[Phe^{21}]big endothelin-1(18–34) and [Ala^{31}]big endothelin-1(18–34) inhibit the human endothelin-converting enzyme-1 (ECE-1) expressed in CHO-K1 cells in a different fashion

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Abstract Endothelin-converting enzyme-1 (ECE-1) is one of the most important enzymes to convert big endothelin-1 (big ET-1) to ET-1. To identify the inhibitors of ECE-1, we examined the effects of variously substituted analogues of big ET-1 on ECE-1 activity using solubilized membranes prepared from human ECE-1-expressed CHO-K1 cells. Among the big ET-1 analogues tested, [Phe^{21}]big ET-1(18–34) and [Ala^{31}]big ET-1(18–34) exhibited a significant inhibition of ECE-1. A kinetic analysis revealed [Phe^{21}]big ET-1(18–34) to be a competitive inhibitor (K_i = 20.6 μM) and [Ala^{31}]big ET-1(18–34) to be a non-competitive inhibitor (K_i = 35.6 μM). These results not only support the concept that ECE-1 recognizes big ET-1 both at the P1 position and at the C-terminal region but also revealed that these two regions are recognized by this enzyme in a different manner.

Key words: Endothelin; Endothelin-converting enzyme; Big endothelin; Big endothelin analogue

1. Introduction

Endothelin-1 (ET-1) is a potent vasoactive peptide consisting of 21 amino acid residues with two disulfide bridges [1]. For the biosynthesis of ET-1, an inactive intermediate form, big ET-1, with 38 amino acid residues in humans is initially produced from pro-ET-1, thereafter big ET-1 is converted into an active form, ET-1, through a proteolytic cleavage between Trp^{21} and Val^{22} by endothelin-converting enzyme-1 (ECE-1). ECE-1 protein was recently purified and its major property as a neutral metalloprotease was clarified [2,3]. The primary structure of ECE-1 and the presence of two isoforms, ECE-1α and ECE-1β, have also been revealed by the cloning of ECE-1 cDNAs [4–10]. The isoforms of ECE-1 were shown to be derived from one gene by alternative splicing based on the genomic structure of the ECE-1 gene [11]. It has recently been shown that ET-1 is essential for the normal development of neural crest-derived tissue in the fetus [12] while it is also involved in a number of disease models of adult animals and in certain pathological conditions in humans [13]. The design of specific inhibitors of ECE-1 may thus lead to the development of new treatments of these diseases, furthermore, these inhibitors may contribute to the clarification of the substrate recognition mechanism of ECE-1.

In the present study, we demonstrated two big ET-1 analogues which show different types of inhibition against ECE-1.

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2. Materials and methods

2.1. Materials

Synthetic human ET-1(1–21) and big ET-1(1–38) were purchased from Peptide Institute (Osaka, Japan). Variously substituted big ET analogues [3], which were synthesized by the solid-phase method, were supplied by Ciba-Geigy International Research Laboratories (Takarazuka, Japan). Porcine ECE was purified from porcine aortic endothelia as previously described [3].

