respectively (Figure 1) which compares with a t_1 of 2 min for substance P, one of the best substrates identified to date for this enzyme (Turner *et al.*, 1987). In contrast, less than 5% of porcine big ET-1 at a similar concentration was degraded by 200 ng E-24.11 in 3 h (Figure 1). Using as much as 4 μ g E-24.11, a t_1 of 150 min was calculated. No such marked differences in half-lives were seen when the peptides were hydrolysed by thermolysin. The respective half-lives under comparable conditions were estimated as porcine big ET-1 (25 min), ET-1 (56 min) and pCTF (47 min) (Figure 2).

When porcine big ET-1 (3 nmol) was incubated with either 200 ng or 4 μ g E-24.11 and the products were resolved by reverse phase h.p.l.c. there was insufficient yield of product with the retention time of ET-1 for quantification by h.p.l.c. However, radioimmunoassay confirmed that under certain conditions of substrate/enzyme ratio, ET-1 immunoreactivity was a detectable product (Table 1). In a subsequent experiment, evaluation of the time course of ET-1 production by E-24.11 (40 ng) from big ET-1 (2 nmol) showed levels of immunoreactivity of 16.1 ± 0.6 , 22.8 ± 1.1 , 30.8 ± 1.8 and 34.0 ± 3.8 pmol $200 \mu\text{l}^{-1}$ incubation volume at 1, 2, 4 and 8 h, compared to control values of <0.06 pmol $200 \mu\text{l}^{-1}$, illustrating the rapid rise in immunoreactivity during 1 h and approximately steady state levels by 4 h.

Characterization of the products by h.p.l.c. combined with RIA showed the presence of authentic ET-1 and hCTF

Figure 2 Hydrolysis of big endothelin-1 (big ET-1), ET-1 and C-terminal fragment by thermolysin. Peptide (3 nmol) was incubated at 37°C, pH 7.4 with thermolysin (50 ng protein) for up to 80 min. All incubations were in the presence of 3 mM CaCl_2 . Products were separated and analysed by reverse phase h.p.l.c. and depletion of substrate was quantified by A_{214} . The symbols correspond to: (□) ET-1; (■) big ET-1; (●) C-terminal fragment. The experiment was repeated twice, with similar results.

(Figure 3). Although insufficient ET-1 was generated for unequivocal identification by mass spectrometry, the identity of hCTF was confirmed ($MH^+ = 1809$) hence demonstrating that E-24.11 is able to cleave the Trp²¹-Val²² bond of big endothelin-1. In addition to ET-1 a second peak of ET-1(16-21) immunoreactivity was observed. This was identified by mass spectrometry to be ET(17-21) ($MH^+ = 659$, LDIIW). The major product of E-24.11 incubations with big ET-1, in terms of A_{280} , eluted in the position of the ET(19-21) standard (IIW) (Figure 3), and this product was also confirmed by mass spectrometry. In agreement with studies using porcine big ET-1, the majority of human big ET-1 remained undigested at the end of the incubation period.

When thermolysin was incubated with big ET-1, both ET-1-like and CTF-like products were detected at 214 nm by h.p.l.c. The formation of ET-1 was confirmed by RIA in amounts approximately 5 fold greater than seen with E-24.11 (Figure 4 and Table 1). As with E-24.11, h.p.l.c. characteriza-

tion of the products from human big ET-1 demonstrated ET-1 and a second immunoreactive product corresponding to ET-1 (17-21). Mass spectrometry confirmed the identity of ET-1, ET-1(17-21) and hCTF (MH^+ of 2493, 659, 1809 respectively). Like E-24.11 the major peak in terms of u.v. absorbance from thermolysin incubations eluted in the position of ET-1(19-21) standard (Figure 4).

