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D-Val[22] containing human big endothelin-1 analog, [D-Val[22]]Big ET-1[16-38], inhibits the endothelin converting enzyme

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Abstract Endothelin converting enzyme (ECE) is essential for generation of the biological effects of endothelin-1 (ET-1) from a precursor, big endothelin-1 (Big ET-1). We synthesized four analogs of human Big ET-1[16–38], substituted with single D-amino acids at P1, P2, P1′ and P2′ positions. ECE activity was determined using an ET-1 specific radioimmunoassay system. None of the D-amino acid containing Big ET-1 analogs were apparently cleaved by ECE, however, one of the synthetic peptides, [D-Val[22]]Big ET-1[16–38], strongly inhibited the ECE activity. Furthermore, when this D-Val[22] containing peptide was preadministrated to rat striatum, it was found to inhibit the dopamine release induced by Big ET-1. This result suggests that the D-Val[22] containing peptide inhibits the ECE activity in vivo. The D-Val[22] containing inhibitor offers hope of developing more potent and highly specific ECE inhibitors of therapeutic significance.

Key words: Endothelin-1; Endothelin converting enzyme; D-Amino acid; Big endothelin-1 analog; Protease inhibitor; Dopamine

1. Introduction

Endothelin-1 (ET-1) is a novel vasoconstrictor peptide consisting of 21 amino acid residues with two disulfide bonds [1]. During the biosynthetic process, prepro-endothelin-1 converts to the putative precursor, big endothelin-1 (Big ET-1), and then Big ET-1 is processed by a novel endopeptidase, named endothelin converting enzyme (ECE) [1,2]. ET-1, Big ET-1 and the C-terminal fragment of Big ET-1 have all been detected in the culture medium [3] and plasma [4]. The in vitro vasoconstrictor activity of Big ET-1 is much lower (>100-fold) than that of ET-1, but the in vivo pressor effects of the two peptides are comparable [5,6]. These findings support the hypothesis that generation of ET-1 by ECE is essential for the potent biological effects of this regulatory peptide system. Development of inhibitors of ECE will provide effective means of preventing production of endothelin under pathogenic conditions [2,7].

Several ECE-like enzyme activities representing different endopeptidase classes have been identified. At first, a chymotrypsin-like enzyme was suggested as a putative ECE [1]. Also cathepsin-like proteases have been considered as candidates for a putative ECE, but can only be induced under selective and unusual conditions [8]. The third class of enzymes as a putative ECE are neutral endopeptidases [9–13]. These enzymes are sensitive to pH, and are inhibited by metal chelating agents, such as EDTA, and by phosphoramidon. The physiological relevance of a phosphoramidon-sensitive ECE activity is indicated by the findings that phosphoramidon also inhibits release of ET-1 from endothelial cells [14].

Substitution with D-configuration amino acids in peptide ligands has been widely used for probing the conformational requirements of ligand–receptor interactions. For example, the low and high affinity receptors of atrial natriuretic peptide can be distinguished by systematic D-amino acid replacements in the ligand [15]. A similar approach has been applied to studies of substrate–enzyme inhibitions. Substitution with D-amino acids in substrates for human spleen fibrinolytic protease and leukocyte elastase-like proteinase caused the specific inhibition of each or both enzymes [16,17]. Recently, short peptide derivatives of ET-1 containing D-amino acid were prepared and found by others to be potent ET-1 receptor antagonists [18–20]. However, these studies on endothelin analogs did not include evaluation of their activities on ECE. Also, none of the many D-amino acid substituted endothelin peptide fragments encompassed the length of Big ET-1, that is the subject of our study here.

In this investigation, we synthesized the human Big ET-1 analogs substituted with systematic single D-amino acids. These analogs were assayed for modulation of ECE activity. Of the four synthetic peptides, one peptide ([D-Val[22]]Big ET-1[16–38]) has shown potent inhibitory activity to the neutral ECE. This peptide was also found to inhibit the dopamine release induced by Big ET-1, in vivo.