2.2. Cloning and transfection of human ECE-1 cDNA

A cDNA library was prepared in γZAP (Stratagene, CA, USA) using poly(A)$^+$ RNA from human umbilical vein endothelial cells (HUVEC). 1.6 kbp of partial cDNA encoding an ECE-1 protein was then obtained from normal human adrenal total RNA by reverse transcription coupled to polymerase chain reaction (RT-PCR) using oligonucleotides with sequences of 5′-AGAACTCCAACAGCAACGTGATCC-3′ and 5′-CCTTACCAGACTTCGCACTTGTGAG-3′ as sense and antisense primers, respectively, which were set based on the bovine ECE-1 cDNA structure [6]. This cDNA was used as a probe for screening a HUVEC cDNA library, and two types of cDNAs, pHECE1A and pHECE1B, corresponding to human ECE-1α and ECE-1β [4,9,11], respectively, were cloned. Nucleotide sequences were determined using a DSQ 1000 DNA autosequencer (Shimadzu Co., Kyoto, Japan). The cDNA insert of pHECE1A consisted of 2662 bp (−11 to +2651; the numbers represent the nucleotide positions by designating the translation start site as +1) and that of pHECE1B consisted of 2793 bp (−143 to +2650). A cDNA portion encoding an entire ECE-1 protein of each ECE-1 clone (1α, −11 to +2359; 1β, −68 to +2374) was subcloned into the SRα promoter-based mammalian expression vector pME18S [14]. The resultant expression plasmid constructs (4 μg), pME18SHECE1A and pME18SHECE1B, were then transfected into CHO-K1 cells cultured in 100 mm dishes using 10 μl of liposomes (Lipofectamine, Life Technologies, Grand Island, NY, USA). The cells (4 dishes), harvested 48 h after transfection, were homogenized with a Polytron homogenizer on setting 7 for 10 s twice in 2 ml of 0.25 M sucrose containing a 25 mM HEPES buffer, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml of pepstatin A and 2 μg/ml of aprotinin (buffer A), and then centrifuged at 221 000×g for 30 min. The resulting membrane fraction was solubilized in 0.4 ml of buffer A containing 0.3% (w/v) Lubrol PX, and then was centrifuged again at 221 000×g for 30 min. The resultant clear supernatant was used for the assay of ECE-1 activity.

2.3. Assay of ECE-1 activity

The solubilized CHO-K1 membranes (3–5 μg) were incubated for 6 h at 37°C in 100 μl of 0.2 M Tris-HCl, pH 7.0, containing varied concentrations of big ET-1 and 0.5 mM *p*-chloromercuribenzoic acid (pCMB) and 0.1 mM actinonin in the presence or absence of 5–100 μM big ET analogues. The reaction was terminated at every 1 h incubation by the addition of 100 μl of radioimmunoassay buffer containing 2 mM EDTA and the amount of ET-1 that had been formed was then measured by a specific radioimmunoassay for the carboxyl-terminal tail of ET-1 using As-N7 antiserum [15] as previously described [3]. As-N7 had no cross-reactivity with the big ET analogues used in this study or their hydrolysis products. For the assay of hydrolysis rates of big ET analogues, 10 μM of each big ET analogue was incubated with the solubilized CHO-K1 membranes in the presence of 0.5 mM pCMB and 0.1 mM actinonin for 30–120 min at 37°C followed by the quantification of the peptide products by

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HPLC on an ODS-120T column (0.46×25 cm, Tosoh) as described previously [3].

3. Results

3.1. Expression of human ECE-1α and ECE-1β in CHO-K1 cells

The CHO-K1 cells which were transfected transiently with pME18S expression vector containing the cDNA insert of pHECE1A showed the phosphoramidon-sensitive but thiorphan-insensitive converting activity from big ET-1 to ET-1 to be characteristic of native ECE [2,3,15,16]. More than 95% of the expressed ECE-1 activity was localized in the membrane fractions, and the Lubrol PX extracts from the membrane fractions displayed ECE-1 activity of 0.192 ± 0.018 nmol of ET-1 formed/h/mg protein (mean ± S.D., $n = 4$) using 0.2 μM big ET-1(1–38) as a substrate, while the ECE-1 activity observed in the extracts prepared from mock-transfected CHO-K1 cells was less than 0.0004 nmol/h/mg. CHO-K1 cells transfected with pME18-SHECE1B showed essentially the same results as those with pME18SHECE1A.

