

# Phosphoramidon-sensitive endothelin-converting enzyme in vascular endothelial cells converts big endothelin-1 and big endothelin-3 to their mature form

Yasuo Matsumura, Yaeko Tsukahara, Kazuko Kuninobu, Masanori Takaoka and Shiro Morimoto

*Department of Pharmacology, Osaka University of Pharmaceutical Sciences, 2-10-65 Kawai, Matsubara, Osaka 580, Japan*

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Incubation of big endothelin-3 (big ET-3<sub>1-41</sub>) with the membrane fraction obtained from cultured endothelial cells (ECs) resulted in an increase in immunoreactive-ET (IR-ET). This increasing activity was markedly suppressed by phosphoramidon, which is known to inhibit the conversion of big ET-1<sub>1-39</sub> to ET-1<sub>1-21</sub>. Reverse-phase HPLC of the incubation mixture of the membrane fraction with big ET-3 revealed one major IR-ET component corresponding to the elution position of synthetic ET-3<sub>1-21</sub>. When the cultured ECs were incubated with big ET-3, a conversion to the mature ET-3, as well as an endogenous ET-1 generation, was observed. Both responses were markedly suppressed by phosphoramidon. By the gel filtration of 0.5% CHAPS-solubilized fraction of membrane pellets of ECs, the molecular mass of the proteinase which converts big ET-1 and big ET-3 to their mature form was estimated to be 300–350 kDa. Phosphoramidon almost completely abolished both converting activities of the proteinase. We conclude that the above type of phosphoramidon-sensitive metalloproteinase functions as an ET-converting enzyme to generate the mature form from big ET-1 and big ET-3 in ECs.

Endothelin-1; Endothelin-3; Big endothelin-1; Big endothelin-3; Metalloproteinase; Phosphoramidon.

## 1. INTRODUCTION

Endothelin-1 (ET-1<sub>1-21</sub>) is produced from a 39-amino acid inactive intermediate form, termed big ET-1<sub>1-39</sub>, through unusual proteolytic processing at the Trp<sup>21</sup>–Val<sup>22</sup> bond by a putative ET-converting enzyme (ECE) [1]. We have proposed that the phosphoramidon-sensitive metalloproteinase is the most plausible candidate for ECE in vascular endothelial cells (ECs) [2–5] and smooth muscle cells [6]. Findings that the pressor activity of big ET-1 (probably by conversion to ET-1) is effectively suppressed by phosphoramidon [7–10], strongly support a functional role of the above type of proteinase as a physiologically relevant ECE.

Subsequent to the original discovery of ET-1 [1], an analysis of a human genomic library showed the possible existence of three structurally distinct isopeptides, termed ET-1, ET-2 and ET-3 [11]. Several studies have indicated the presence of immunoreactive (IR)-ET-1, IR-ET-2 and IR-ET-3 in mammalian tissues [12–14]. Based on findings that ET-2 and ET-3 are derived from big ET-2<sub>1-37</sub> and big ET-3<sub>1-41</sub>, as deduced from sequence analyses of cDNA [15–17], one can speculate on the possible biosynthetic pathway for ET-2 and ET-3 analogous to ET-1.

We report here that the phosphoramidon-sensitive

and membrane-bound metalloproteinase derived from cultured porcine aortic ECs, of which the molecular mass is estimated to be 300–350 kDa, can convert both big ET-1 and big ET-3 to their mature form.

## 2. MATERIALS AND METHODS

### 2.1. Materials

ET-1<sub>1-21</sub>, ET-3<sub>1-21</sub>, porcine big ET-1<sub>1-39</sub>, human big ET-3<sub>1-41</sub> (amide) and phosphoramidon were purchased from Peptide Institute Inc. (Osaka, Japan). [<sup>125</sup>I]ET-1 and [<sup>125</sup>I]ET-3 were obtained from Amersham, Japan.

