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Phosphoramidon inhibits the generation of endothelin-1 from exogenously applied big endothelin-1 in cultured vascular endothelial cells and smooth muscle cells

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When cultured porcine aortic endothelial cells (ECs) were incubated with porcine big endothelin-1 (bit ET-1_{1-39}), there was a time-dependent increase in immunoreactive (IR)-ET in the culture supernatant, in addition to an endogenous IR-ET release fron the cells. Reverse-phase HPLC of the culture supernatant revealed one major IR-ET component corresponding to the elution position of synthetic ET-1, thereby indicating that the additional increase in IR-ET was due to the conversion of big ET-1 to mature ET-1_{1-21}. Phosphoramidon, a metalloproteinase inhibitor, strongly suppressed this increase in IR-ET as well as the endogenous IR-ET release. Cultured vascular smooth muscle cells (VSMCs) also released IR-ET. The apparent conversion of exogenously applied big ET-1 to ET-1 and its inhibition by phosphoramidon were observed using cultured VSMCs, although the enzyme inhibitor did not influence the basal secretion of IR-ET from VSMCs. These results suggest that both cultured ECs and VSMCs can generate ET-1 from exogenously applied big ET-1 via action of the same type of phosphoramidon-sensitive metalloproteinase, which is also involved in the endogenous ET-1 generation in ECs.

Endothelin-1; Big endothelin-1; Metalloproteinase; Phosphoramidon; Endothelial cell; Vascular smooth muscle cell

1. INTRODUCTION

Endothelin-1 (ET-1_{1-21}) is produced from a 39-amino acid intermediate form, tested big ET-1, through an unusual proteolytic processing at the Trp^{21}–Val^{22} bond by a putative ET converting enzyme (ECE) [1]. Several types of proteinases have been reported to cleave the Trp^{21}–Val^{22} bond in the big ET-1 molecule. We [2,3] and others [4] noted that cathepsin D-like aspartic proteinase may be involved in the conversion of big ET-1 in vascular endothelial cells (ECs). However, since cathepsin D cleaves not only the Trp^{21}–Val^{22} bond but also the Asp^{18}–Ile^{19} bond [5,6], the physiological relevance of the conversion by this type of enzyme is unclear. We obtained evidence for the presence of phosphoramidon-sensitive metalloproteinase which exists in a membrane-bound form and can convert big ET-1 to ET-1 via a single cleavage between Trp^{21} and Val^{22}, in cultured vascular ECs [7]. Furthermore, we found that phosphoramidon caused a decrease in ET-1 secretion and an increase in big ET-1 secretion from cultured ECs, as a result of the inhibition of ECE by phosphoramidon [8]. These findings strongly suggest that phosphoramidon-sensitive metalloproteinase is responsible for the conversion of big ET-1 in vascular ECs.

In the present study, we examined whether intact cultured ECs and vascular smooth muscle cells (VSMCs) can convert exogenously applied big ET-1 to the mature ET-1. Since results clearly revealed the generation of ET-1 from exogenous big ET-1 in both species of cells, we evaluated the effect of phosphoramidon on this generation of ET-1, and the effect was compared with that on endogenous ET-1 generation in cell species.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

ECs and VSMCs isolated from fresh porcine thoracic aortas were cultured as described [9,10]. The cells (5–10 passages) grown in 60-mm Petri dishes (ECs, about 10^6 cells; VSMCs, about 2×10^6 cells) 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^{-4} M) and big ET-1 (70 pmol), at 37°C in CO_2 incubator for 3–24 h. After the incubation, medium was aspirated off, boiled for 5 min, and the preparation was centrifuged at 8000 × *g* for 5 min. The resulting supernatant served as sample for the radioimmunoassay (RIA) and reverse-phase high performance liquid chromatography (RP-HPLC). Phosphoramidon was purchased from Peptide Institute Inc. (Osaka, Japan).

2.2. *Radioimmunoassay (RIA)*

RIAs for ET and the C-terminal fragment (CTF_{22-39}) of big ET-1 were performed as described [2,3]. ET-1 antiserum (a generous gift from Dr M.R. Brown, Department of Medicine, University of California, San Diego) did not cross-react with big ET-1, as described [11]. Antiserum to the CTF (Peptide Institute Inc., Osaka, Japan) had a 100% cross-reactivity with big ET-1 and no cross-reactivity with ET-1.