2. Materials and methods

2.1. Synthesis of peptides

The peptides were synthesized by the solid-phase method on a Biosearch automated peptide synthesizer Model 9600 based on *t*-butyloxycarbonyl strategy. Deprotection and cleavage from the resin was achieved by the two-step method using trimethylsilyl bromide-thioanisole and HF [21,22]. The crude peptides were purified by gel-filtration followed by reverse phase HPLC. The purity and identity of the peptides were confirmed by analytical HPLC and by amino acid analysis. Big ET-1, ET-1 and phosphoramidon were purchased from Peptide Institute Inc. (Osaka, Japan).

2.2. Isolation of ECE

Bovine aortic endothelial cells were prepared by the method described by Gospodarowicz et al. [23]. Confluent cells were washed with PBS and scraped in PBS by rubber policeman. Cells were homogenized followed by centrifugation at 100,000 × *g* for 1 h. After washing with

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Abbreviations: ET-1, endothelin-1; Big ET-1, big endothelin-1; ECE, endothelin converting enzyme; DA, dopamine.

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PBS three times, the pellet was rehomogenized in PBS containing 1% CHAPS, and then stirred for 4 h. After centrifugation at 100,000 × *g* for 1 h, the supernatant was concentrated by ultrafiltration through a UK-10 filter (Advantec Tokyo, at molecular weight cut-off 10,000). The concentrate was passed through a column of Immobilized Ovoinhibitor (Pierce, Rockford), in order to remove serine proteases. The resulting enzyme preparation was used for the assays. The activity of ECE in this preparation was stable during storage at −80°C for over 1 month (Okitsu, M. et al., unpublished data).

2.3. Enzyme assay

The enzyme preparation containing 0.2 mg protein was incubated with Big ET-1 at a concentration of 1.0×10^{-5} M for 2 h at 37°C in 20 μl of the reaction mixture. The reaction mixture consisted of 50 mM HEPES, pH 7.0 and 2.5 mM *N*-ethylmaleimide (NEM). NEM inhibits non-specific degradation of peptides produced by the ECE preparation [24]. After stopping the reaction by boiling at 100°C for 5 min, the resultant supernatant was diluted 1/100 and used directly for radioimmunoassay (RIA). In experiments testing the proteolytic activity of the enzyme on our synthetic peptides, substrate concentrations were kept at 1.0×10^{-5} M. In the evaluation of synthetic peptides as inhibitors the enzyme fraction was co-incubated with 1.0×10^{-5} M of Big ET-1 and 1.0×10^{-3} M of each synthetic peptide. In the kinetic experiments, both the concentration of Big ET-1 and synthetic peptides were varied. One unit of enzyme activity is the quantity which catalize the formation of 1 pmol of the product per hour at 37°C. Protein concentrations were determined with the Micro BCA Protein Assay Reagent (Pierce, Rockford) [25], and bovine serum albumin as standard.

2.4. Radioimmunoassay (RIA) for ET-1

RIA was performed as described by Ando et al. [26], with some modifications. The assay buffer for RIA was 0.01 M phosphate buffer, pH 7.4, containing 0.14 M NaCl, 0.01 M EDTA, 0.02 M glycine, 0.01 M ε-aminocaproic acid, 1 mM sodium azide, 0.1 mg/ml human serum albumin (Sigma), and 0.1% Triton X-100. This assay buffer was used to dissolve all reagents. The incubation mixture consisted of 0.1 ml sample or standard solution, 0.1 ml assay buffer, 0.1 ml anti-Lys-Lys-ET[15–21] antiserum (final dilution; 1:30,000 – Immuno Biological Laboratories (Gumma, Japan)), 0.1 ml of 0.185 kBq of [^{125}I]ET-1 (74 TBq/mmol, Amersham) and 1 μl of normal rabbit serum (Calbiochem). Incubations were carried out at 4°C for 24 h. In order to separate the bound ligands from the free ones, 0.1 ml of 5 units/ml goat anti-rabbit γ-globulin was added following the incubation at 4°C for 4 h. After centrifugation at 10,000 × *g* for 5 min, the radioactivity in the precipitate was measured by γ-counter. The amount of immunoreactive-peptide in the unknown sample was extrapolated from the standard curve linearized by a logit-log transformation. In order to compare our RIA system to that used by Ando et al. [26], we also used the antiserum provided from them.