### 2.2. Cell culture and preparation of membrane fraction

ECs isolated from fresh porcine aortas were cultured as described [18,19], and used between the 5th and 10th passages. To obtain the membrane fraction of ECs, the confluent cells were scraped with a Cell Lifter (Costar, MA). After washing with phosphate-buffered saline, the cells were homogenized in ice-cold 20 mM Tris-HCl buffer (pH 7.2) containing 5 mM MgCl<sub>2</sub>, 0.1 mM E-64 and 0.1 mM *p*-APMSF, and then the preparation was centrifuged at 105,000 × *g* for 30 min. The pellet was washed with Tris-HCl buffer, resuspended in the same buffer and the preparation used as the membrane fraction. For anion-exchange HPLC and gel filtration HPLC, the membrane pellet was resuspended in the Tris-HCl buffer containing 0.5% CHAPS. After 36 h, the suspension was centrifuged at 105,000 × *g* for 30 min, and the resulting supernatant served for HPLC. In some experiments, the cultured intact ECs grown in 60-mm gelatin-coated Petri dishes were incubated with 3 ml of serum-free Dulbecco's modified Eagle's medium containing 0.01% heat-inactivated bovine serum albumin, in the absence or presence of phosphoramidon (10<sup>−4</sup> M) and big ET-3 (70 pmol), at 37°C in a CO<sub>2</sub> incubator for 12 h. After the incubation, ETs in the medium were extracted with a Sep-Pak C<sub>18</sub> cartridge (Waters, MA), as described [3]. The eluates were subjected to reverse-phase (RP)-HPLC coupled with a radioimmunoassay (RIA) for ETs.

Correspondence address: S. Morimoto, Department of Pharmacology, Osaka University of Pharmaceutical Sciences, 2-10-65 Kawai, Matsubara, Osaka 580, Japan. Fax: (81) (723) 329 929.

### 2.3. Anion-exchange HPLC

Detergent-solubilized fractions (about 6 mg of protein) of the membrane pellet derived from about  $7.5 \times 10^7$  cells were applied to a COSMOGEL QA column ( $8 \times 75$  mm, Nakalai Tesque Ltd., Kyoto, Japan) using a Waters HPLC system (Model 650E). Elution was performed in the same manner as described [4], except that the buffer contained 0.5% CHAPS. Active fractions were subjected to a gel filtration HPLC.

### 2.4. Gel filtration HPLC

A Superdex 200 pg (HiLoad 16/60, Pharmacia) was used to estimate the molecular weight of ECE. The column was eluted with 20 mM Tris-HCl buffer (pH 7.2) containing 0.2 M NaCl and 0.5% *n*-octyl- $\beta$ -D-thioglycoside. The flow rate was 1 ml/min and 1 ml fractions were collected.

### 2.5. Measurement of ET-converting activity

ET-converting activities of the membrane fraction of ECs and those of each fraction from anion-exchange HPLC and gel filtration HPLC were determined as described [2]. 50  $\mu$ l of the sample and 0.05 ml of enzyme inhibitor solution were mixed with 0.35 ml of 50 mM sodium phosphate buffer (pH 6.5). After pre-incubation at 37°C for 30 min, 0.05 ml of big ET-1 or big ET-3 (final concentrations: 100 ng big ET-1/ml; 112 ng big ET-3/ml) was added to the mixture and the preparation was incubated at 37°C for 0.5–12 h. The reaction was stopped by boiling for 10 min. The sample were neutralized and centrifuged at 8,000  $\times$  g for 5 min. The resulting supernatant was used for the RIA and RP-HPLC.

### 2.6. Radioimmunoassay (RIA)

RIAs for ET-1 and ET-3 were performed as described [18,19]. ET-1 antiserum (a generous gift from Dr. M.R. Brown, Department of Medicine, University of California, San Diego) had a 50% cross-reactivity with ET-3 and no cross-reactivity with big ET-1 and big ET-3.

### 2.7. Reverse-phase (RP)-HPLC

RP-HPLC was performed using a Capcell-Pak 5C<sub>18</sub>-SG300 column ( $4.6 \times 250$  mm, Shiseido, Tokyo, Japan) eluted with a linear gradient from 0 to 35% CH<sub>3</sub>CN in 0.02% TFA for 15 min, followed by isocratic elution at 35% CH<sub>3</sub>CN in 0.02% TFA for 15 min and a linear gradient from 35 to 63% CH<sub>3</sub>CN in 0.02% TFA for 15 min. The flow rate was 0.5 ml/min. Each fraction was evaporated and assayed for IR-ET using RIA.

