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Endothelin-converting enzyme activity in human serum lipoprotein fraction

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Endothelin-1 (ET-1)-converting enzyme (ECE) activity in the human serum lipoprotein fraction was studied using a sensitive enzyme immunoassay and reverse-phase high performance liquid chromatography. The ECE activity of cleaving synthetic human big ET-1 into ET-1 by the serum lipoprotein fraction was about 14-times greater than that by whole serum, and the activity was closely associated with lipoprotein itself. The lipoprotein ECE activity, which was optimal at pH 7.0, was inhibited by EDTA, *o*-phenanthroline, phosphoramidon, thiorphan, phenylmethanesulfonyl fluoride and chymostatin, but not by cysteine or aspartic proteinase inhibitors, suggesting metalloproteinase- and chymotrypsin-like properties. These results suggest that the serum lipoprotein ECE may be involved in the processing of big ET-1 to ET-1 in the circulatory system.

Endothelin; Lipoprotein; Endothelin converting enzyme

1. INTRODUCTION

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide with 21 amino acid residues originally isolated from the culture supernatant of porcine aortic endothelial cells (ECs) [1]. Human ET-1 is generated from a 38-residue intermediate form, termed big ET-1, through a specific cleavage at the Trp^{21}–Val^{22} bond by a putative ET-converting enzyme(s) (ECE). Since the vasoconstrictor activity of big ET-1 is much lower than that of ET-1 [2], the conversion of big ET-1 to ET-1 in vivo is essential for physiological functions of ET-1.

It was initially suggested that ECE could be a chymotrypsin-like proteinase(s). Indeed, chymotrypsin has been shown to generate ET-1 from big ET-1 [3]. Recently, several groups have reported that ECE present in cultured ECs [4,5] and vascular smooth muscle cells [6] was a phosphoramidon-sensitive neutral metalloproteinase(s). It has been shown that intravenous injection of big ET-1 to anesthetized rats produced a pressor effect similar to ET-1, and this effect was inhibitable with phosphoramidon [7–10]. These findings suggest that phosphoramidon-sensitive ECE is responsible for the conversion of big ET-1 to ET-1 in vivo, however, it remains unknown whether conversion of big ET-1 to ET-1 in vivo is due to membrane-bound enzyme(s) or circulating one(s). Therefore, the present study was aimed at elucidating whether human serum has any ECE activities, and to characterize serum components responsible for ECE activity.

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Abbreviations: ET-1, endothelin-1; big ET-1, big endothelin-1; IR-ET-1, immunoreactive endothelin-1; ECE, endothelin converting enzyme.

2. MATERIALS AND METHODS

2.1. *Materials*

Synthetic ET-1 (1–21), ET-2 (1–21), ET-3 (1–21), human big ET-1 (1–38), human big ET-2 (1–37), human big ET-2 (1–38) and human big ET-3 (1–41, amide) were purchased from Peptide Institute Inc. (Osaka, Japan), sandwich enzyme immunoassay (EIA) kits for ET-1/ET-2 and for ET-3 from IBL Co. (Gunma, Japan), dextran sulphate (molecular weight 500,000 Da) from WAKO Pure Chemical Co. (Osaka, Japan), EDTA, phosphoramidon, chymostatin and E-64 from Boehringer-Mannheim GmbH (Mannheim, Germany), thiorphan, pepstatin A and captopril from Sigma Chemical Co. (St. Louis, USA), phenylmethanesulfonyl fluoride (PMSF) and *N*-methylmaleimide from Nacalai Tesque Inc. (Kyoto, Japan), 1,10-phenanthroline (*o*-phenanthroline) from Merck Co. (Rahway, USA), Sep-Pak C_{18} cartridge from Waters Assoc. (Milford, USA), NAP-5 column, heparin-Sepharose CL-6B and blue-Sepharose CL-6B from Pharmacia Co. (Uppsala, Sweden), and goat anti-lipoprotein ($\alpha + \beta$) antiserum from Nordic Immunological Laboratories (Tilburg, The Netherlands).