2.5. Measurement of dopamine release from the striatum

Male Wistar rats 250–350 g were anesthetized and the guide cannula for penetration of microdialysis probe into the striatum was stereotaxically implanted as described by Paxinos and Watson [27]. After allowing at least three days for recovery from the surgery, microdialysis experiments were carried out with a freely moving rat placed in a acrylic home cage. On the day before the dialysis experiment, the dummy cannula was removed from the guide cannula, and the dialysis probe was inserted into the guide cannula. This dialysis probe (MI-BDP-I-8–03, Eicom, Japan) had a dialysis membrane (at molecular weight cut-off 5,000) and a side tube in order to microinject sample from outside. The probe was perfused with the Ringer solution (147 mM Na^+, 4 mM K^+, 2.3 mM Ca^{2+}, and 155.6 mM Cl^-, pH 6.0) at a rate of 2 μl/min (EP-60; Eicom, Japan). In order to observe the effect of Big ET-1, phosphoramidon and EV-D, 10 μl of sample were microinjected via the side tube.

The releasable dopamine (DA) was separated by the reverse-phase HPLC using MA-ODS Column (5 μm particles, diam. 4.6 × 250 mm; Eicom, Japan) and detected with electrochemical detector (CB-100; Eicom, Japan). The mobile phase was composed by 0.1 M citric acid buffer, pH 3.9, containing 12% methanol, 160 mg/l sodium-1-octanesulfate and 20 mM EDTA. The graphite electrode (WE-3G; Eicom, Japan) was set at +0.65 V (vs. Ag/AgCl). The perfusate from the striatum (40 μl) was injected into the HPLC every 20 min by an automatic injector (AS-10; Eicom, Japan). The level of DA was expressed as% of the basal level.

3. Results and discussion

3.1. Synthetic peptides

We previously reported the method for the synthesis of Big ET-1[1–38] and related peptides [22]. Utilizing this method, we synthesized several peptides listed in Fig. 1. Since Big ET-1 contains 2 specific disulfide bonds and a long C-terminal segment, synthesis of numerous analogs poses a cumbersome challenge. Therefore, the design of shorter yet still suitable cleavage site segments are advantageous. However, Wu-Wong et al. found that a neutral ECE did not cleave a decapeptide substrate that contains five amino acids on both sides of the scissile bond [12]. This suggested that portions of the substrate sequence far removed from the scissile bond may be essential for enzyme recognition. Okada et al. [13] reported that the C-terminal residues 27–34 of Big ET-1 are important for conversion by a neutral ECE from bovine endothelial cells. This region may be critical for enzyme recognition. They also found that Big

Peptide Name	Amino Acid Sequences
	P_2 P_1 P_1' P_2'
	16 21 22 25 35 38
ET-L: BigET-1(16-38);	H-H L D I I W V N T P E H V V P Y G L G S P R S-OH
EI-D: [D-Ile^{20}]BigET-1(16-38);	H-H L D I i W V N T P E H V V P Y G L G S P R S-OH
EW-D: [D-Trp^{21}]BigET-1(16-38);	H-H L D I I W V N T P E H V V P Y G L G S P R S-OH
EV-D: [D-Val^{22}]BigET-1(16-38);	H-H L D I I W v N T P E H V V P Y G L G S P R S-OH
EN-D: [D-Asn^{23}]BigET-1(16-38);	H-H L D I I W V n T P E H V V P Y G L G S P R S-OH
BigET-1(16-21);	H-H L D I I W-OH
[D-Ile^{20}]BigET-1(16-21);	H-H L D I i W-OH
[D-Trp^{21}]BigET-1(16-21);	H-H L D I I W-OH

Fig. 1. Amino acid sequences of synthetic peptides. Each amino acid is shown by a one-letter symbol and D-amino acid shown by small letters are underlined. The bracketed region indicates the scissile bond of each peptide, which is cleaved by ECE.