## 3. RESULTS AND DISCUSSION

When big ET-1 was incubated with the membrane fraction of cultured ECs at pH 6.5, a time-dependent increase in IR-ET in the reaction mixture was observed (Fig. 1). This increasing response was markedly inhibited by phosphoramidon, as described [2,4]. In addition, we have already confirmed by RP-HPLC that the major IR-ET component is ET-1 converted from big ET-1 [2]. Incubation of big ET-3 with the membrane fraction also increased IR-ET in a time-dependent manner (Fig. 2). The optimum pH of this increase was 6.5, which is the same value seen with big ET-1 as a substrate [2]. In the presence of  $10^{-4}$  M phosphoramidon there was no notable increase in IR-ET.

Using RP-HPLC coupled with RIA for ET, we characterized the IR-ET in the reaction mixture of big ET-3 with the membrane fraction. The elution profiles revealed one major IR-ET component corresponding to the elution position of synthetic ET-3 (Fig. 3A). The

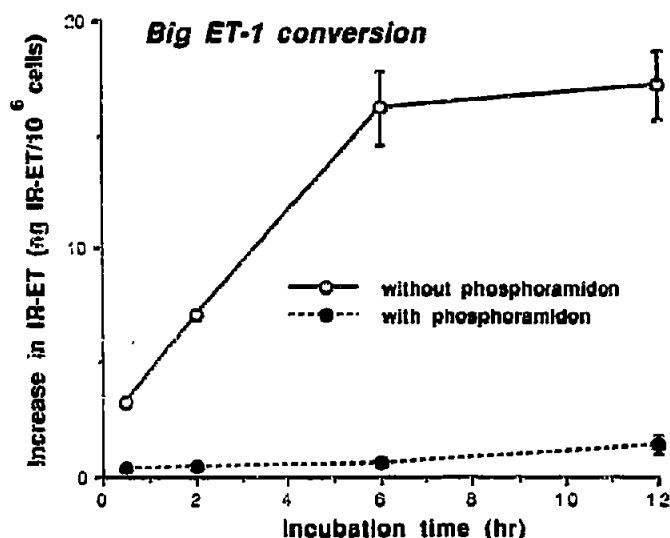


Fig. 1. Time-course of the increase in IR-ET during incubation of big ET-1 with the membrane fraction of cultured ECs (derived from  $4 \times 10^5$  cells). Incubation was performed with 1 mM *N*-ethylmaleimide, in the presence or absence of 0.1 mM phosphoramidon. Values represent the mean  $\pm$  S.E.M. from four separate experiments.

addition of phosphoramidon to the reaction mixture greatly decreased the production of ET-3-like materials (Fig. 3B).

As described [3,5], cultured ECs release ET-1 in a time-dependent manner, and the amount of ET-1 is remarkably decreased by phosphoramidon and accompanied by an increase in the release of big ET-1, thereby strongly suggesting that phosphoramidon suppresses the release of ET-1 from ECs by inhibiting ECE. In the