Discussion

The nature and identity of the physiological ECE has generated considerable controversy in the literature (Opengorth *et al.*, 1992; Turner, 1993). The consensus is that a phosphoramidon-sensitive membrane metallopeptidase effects this conversion. However, the cellular and subcellular location of this activity is still unclear as is the cell biology of endothelin processing itself (Harrison *et al.*, 1993). The sensitivity of ECE to phosphoramidon suggests a similarity with the well characterized metallopeptidases E-24.11 and thermolysin. However, Abassi *et al.* (1993) failed to detect production of ET-1 from big ET-1 by recombinant E-24.11. The ability of thermolysin to generate ET-1 from its precursor has not previously been examined. A possible candidate for the physiological ECE has recently been isolated from rat lung and porcine aortic endothelial cells and is characterized by sensitivity to phosphoramidon (IC_{50} in the range 0.1–1 μM) but not by another E-24.11 inhibitor, thiorphan (Takahashi *et al.*, 1993; Ohnaka *et al.*, 1993).

In the present study, a marked difference was seen in the abilities of E-24.11 and thermolysin to metabolize big ET-1. Thermolysin degraded big ET-1, ET-1 and CTF at broadly similar rates. When big ET-1 was the substrate, both ET-1 and CTF were detected by RIA and h.p.l.c. as intermediates and their production was confirmed by mass spectrometry. In contrast, E-24.11 degraded big ET-1 approximately two orders of magnitude more slowly than the product peptides.

Table 1 RIA values for endothelin-1 (ET-1) production from big ET-1 catalysed by E-24.11 and thermolysin

	Endothelin-1 like immunoreactivity (pmol 200 μl^{-1})		
	20 ng ml^{-1}	200 ng ml^{-1}	2 μg ml^{-1}
<i>E-24.11</i>			
big ET-1 (1 μM)	1.7 $\pm$ 0.1	6.1 $\pm$ 0.1	2.3 $\pm$ 0.1
big ET-1 (10 μM)	7.5 $\pm$ 0.1	27.6 $\pm$ 0.3	33.4 $\pm$ 0.4
<i>Thermolysin</i>			
big ET-1 (10 μM)		161 $\pm$ 4	121 $\pm$ 4

Values are mean $\pm$ s.e.mean; $n = 3$ for each 2 h incubation.

Figure 3 High performance liquid chromatography (h.p.l.c.) characterization of products of E24.11 incubation with human big endothelin-1 (big ET-1). H.p.l.c. was performed on the pooled material obtained after 8 h incubation of E.24.11 and human big ET-1; equivalent to 100 ng enzyme and 5 nmol peptide. (a) u.v. absorption at 280 nm; (b) hatched columns = hCTF immunoreactivity; solid columns = ET-1(16-21) immunoreactivity. The elution positions of the standard peptides, hCTF, IIW, human big ET-1 and ET-1 are indicated.

Figure 4 High performance liquid chromatography (h.p.l.c.) characterization of products of thermolysin incubation with human big endothelin-1 (big ET-1). H.p.l.c. was performed on the pooled material obtained after 2 h incubation of thermolysin and human big ET-1; equivalent to 120 ng enzyme and 6 nmol peptide. (a) u.v. absorption at 280 nm; (b) hatched columns = hCTF immunoreactivity; solid columns = ET-1(16-21) immunoreactivity. The elution positions of the standard peptides, hCTF, IIW, human big ET-1 and ET-1 are indicated.

Nevertheless, low, steady state levels of ET-1 were clearly detected by RIA and confirmed by h.p.l.c. In addition, confirmation of the Trp²¹-Val²² cleavage was obtained by the production of ET-1 and CTF, with the identity of the latter being verified by mass spectrometry. However, the levels of detectable ET-1 depend critically on the enzyme:big ET-1 ratio as the generated ET-1 is degraded rapidly to the 17-21 and 19-21 fragments. The failure of Abassi *et al.* (1993) to detect ET-1 production from big ET-1 by recombinant E-24.11 may reflect not only the lack of sensitivity of their assay system and relatively poor resolution of substrate and product peptides by h.p.l.c., but also the ratio of enzyme to substrate used, which would have favoured degradation of any ET-1 generated. Earlier reports of the hydrolysis of ET-1 by E-24.11 described the formation of ET-1(17-21) (LDIIW) and ET-1(19-21) (IIW), with the latter being the predominant fragment obtained from metabolism of the C-terminal hexapeptide sequence of ET-1 (Vijayaraghavan *et al.*, 1990; Fagny *et al.*, 1991). Under our conditions, the major product in terms of A₂₈₀ from thermolysin or E-24.11 incubations eluted in the position of ET-1(19-21) (Figures 3 and 4). The identification of this fragment, and the observations from the time course of ET-1 production by E-24.11 suggest that ET-1 reaches a critical concentration, and then degradation matches the formation of fresh ET-1, hence the large peak of IIW compared to the ET-1 peak. We predict that the formation of this fragment required the initial cleavage of big ET-1 at Trp²¹-Val²² to form ET-1, which then underwent hydrolysis in the manner described for ET-1.