2.2. *Isolation of lipoprotein fraction*

Serum was obtained from 6 healthy male donors 4 h after a meal, and a density fraction up to 1.210 g/ml of serum was prepared by ultracentrifugation. Briefly, the solvent density of serum was adjusted to 1.210 g/ml by adding solid KBr. After adjusting the pH to 7.0, the solution was centrifuged at 100,000 × *g* for 20 h at 4°C. The lipoprotein fraction was removed and dialysed against 150 mM NaCl/10 mM Tris-HCl buffer (pH 7.0). Determination of protein concentration was performed by the modified method of Lowry et al.

2.3. *Precipitation of lipoprotein*

A solution of 10% dextran sulfate and/or 1 M $MnCl_2$ was added to 0.5 ml serum lipoprotein fraction so as to provide final concentrations of 0.65% and 0.2 M, respectively. After leaving at room temperature for 2 h, the lipoprotein precipitate was removed by centrifugation at

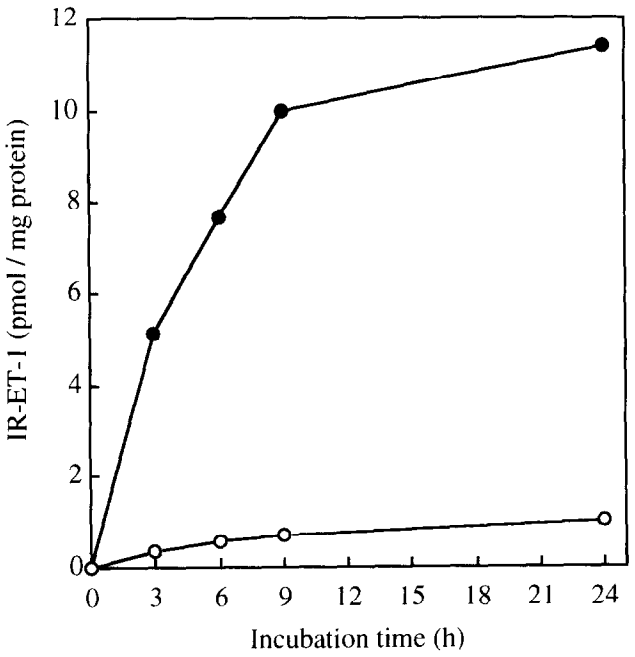

Fig. 1. Conversion of big ET-1 to ET-1 by human serum and lipoprotein fraction. Dialysed lipoprotein fraction (●) or dialysed serum (○) (0.5 ml) was incubated with big ET-1 (0.5 nmol) at 37°C for the indicated times.

20,000 × *g* for 30 min at 4°C. The resulting supernatant (0.5 ml) was subjected to a NAP-5 column equilibrated with 150 mM NaCl/10 mM Tris-HCl buffer (pH 7.0), and eluted with the same buffer.

2.4. *Measurement of ECE activity*

Big ET-1 (1 nmol/ml) was incubated with the lipoprotein fraction in 150 mM NaCl/10 mM Tris-HCl buffer (pH 7.0) at 37°C for 6 h. The reaction was stopped by adding of 2 mM EDTA. Immunoreactive (IR) ET-1 generated during incubation was measured. IR-ET-1 generated by the whole serum was extracted with a Sep-Pak C_{18} cartridge as described [11].

2.5. *Sandwich enzyme immunoassay (EIA)*

EIA for ET-1/ET-2 was performed by a sandwich EIA kit using anti-ET-1 (15–21) (rabbit IgG) and anti-ET-1 (rabbit IgG Fab'-horseradish peroxidase labeled). EIA for ET-3 was performed by a sandwich EIA kit using anti-ET-1 (15–21) (rabbit IgG) and anti-ET-3 (rabbit IgG Fab'-horseradish peroxidase labeled). These assay kits are sensitive enough to detect as little as 10 pg/ml; the cross-reactivities of the antibodies with big EG-1, big ET-2 or big ET-3 in each EIA were less than 0.1%.