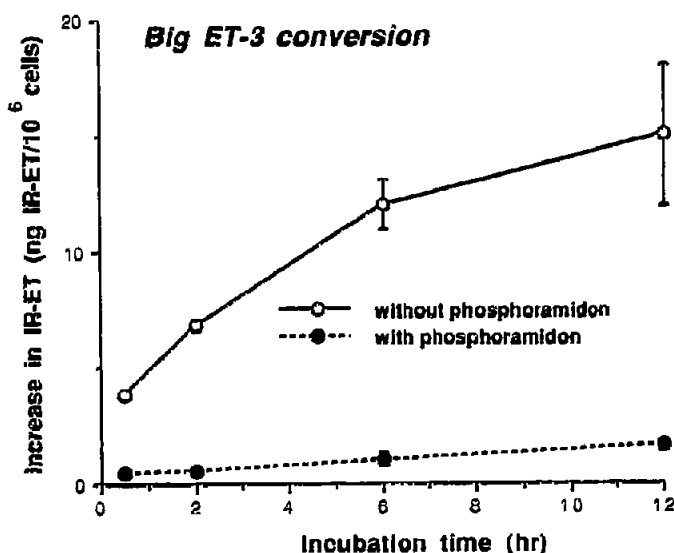


Fig. 2. Time-course of the increase in IR-ET during incubation of big ET-3 with the membrane fraction of cultured ECs (derived from  $4 \times 10^5$  cells). Incubation was performed with 1 mM *N*-ethylmaleimide, in the presence or absence of 0.1 mM phosphoramidon. Values represent the mean  $\pm$  S.E.M. from four separate experiments.

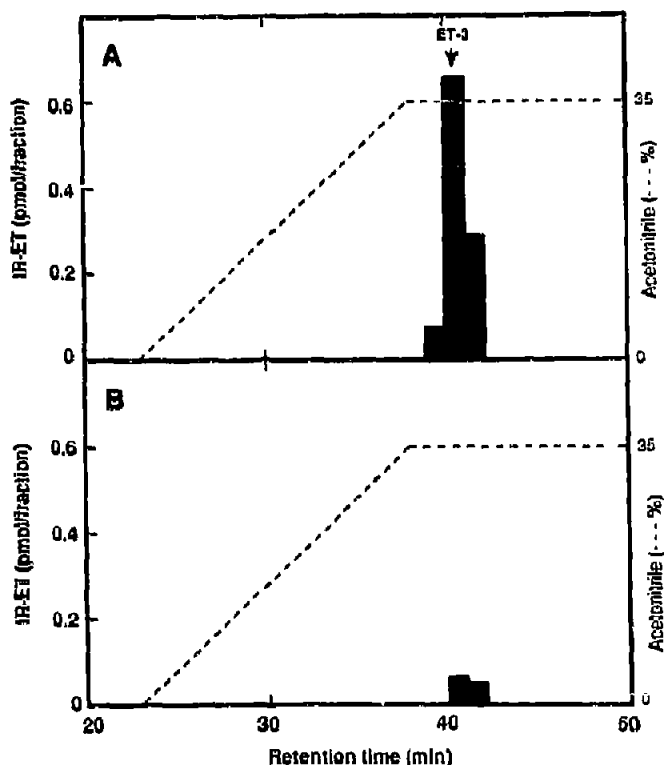


Fig. 3. RP-HPLC profiles of IR-ET in the reaction mixture of big ET-3 with the membrane fraction. The incubation was carried out at pH 6.5 for 6 h with 1 mM *N*-ethylmaleimide. A, without phosphoramidon; B, with 0.1 mM phosphoramidon. The arrow indicates the elution position of synthetic ET-3.

present study, when the intact cultured cells were incubated with big ET-3 (70 pmol/ $10^6$  cells) for 12 h, there was a gradual additional increase in IR-ET in the culture medium (data not shown). Consistent with the case of endogenous IR-ET release (derived from ET-1), phosphoramidon markedly suppressed the IR-ET increase by the exogenous application of big ET-3. Using RP-HPLC and RIA, we examined the IR-ET in the culture medium with exogenous big ET-3 application. As shown in Fig. 4A, there were two major components of IR-ET, which corresponded to elution positions of synthetic ET-1 and ET-3, respectively, thereby indicating that the additional increase in IR-ET by exogenous big ET-3 application is due to production of the mature ET-3. Phosphoramidon potently suppressed this ET-3 production, as it did the endogenous ET-1 production (Fig. 4B).