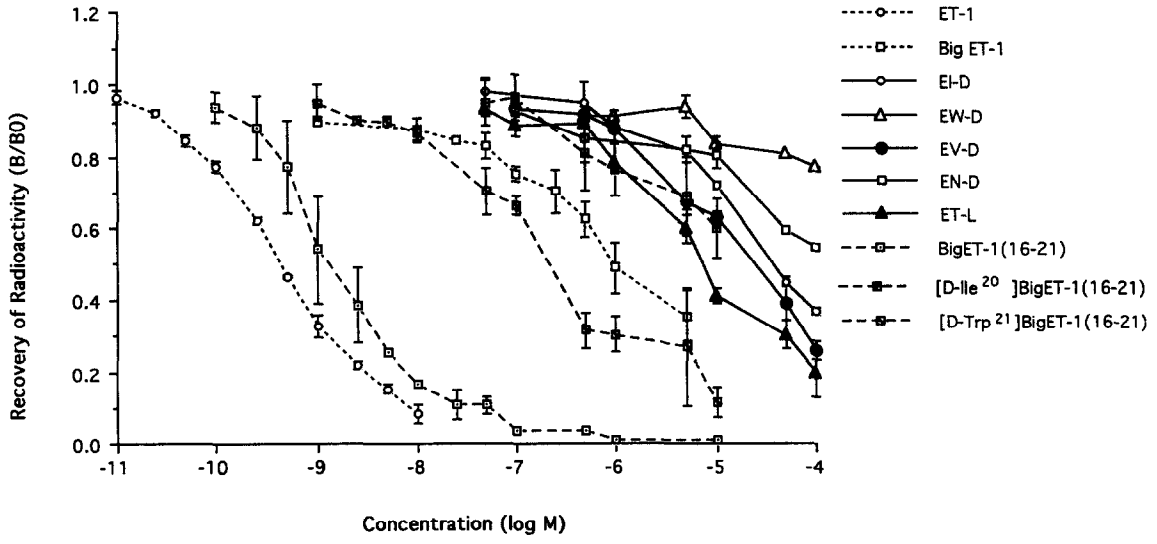

Fig. 2. Cross-reactivity of the synthetic peptides to antiserum. Each curve was constructed using B/B_0 vs. log$M$ for each synthetic peptide. Vertical bars indicate means ± S.D. (n = 3).

ET-1[16–37] has a high sensitivity to a neutral ECE [13]. Therefore, we chose to synthesize ET-L (Big ET-1[16–38]), a 23-mer, as a suitable substrate for a neutral ECE, which has shorter length than Big ET-1 and does not contain disulfide bonds. We also prepared another four ET-L analogs substituted with systematic single D-amino acid replacement, such as EI-D ([D-Ile^{20}]Big ET-1[16–38]), EW-D ([D-Trp^{21}]Big ET-1[16–38]), EV-D ([D-Val^{22}]Big ET-1 [16–38]), and EN-D ([D-Asn^{23}]Big ET-1[16–38]). Other peptides listed in Fig. 1 are fragments of peptides described above, which are the expected cleavage products by the neutral ECE.

3.2. Radioimmunoassay

The RIA system in this investigation was the same as described by Ando et al. [26], and the titer and specificity of antiserum was also the same (data not shown). Antiserum against Lys-Lys-ET[15–21] recognized the sequence of ET[15–21] including the free C-terminal Trp residue. Immunoreactivities of the synthetic peptides were determined using our antiserum (Fig. 2). The detection limit of ET-1 and Big ET-1 was 2.5×10^{-11} M and 2.5×10^{-7} M, respectively. The immunoreactivity of ET-L to our antiserum was less potent (1.0×10^{-6} M) than that of Big ET-1. The four Big ET-1 analogs substituted with D-amino acids (EI-D, EW-D, EV-D and EN-D) reacted with our antiserum only at the concentrations higher than 1.0×10^{-5} M. The other peptides (ET-1[16–21], [D-Ile^{20}]ET-1[16–21] and [D-Trp^{21}]ET-1[16–21]) are putative fragments after ECE digestion of ET-L, EI-D and EW-D. ET-1[16–21] was immunoreactive at the concentration of more than 5×10^{-10} M. This immunoreactivity is comparable to that of ET-1. [D-Ile^{20}]ET-1[16–21] and [D-Trp^{21}]ET-1[16–21] were immunoreactive at concentrations of more than 1.0×10^{-8} M and 2.5×10^{-7} M, respectively. We determined the appropriate peptide concentrations in the enzyme assays on the basis of results in Fig. 2.