3.2. Effects of big ET analogues on ECE-1 activity

We previously showed that a linear peptide of big ET-1(18–34) has the full substrate activity for the purified porcine aortic ECE and various substitutions for the amino acid residues of this linear peptide reduced its hydrolysis rate [3]. The human ECE-1α expressed in CHO-K1 cells also effectively hydrolyzed the big ET-1(18–34). We then examined whether or not these variously substituted analogues of big ET-1(18–34) have any inhibitory activity against ECE-1. Among the various big ET analogues examined, big ET-1(18–34) itself, [Phe^{21}]big ET-1(18–34) and [Ala^{31}]big ET-1(18–34) both showed a significant inhibition of the ECE-1 activity expressed in the CHO-K1 membrane fractions (Table 1). [Phe^{21}]big ET-1(18–34) and [Ala^{31}]big ET-1(18–34) suppressed the conversion of big ET-1(1–38) to ET-1 dose-dependently, and then decreased to 10–14% of the original rates at 100 μM (Fig. 1). [D-Val^{22}]big ET-1(16–38), which was reported to have an inhibitory activity against ECE [17], did not show any inhibition up to 100 μM, but reduced to 70% of the original rates at 0.75 mM (Fig. 1). The hydrolysis rates of big ET analogues themselves were 7.18 ± 0.58 and 2.01 ± 0.12 nmol/h/mg protein (mean ± S.D., $n = 3$) for big ET-1(18–34) and [Phe^{21}]big ET-1(18–34), respectively, at 10 μM, but [Ala^{31}]big ET-1(18–34) was not hydrolyzed by the expressed ECE-1α. A kinetic analysis of the hydrolysis rates of big ET-1(1–38) in the presence of different concentrations of those big ET-1 analogues revealed [Phe^{21}]big ET-1(18–34) to be a competitive inhibitor ($K_i = 20.6 \pm 3.8$ μM), and [Ala^{31}]big ET-1(18–34) to be a non-competitive inhibitor ($K_i = 35.6 \pm 8.1$ μM) (Fig. 2). Essentially similar kinetic results were thus obtained using purified porcine aortic ECE (data not shown). The apparent Michaelis constant (K_m) for big ET-1(1–38) in the expressed ECE-1α was 4.5 ± 1.2 μM (mean ± S.D., $n = 3$), which closely agreed with the findings ($K_m = 3.3 \pm 0.3$ μM) obtained in the purified porcine aortic ECE [3].

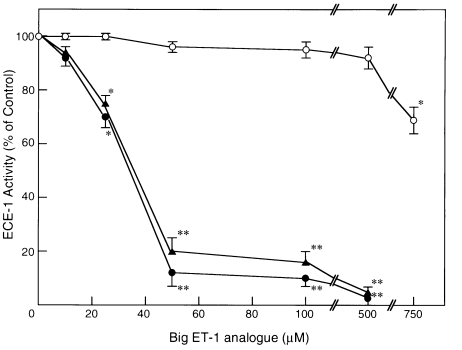

Fig. 1. Concentration-dependent inhibition of the ECE-1 activity in solubilized membranes prepared from ECE-1α cDNA-transfected CHO-K1 cells. The solubilized membrane fractions were incubated with 0.2 μM human big ET-1(1–38) in the presence of various concentrations of [Phe^{21}]big ET-1(18–34) (●), [Ala^{31}]big ET-1(18–34) (▲) and [D-Val^{22}] big ET-1(16–38) (○). The amounts of ET-1 produced were assayed at every 1 h incubation as described in Section 2. Data are represented as the mean ± S.D. of four independent experiments in values relative to the activity in the absence of the big ET-1 analogues. *$P < 0.05$; **$P < 0.001$ vs. the activity in the absence of the big ET-1 analogues, Student's t-test.

Table 1
Effects of various big ET analogues on the conversion rate of human big ET-1(1–38) by solubilized membranes prepared from human ECE-1α cDNA-transfected CHO-K1 cells

Big ET analogue	Hydrolysis rate of big ET-1(1–38) (%)
Big ET-1(1–38)	100
+big ET-1(18–34)	25
+big ET-1(17–26)	104
+[A^{20}]big ET-1(18–34)	92
+[A^{21}]big ET-1(18–34)	95
+[F^{21}]big ET-1(18–34)	10
+[A^{22}]big ET-1(18–34)	105
+[F^{22}]big ET-1(18–34)	108
+[Q^{27},T^{28},A^{29}]big ET-1(18–34)[a]	109
+[F^{21}]big ET-2(18–34)	98
+[D-W^{21},D-V^{22}]big ET-1(18–34)	105
+[D-V^{22}]big ET-1(16–38)	96
+[A^{31}]big ET-1(18–34)	14