We further characterized the phosphoramidon-sensitive ECE solubilized from the membrane fraction of ECs, using anion-exchange HPLC and gel filtration HPLC. The anion-exchange HPLC of the detergent-solubilized fraction gave one major peak of big ET-1-converting activity in the eluate at 0.2 M NaCl. Qualitatively similar elution patterns were observed with big ET-3-converting activity (data not shown). Active frac-

tions obtained from anion-exchange HPLC were applied to a Superdex 200 pg column in order to estimate the molecular weight of the enzyme. As shown in Fig. 5 (upper panel), the big ET-1 converting activity was observed as a single major peak and corresponded to an apparent molecular mass of 300–350 kDa. The addition of phosphoramidon abolished the converting activity in the peak fraction, although phosphoramidon-resistant small activities were detected in later fractions. Phosphoramidon-sensitive big ET-3-converting activity was also observed as a single major peak in the same fractions as seen with big ET-1 converting activity (Fig. 5, lower panel).

There is accumulating evidence that big ET-1 is converted to ET-1 in various cells and tissues by phosphoramidon-sensitive metalloproteinase [2–6,20–27]. On the other hand, results with regard to the conversion of big ET-3 are conflicting. Okada et al. [20] reported that a membrane-bound and phosphoramidon-sensitive ET-1 converting enzyme of cultured bovine ECs is much less susceptible to big ET-3. Takada et al. [28] also found no substantial big ET-3-converting activity of cytosolic ET-1-converting enzyme obtained from cul-

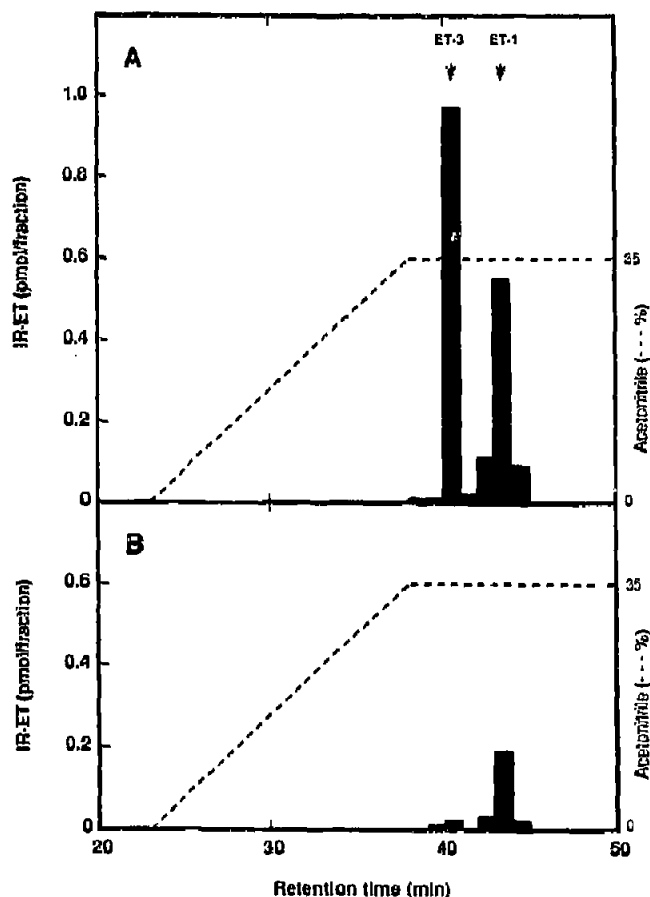


Fig. 4. RP-HPLC profiles of IR-ET in culture medium of ECs. ECs were incubated with synthetic big ET-3 for 12 h, in the absence (A) or presence (B) of 0.1 mM phosphoramidon. Arrows indicate the elution position of synthetic ET-1 and ET-3.