2.6. *Reverse-phase high performance liquid chromatography (RP-HPLC)*

RP-HPLC was performed as described [12] using a Capcell-Pak C_{18}-SG120 column (4.6 × 250 mm, Shiseido, Tokyo, Japan). Fractions were evaporated and assayed for IR-ET-1.

3. RESULTS AND DISCUSSION

As shown in Fig. 1, incubation of synthetic human big ET-1 with human serum and the lipoprotein fraction (density fraction <1.210 g/ml) resulted in a time-dependent increase in IR-ET-1, which reached a plateau after 9 h. The amounts of IR-ET-1 generated by the lipoprotein fraction were about 14-fold greater than those by the serum. Characterization of IR-ET-1 material in the reaction mixture of big ET-1 with the lipoprotein fraction by RP-HPLC revealed that a major peak of IR-ET-1 eluted at the position of synthetic ET-1, while a minor peak corresponding to human big ET-1 was also observed (Fig. 2). These data suggest that the ECE activity in human serum was mainly concentrated in the lipoprotein fraction.

To ascertain whether lipoprotein itself had any ECE activities, serum lipoprotein was removed by precipitation with dextran sulphate and $MnCl_2$ to prepare lipoprotein-free supernatant, while lipoprotein-containing supernatant was obtained by adding either one of them to the lipoprotein fraction [13]. Human big ET-1 was incubated with the lipoprotein-containing and the lipoprotein-free supernatant and the IR-ET-1 generated was measured by a sandwich EIA for ET-1. As shown in Fig. 3, the ECE activity was negligible in the lipoprotein-free supernatant, whereas the ECE activity remained unchanged in the lipoprotein-containing supernatant after treatment with either dextran sulphate or $MnCl_2$ alone. We also found that the ECE activity of the lipoprotein fraction was adsorbed on heparin-Sepharose CL-6B, blue-Sepharose CL-6B and anti-human lipoprotein ($\alpha + \beta$) IgG-Sepharose 4B (data not shown), indicating that the ECE activity in the lipoprotein fraction is closely associated with lipoprotein itself.

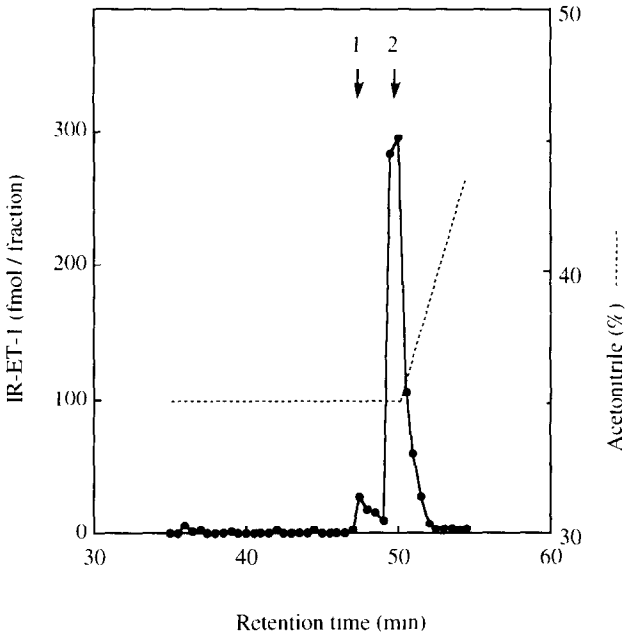

Fig. 2. Reverse-phase HPLC profile of immunoreactive (IR)-ET-1 after incubation of big ET-1 with serum lipoprotein fraction. The lipoprotein fraction (11.6 mg protein) was incubated with big ET-1 (1 nmol) for 6 h at 37°C. Amounts of IR-ET-1 in each fraction are shown by filled circles and the gradient of acetonitrile is shown by a dotted line. Arrows indicate the elution positions of synthetic human big ET-1 (1) and ET-1 (2).