[a]Big ET-2(18–34). Effects of big ET analogues on the conversion rate of big ET-1(1–38) (NH_2-C^{1}SCSSLMDKECVYFCHLDII^{20}W^{21}V^{22}N-TPEH^{27}V^{28}V^{29}PY^{31}GLGSPRS^{38}-COOH) were estimated by incubating 0.2 μM big ET-1(1–38) with the solubilized membranes in the presence of 100 μM of each big ET analogue followed by an assay of ET-1 produced by a specific radioimmunoassay. The conversion rate is expressed as the percent of that in the absence of an analogue, which was 0.21 ± 0.01 nmol/h/mg protein (mean ± S.D., $n = 3$).

4. Discussion

Recent studies have suggested that ECE-1 recognizes a relatively long C-terminal amino acid sequence in addition to the processing site of big ET-1 as follows. Not only in vitro [2,3,16,18–20] but also in vivo [21] investigations have shown that phosphoramidon-sensitive ECE hydrolyzes big ET-1 more efficiently than big ET-2 or big ET-3. This difference in the hydrolysis rate among big ET isopeptides has been suggested to be mainly due to the difference in their C-terminal sequence, namely, it has been shown by measuring the hydrolysis rates of variously substituted analogues of big ET-1 that ECE recognizes predominantly the sequence from His^{27} to Gly^{34} in the C-terminal of big ET-1 [3,19]. The importance of Trp^{21} in the processing of big ET-1 has also been demonstrated using purified ECE [3] and by site-directed mutagenesis of prepro-ET-1 [22]. The mode of substrate recog-