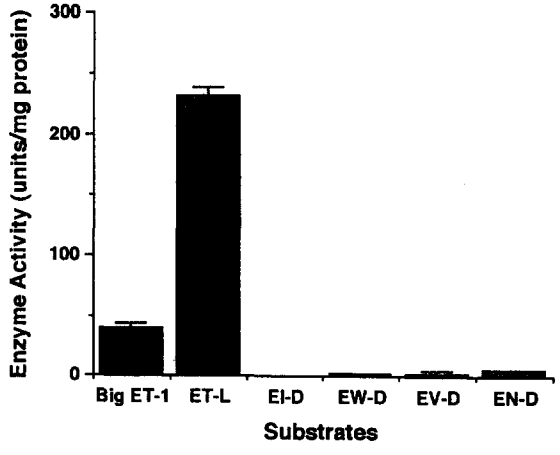

Fig. 3. Cleavage of Big ET-1 analogs by Immobilized Ovoinhibitor purified ECE. Peptide substrate concentration: 1.0×10^{-5} M. To determine the concentration of the hydrolyzed fragments, standard curves were used for each peptide: ET-1 for Big ET-1, [D-Ile^{20}]ET-1[16–21] for EI-D, [D-Trp^{21}]ET-1[16–21] for EW-D and ET-1[16–21] for EV-D, EN-D and ET-L. Vertical bars indicate means ± S.D. (n = 3).

3.3. Inhibition of ECE by synthetic peptides

The various shortened Big ET-1 analogs were evaluated as substrates for the enzyme. ET-L was found to be cleaved efficiently, as measured by RIA (Fig. 3). In this case, the immunoreactive-peptide is most probably ET-1[16–21]. The four D-amino acid containing human Big ET-1 analogs (EI-D, EW-D, EV-D and EN-D) were not converted to the immunoreactive-peptide by ECE (Fig. 3). These result suggest that ECE does not cleave Big ET-1 analogs substituted by D-amino acid at the P1, P2, P1′ and P2′ positions.

The ECE activity of the Immobilized Ovoinhibitor column purified enzyme was inhibited by EDTA, a metal chelator, and by phosphoramidon, a neutral protease inhibitor (Fig. 4). These results confirmed the previous findings of others [9–11]. Our kinetic studies of the enzyme, using Big ET-1 as the substrate, yielded a K_m of 6.9×10^{-6} M and V_{max} of 189 units/mg protein. These properties of the enzyme were very similar to those obtained by Ohnaka et al. [9]. Therefore, our ECE activities detected in this investigation also result from the existence of a phosphoramidon-sensitive neutral metal protease.

The ECE activity was apparently inhibited by all four human Big ET-1 analogs substituted with systematic single D-amino acids (EI-D, EW-D, EV-D and EN-D), at the concentration of 1.0×10^{-3} M (Fig. 4). EN-D showed the weakest inhibitory activity, although EI-D and EW-D apparently inhibited the ECE activity (Fig. 4). EV-D showed the strongest inhibitory activity with an apparent 85% inhibition of enzyme activity (Fig. 4). We performed kinetic studies using EV-D and found that it acted through a competitive mode of inhibition mechanism with apparent K_i value of approximately 2.5×10^{-5} M.

3.4. Inhibition of dopamine release in vivo

It has been postulated that ET-1 may function as neuropeptide in controlling of neural function [2]. Using microdialysis technique, which is a new technique to monitor levels of chemical agents in the extracellular space [28], Kurosawa et al. was reported that ET-1 induced dopamine (DA) release from the striatum of rats [29]. Recently, we suggested that ET-1 directly induced the dopamine release (Horie, K. et al., unpublished data). When Big ET-1 (50 pmol) was microinjected into the rat striatum, the DA release from the striatum was dramatically increased to 17,000% of the basal level (Fig. 5). However, when 10 pmol of phosphoramidon was preinjected, the DA release by Big ET-1 was inhibited (Fig. 5). The dose for 50% inhibiton of this effect of phosphoramidon is about 1.5 pmol. These results suggest that Big ET-1 is converted to ET-1 by ECE in the rat striatum, and the produced ET-1 increases the DA release. Our new potent ECE inhibitor, EV-D, was found to inhibit the DA release induced by Big ET-1 (Fig. 5). The dose for 50% inhibiton of this effect of EV-D is about 0.33 pmol. These parallel results implicate that EV-D inhibits ECE in the rat striatum, prevents the production of ET-1, and thus abolishes the inducing DA release.