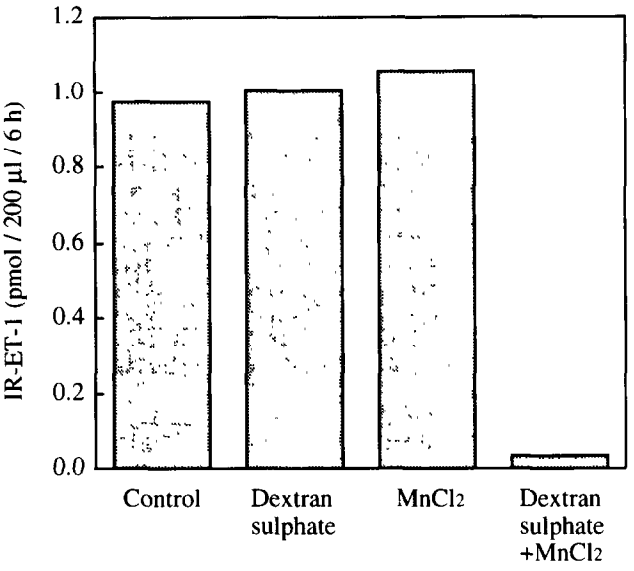

Fig. 3. The ECE activity of lipoprotein-containing and lipoprotein-free supernatant. The lipoprotein-containing supernatant after treatment with either dextran sulphate or $MnCl_2$, and the lipoprotein-free supernatant after precipitation with dextran sulphate and $MnCl_2$ (200 μl) were incubated with big ET-1 (0.2 nmol) for 6 h at 37°C. Each column represents the mean of two experiments.

We next examined enzymatic properties of the ECE activity of serum lipoprotein. The pH profile of the lipoprotein ECE activity is shown in Fig. 4. The effect of various proteinase inhibitors on the lipoprotein ECE activity were studied (Table I). The lipoprotein ECE

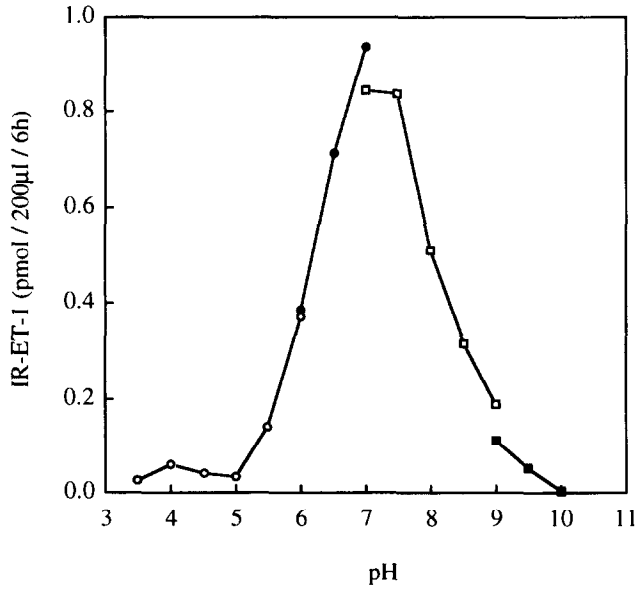

Fig. 4. The pH profile of the ECE activity of serum lipoprotein. The lipoprotein fraction (0.37 mg protein) was incubated big ET-1 (0.2 nmol) in 50 mM of different buffers for 6 h at 37°C. Buffers used were sodium-acetate, pH 3.5–6.0 (○), PIPES-NaOH, pH 6.0–7.0 (●), Tris-HCl, pH 7.0–9.0 (□) and sodium bicarbonate, pH 9.0–10.0 (■). Each point is the mean of two experiments.

activity was inhibited by EDTA, *o*-phenanthroline and metalloproteinase inhibitors (phosphoramidon, thiorphan) and serine proteinase inhibitors (PMSF, chymostatin). Captopril had a slight inhibitory effect, but cysteine proteinase inhibitors (*N*-ethylmaleimide, E-64) or aspartic proteinase inhibitor (pepstatin A) had no effect.