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

About 5 ml of culture supernatant after incubation for 12 h (ECs)

or 24 h (VSMCs), with or without big ET-1 and phosphoramidon, was applied to a Sep-Pak C_{18} cartridge (Waters, MA). The adsorbed materials were eluted with 3 ml of 60% CH_3CN in 0.09% trifluoroacetic acid (TFA), and evaporated in a centrifugal concentrator. The residual materials were dissolved in 0.5 ml of 0.02% TFA and 0.4 ml portions were then applied on a Capcell-Pak $5C_{18}$-SG300 column (4.6 × 250 mm, Shiseido, Tokyo, Japan) using a Waters HPLC system (Model 600E). Elution was performed using 0.02% TFA in water (solvent A) and 0.02% TFA in CH_3CN (solvent B). The gradient consisted of a linear one from 0–35 vol./vol.% solvent B for 15 min, followed by isocratic elution at 35 vol./vol.% solvent B for 15 min and a linear gradient from 35–63 vol./vol.% solvent B for 15 min. The flow rate was 0.5 ml/min. Each fraction was evaporated and assayed for immunoreactive (IR)-ET by RIA.

2.4. *Peptides*

Synthetic porcine $ET\text{-}1_{1-21}$ and big $ET\text{-}1_{1-39}$ were obtained from Peptide Institute Inc. (Osaka, Japan). The CTF_{22-39} was prepared by solid phase synthesis. The homogeneity was confirmed by RP-HPLC and by amino acid analysis.

3. RESULTS AND DISCUSSION

Cultured ECs released IR-ET and IR-CTF in a time-dependent manner (2.65 ± 0.18 pmol of IR-ET/10^6 cells/24 h and 3.05 ± 0.20 pmol of IR-CTF/10^6 cells/24 h, respectively), and the amount of IR-ET was markedly decreased by phosphoramidon (10^{-4} M). As described earlier [8], the decreased release of IR-ET observed with phosphoramidon is probably due to inhibition of ECE by the agent.

When the cells were incubated with synthetic big ET-1 (70 pmol/10^6 cells), a time-dependent additional increase in IR-ET in medium was observed (Fig. 1). With incubation for 12 h, the increase in IR-ET was 0.60 ± 0.07 pmol/10^6 cells. Consistent with the case of endogenous IR-ET release, phosphoramidon remarkably suppressed (80–90%) the IR-ET increase by exogenous application of big ET-1.

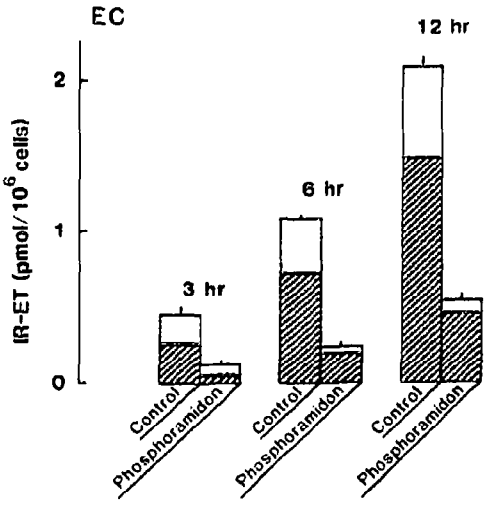

Fig. 1. Changes in IR-ET content in culture medium of ECs after incubation with or without phosphoramidon (10^{-4} M) for 3–12 h: (Hatched column) IR-ET content in the absence of the exogenous application of synthetic big ET-1 (endogenous IR-ET release); (open column) additional increase in IR-ET by the exogenous application of synthetic big ET-1. Values represent the mean ± SE from 7 separate experiments.

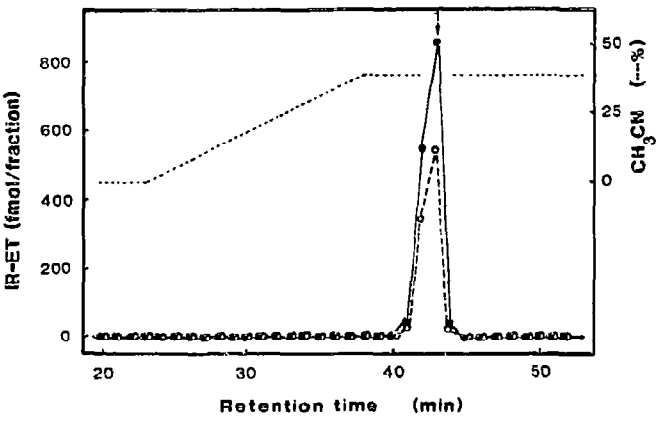

Fig. 2. RP-HPLC profiles of IR-ET in culture medium of ECs. ECs were incubated in the absence (○) or presence (●) of synthetic big ET-1 for 12 h. Arrow indicates the elution position of ET-1.