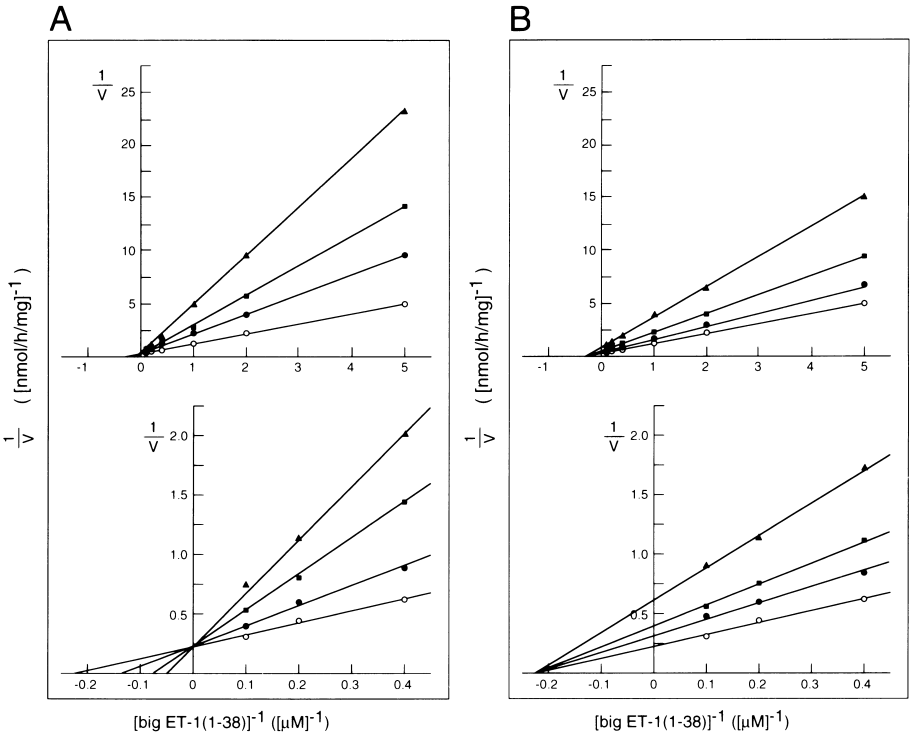

Fig. 2. Kinetic analyses of inhibition by [Phe^{21}]big ET-1(18–34) and [Ala^{31}]big ET-1(18–34) of the ECE-1 activity in solubilized membranes prepared from ECE-1α cDNA-transfected CHO-K1 cells. The solubilized membrane fractions were incubated with various concentrations of human big ET-1(1–38) in the absence (○) or presence of 30 (●), 50 (■) or 75 (▲) μM [Phe^{21}]big ET-1(18–34) (A), or [Ala^{31}]big ET-1(18–34) (B). The production rates of the ET-1 (V) and big ET-1 concentrations used are reciprocally plotted.

nition by ECE-1α is quite unique, because neutral endopeptidase 24.11 (NEP), which is thought to belong to the same family as that of ECE-1 [5,6,11], recognizes only the hydrophobic amino acid residues at the processing site. The unique character of the substrate recognition by ECE-1 also strongly suggests that the variously substituted analogues of the big ET-1 can inhibit the ECE-1 activity, furthermore, an analysis of the inhibitory effects of these analogues would thus be able to assess the processing mechanism of ECE-1. The membrane fractions prepared from CHO-K1 cells transfected with an expression vector containing ECE-1α or -1β cDNA exhibited approximately 10 times as much ECE-1 activity as the crude membrane fractions prepared from vascular endothelial cells [3,15], and the membranes prepared from the mock-transfected cells showed substantially no ECE-1 activity. Thus, the membrane extracts of pME18SHECE1A/B-transfected CHO-K1 cells seem to be an appropriate ECE-1 source for a study of the processing mechanism of ECE-1. In the present study, the two big ET-1 analogues, [Phe^{21}]big ET-1(18–34) and [Ala^{31}]big ET-1(18–34), in which the amino acid residue of big ET-1(18–34) was replaced either at the P1 position (Phe^{21}) or at the C-terminal region (Ala^{31}), showed an inhibitory activity against ECE-1, although the affinity of these inhibitors to ECE-1 was 5–8 times less than that of big ET-1(1–38). [Phe^{21}]big ET-1(18–34) behaved as a competitive inhibitor, while [Ala^{31}]big ET-1(18–34) acted as a non-competitive inhibitor. The difference in the inhibitory fashion between these two analogues may suggest that Phe^{21} at the P1 position interacts with the processing center of ECE-1 while Ala^{31} in the C-terminal region interacts with a site indispensable for the ECE-1 activity, but which is different from the processing center. It seems that phosphoramidon also interacts with the processing center, because it has been shown to be a competitive inhibitor of ECE-1 [20]. Morita et al. [17] reported the inhibitory activity of [D-Val^{22}] big ET-1(16–38) using the crude extract of bovine aortic endothelial cells, and showed an 85% inhibition of ECE by the addition of 1 mM [D-Val^{22}] big ET-1(16–38) when assayed using big ET-1(1–38) at 1 μM. However, the inhibitory activity of [D-Val^{22}] big ET-1(16–38) was far less potent than that of [Phe^{21}]big ET-1(18–34) or [Ala^{31}]big ET-1(18–34) (Fig. 1). The [Ala^{31}]big ET-1(18–34) demonstrated in the present study may be a useful inhibitor for the ECE-1 study, because this analogue was not substantially hydrolyzed by ECE-1.

In conclusion, we demonstrated that a modification of amino acid residues at the P1 position and the C-terminal region of big ET-1 produced effective inhibitors of ECE-1, while the big ET-1 analogues synthesized in this way exhibited a different manner of inhibition, suggesting that ECE-1 recognizes big ET-1 at the two sites. More detailed analyses by further modifying the big ET sequence in combination with site-directed mutagenesis of ECE-1 would be expected to clarify the processing mechanism of this unique enzyme.

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