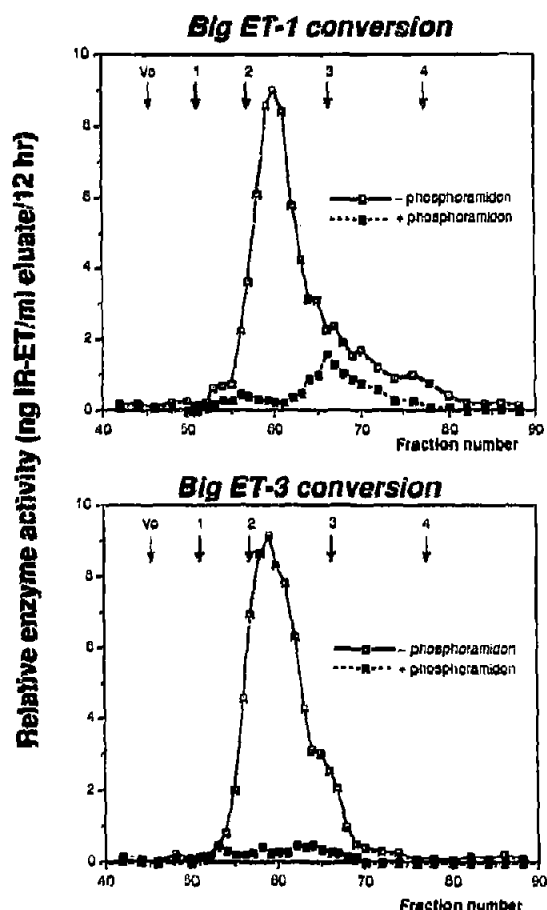


Fig. 5. Elution profiles of phosphoramidon-sensitive ECE by gel filtration on a Superdex 200 pg column. Each eluate was incubated with big ET-1 or big ET-3 in the presence of 1 mM *N*-ethylmaleimide for 12 h. Arrows indicate the elution positions of void volume (Vo) and molecular weight standards: 1, thyroglobulin (669 kDa); 2, ferritin (440 kDa); 3, catalase (232 kDa); 4, bovine serum albumin (67 kDa).

tured bovine ECs. Moreover, D'Orléans-Juste et al. [29] found that, unlike big ET-1, big ET-3 induced no pressor responses in anesthetized guinea-pigs, thereby suggesting that ECE is specific for big ET-1 and may not convert big ET-3 to the mature form. In contrast, using intact cultured bovine ECs, Ohnaka et al. [30] noted that exogenously applied big ET-3 is converted to the mature ET-3 at a high affinity and that this conversion is abolished by phosphoramidon, in the same manner as seen with big ET-1. Most recently, we noted that intravenous injection of big ET-3 (3 nmol/kg) to anesthetized rats produced a long-lasting hypertensive effect, although the magnitude was slightly less potent than that of big ET-1. The pressor response to big ET-3 was markedly attenuated by the pretreatment with phosphoramidon (5 mg/kg, i.v.), thereby suggesting that big ET-3 is converted to biologically active ET-3 by phosphoramidon-sensitive metalloproteinase *in vivo*, in the same manner as the case seen with big ET-1 (Matsumura et al., submitted). Thus, taken together

with the findings of Ohnaka et al. [30] and ours, the phosphoramidon-sensitive metalloproteinase appears to be involved at least in the extracellular conversion of big ET-3 to the mature form. The reason for the discrepancy between our findings and those previously published is unclear but may be partly due to differences in species and experimental conditions.

In the present study, we characterized for the first time the big ET-3-converting enzyme in ECs. The results clearly indicate that cultured porcine ECs contain phosphoramidon-sensitive metalloproteinase which converts big ET-3 to ET-3, as seen in the case of the conversion of big ET-1 in the same cells. Thus, big ET-3, as well as big ET-1, is a good substrate for phosphoramidon-sensitive ECE in ECs. Taken together with the chromatographic analysis, we conclude that the phosphoramidon-sensitive ECE with an apparent molecular weight of 300–350 kDa can convert both big ET-1 and big ET-3 to their mature form.

A possible relationship between an accelerated formation of ET-1 and various vascular diseases has been proposed [31,32]. Thus, a specific inhibitor of ECE may have beneficial effects in the prevention and/or treatment of certain diseases. Most recently, we found that intracisternal administration of phosphoramidon effectively suppressed the development of cerebral vasospasm following subarachnoid hemorrhage in the canine 'two-hemorrhage' model and suggested that ET-1 may be a causal factor in the cerebral vasospasm [33]. The pathophysiological significance of ET-3 remains the subject of ongoing studies.

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