The marked differences in the relative rates of metabolism of big ET-1 and ET-1 by E-24.11 and thermolysin can be rationalised in terms of their individual specificity requirements. Both Hersh & Morihara (1986) and Poszgay *et al.* (1986) have compared some of the properties of E-24.11 and thermolysin in detail. In summary, a major difference was seen between the active site of E-24.11 and thermolysin in terms of hydrophobicity. Thermolysin appears to utilise hydrophobic interactions as a major binding force whereas E-24.11 utilises weaker hydrophobic binding interactions but also make use of an ionic interaction with the C-terminal carboxylate of its substrates. Thus, E-24.11 prefers small peptide substrates and exhibits some of the characteristics of a carboxydipeptidase as in the cleavage of the Gly³-Phe⁴ bond in [Leu⁵]-enkephalin. In addition, thermolysin prefers a bulky hydrophobic residue in the P₁ position and a small hydrophobic residue in the P₂ position (Poszgay *et al.*, 1986). Big ET-1 fits these requirements for thermolysin with the

presence of Trp at the P₁ position and Ile at the P₂ position. Thus, although thermolysin further degrades ET-1 and cannot be regarded as an endothelin converting enzyme in the strict sense of the term, it may represent a more useful model for ECE than E-24.11. In particular, it is readily available in homogeneous form and X-ray crystal structures of inhibitor-thermolysin complexes are available (Weaver *et al.*, 1977; Monzingo & Matthews, 1982; 1984). In the case of the phosphoramidon-thermolysin complex, the inhibitor is bound with its phosphoramidate function mimicking the proposed tetrahedral transition state for peptide bound hydrolysis (Weaver *et al.*, 1977). Unlike E-24.11, which is equally sensitive to phosphoramidon and thiorphan, thermolysin is much less sensitive to inhibition by thiorphan (I₅₀ = 60 µM; Fulcher *et al.*, 1982), reinforcing the similarity with ECE.

The relative stability of big ET-1 in the presence of E-24.11 compared to ET-1 and CTF is consistent with the known preference of the enzyme for oligopeptides. The present studies provide little evidence of metabolism of big ET-1 at the cleavage sites of ET-1 without first undergoing the initial cleavage at the Trp²¹-Val²² bond. This indicates that the conformation of big ET-1 under these conditions favours this particular cleavage, and protects against hydrolysis of other bonds.

An enzyme-linked immunoadsorbent assay has been used to demonstrate that E-24.11 is widely distributed on mammalian cells and tissues although the enzyme is predominantly localized to the brush border epithelial cells of intestine and kidney (Howell *et al.*, 1991). Low levels of the enzyme have, however, been detected on endothelial cells of venous or arterial origin (Llorens-Cortes *et al.*, 1992). Thus, E-24.11 has the required specificity, cellular localization and topology to function in the processing and metabolism of big ET-1. The kinetics of metabolism of big ET-1 and ET-1 by E-24.11 (Figure 1) would tend to favour a role for the enzyme in ET-1 inactivation rather than the processing of big ET-1. Nevertheless, the presence of E-24.11 in endothelial plasma membranes or in partially purified preparations of ECE may lead to erroneous estimates of endothelin converting activity. E-24.11 activity can be eliminated either by immunoabsorption (Murphy *et al.*, 1993) or by the routine inclusion of thiorphan in assays of ECE.

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