In this paper, we have shown that EV-D, [D-Val^{22}]Big ET-1[16–38], inhibits the ECE activity in in vitro and in vivo experiments. These inhibitory effects of EV-D were comparable with those of phosphoramidon. Phosphoramidon has the phosphoryl-Leu moiety, which contributes the majority of its binding energy to the enzyme. Many enzymes, such as thermolysin and neutral endopeptidase 24.11, are inhibited by phosphoramidon [2]. On the other hand, EV-D may be bound to ECE through specific peptide chain interactions, including amino acid residues 27–34 [13], but cannot be hydrolyzed at the scissile site. These suggest that EV-D is a more specific ECE inhibitor compared to phosphoramidon. EV-D is potentially a useful ECE inhibitor for studying biological role and mechanism of endothelin precursors. Structure–activity relationship studies of EV-D will be expected to produce stronger and more specific ECE inhibitors of therapeutic significance.

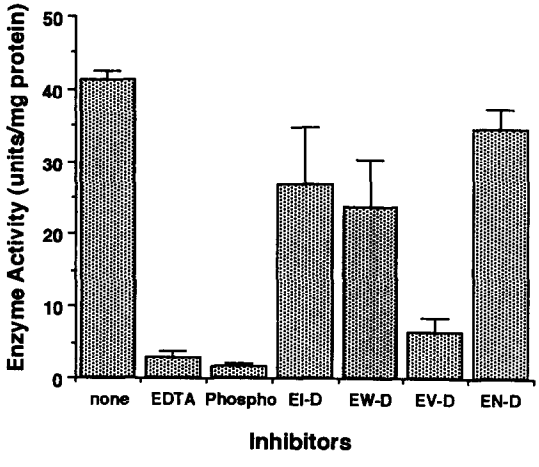

Fig. 4. Inhibitory effect of Big ET-1 analogs containing D-amino acid on ECE activity. Concentration of phosphoramidon and EDTA was 0.25 mM and 0.125 mM, respectively. Concentration of each Big ET-1 analog was 1.0×10^{-3} M in the enzyme assay. Vertical bars indicate means ± S.D. ($n = 3$).

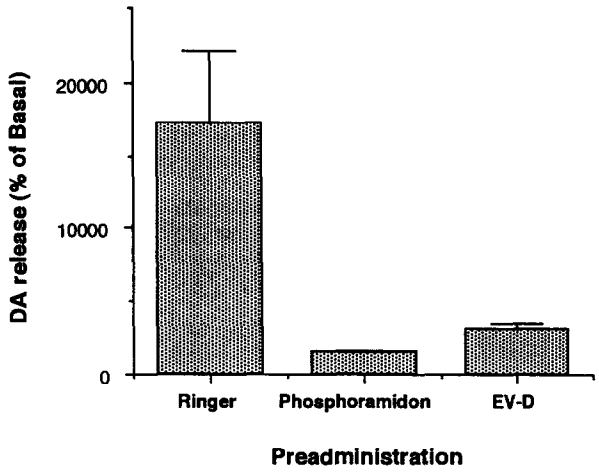

Fig. 5. EV-D suppresses the Big ET-1 induced dopamine release from the striatum of rat. At one hour after pre-administration of Ringer, phosphoramidon (10 pmol/10 μl Ringer), or EV-D (1 pmol/10 μl Ringer), Big ET-1 (50 pmol/10 μl Ringer) was microinjected. Averaged values of DA contents at 20, 40, 60 and 80 min were expressed as % of the basal levels. Vertical bars indicate means ± S.D. ($n = 4$ for Ringer, $n = 1$ for phosphoramidon, $n = 3$ for EV-D).

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