Our data suggest that the lipoprotein ECE activity was sensitive not only to metalloproteinase inhibitors but also to serine proteinase inhibitors. Recently, Wypij et al. [14] reported that there were two pertinent ECE activities, a chymotrypsin-like proteinase (chymase) and a phosphoramidon-sensitive metalloproteinase, in the lung particulate fraction of the rat. Since the lipoprotein fraction is composed of heterogeneous molecules, it seems possible that the ECE in the lipoprotein fraction may consist of a mixture of metalloproteinase- and chymotrypsin-like proteinase. Alternatively, the ECE in the lipoprotein fraction may be a single enzyme with both metalloproteinase- and chymotrypsin-like proteinase properties. It should be noted that human apolipoprotein (a) contains a plasminogen-like serine proteinase domain within the molecule [15], the proteolytic activity of which is inhibitable with PMSF, but not with *o*-phenanthroline [16].

The lipoprotein ECE did not significantly cleave big ET-2 (1–37) or big ET-2 (1–38) into ET-2, as measured by EIA for ET-1/ET-2, whereas its converting activity for big ET-3 as measured by EIA for ET-3 was one-third as much as that for big ET-1 (data not shown). Although the amino acid sequence of the cleavage site (Trp^{21}–Val^{22}) of big ET-2 (1–37) and big ET-2 (1–38) is identical to that of big ET-1, the possible cleavage site (Trp^{21}–Ile^{22}) of big ET-3 is different from that of big ET-1 and big ET-2. However, the C-terminal region of big ET-3 ($\underline{Gly}^{38}$-$\underline{Ser}^{39}$-Phe^{40}-$\underline{Arg}^{41}$) is very similar to that of big ET-1 ($\underline{Gly}^{34}$-$\underline{Ser}^{35}$-Pro^{36}-$\underline{Arg}^{37}$). It is possible to

Table I

Effects of various proteinase inhibitors on ECE activity of serum lipoprotein

Inhibitor	Concentration (mM)	Inhibition (%)
EDTA	1.0	100
o-Phenanthroline	0.1	50
	0.5	98
Phosphoramidon	0.1	96
Thiorphan	0.1	95
PMSF	0.1	29
	1.0	84
Chymostatin	0.01	79
E-64	0.1	0
Captopril	0.1	32
N-Ethylmaleimide	1.0	4
Pepstatin A	0.01	0

The lipoprotein fraction (0.28 mg protein) was incubated with big ET-1 (0.2 nmol) for 6 h at 37°C. The amount of IR-ET-1 generated during 6 h in the absence of proteinase inhibitors was 1.39 pmol.

speculate that the C-terminal region (34–37) of big ET-1 may be the recognition site by the lipoprotein ECE. A similar finding has been reported on the phosphoramidon-sensitive ECE from bovine ECs [17], showing that the C-terminal region (Gly^{32}-Leu^{33}-Gly^{34}-Ser^{35}-Pro^{36}-Arg^{37}) of big ET-1 is important for conversion by the endothelial ECE.

Recently, it has been reported that patients with atherosclerosis had raised plasma IR-ET-1 levels [18], and that plasma IR-ET-1 levels showed positive correlation with the extent of the atherosclerotic vascular lesions [19]. Furthermore, it has been reported that the plasma IR-ET-1 concentration showed positive correlation with plasma total cholesterol levels, and administration of clinofibrate reduced plasma IR-ET-1 levels in hypercholesterolemic rat [20]. Therefore, it is possible to postulate that the enhanced activity of the lipoprotein ECE in hypercholesterolemia may increase circulating IR-ET-1 levels. The question as to which fraction of the serum lipoprotein has ECE activity, and whether the lipoprotein ECE is involved in the development and/or progression of atherosclerosis, need to be answered.

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