Using RP-HPLC coupled with RIA for ET, we examined the IR-ET in the culture medium, with or without exogenous big ET-1 application. As shown in Fig. 2, in both cases, the RP-HPLC profiles clearly revealed one major IR-ET component corresponding to the elution position of synthetic ET-1, thereby indicating that the additional increase in IR-ET by exogenous big ET-1 application, as well as the endogenous release of IR-ET, are due to production of the mature ET-1. Phosphoramidon is likely to suppress ET-1 production in both cases by inhibiting the conversion of big ET-1 to ET-1.

Only a small fraction of exogenously added big ET-1 can be converted to the mature ET-1. When incubating 70 pmol big ET-1 with 10^6 cells for 12–24 h, 1–2% of the added big ET-1 was converted to ET-1. On the other hand, with the same cultured cells and under the same conditions, the ratio of the amount of endogenously released ET-1 and big ET-1 was 4–9:1 on a molar basis [3,8]. If exogenously added big ET-1 is converted to ET-1 in the same manner as the endogenously generated big ET-1 is processed to the mature ET-1, 80–90% of the added big ET-1 would be converted to ET-1. By way of explanation, we assume that generation of endogenous ET-1 is an intracellular event, whereas the conversion of exogenously added big ET-1 is an extracellular one (probably via the action of ectoenzyme). Alternatively, if part of the added big ET-1 can enter the cell, a small amount of big ET-1 may be converted to ET-1 by an intracellular processing enzyme, followed by the release of generated ET-1 into the extracellular space. In addition, based on the assumption that endogenously generated big ET-1 is converted to ET-1 by an intracellular enzyme, the ECE inhibitor phosphoramidon would penetrate the plasma membrane of ECs. Further

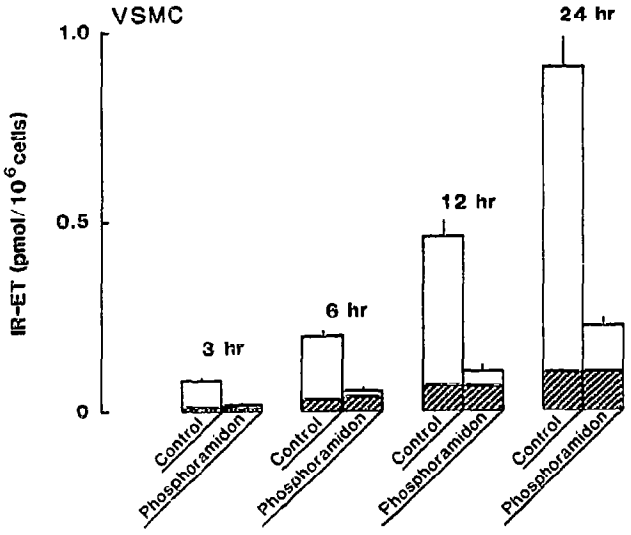

Fig. 3. Changes in IR-ET content in culture medium of VSMCs after incubation with or without phosphoramidon (10^{-4} M) for 3–24 h: (Hatched column) IR-ET content in the absence of the exogenous application of synthetic big ET-1 (endogenous IR-ET release); (open column) additional increase in IR-ET by the exogenous application of synthetic big ET-1. Values represent the mean ± SE from 10 separate experiments.

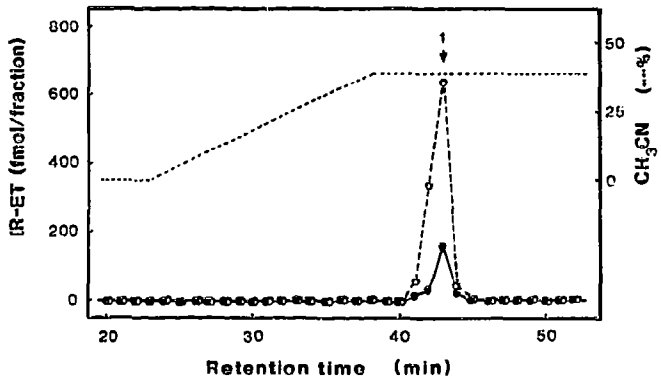

Fig. 4. RP-HPLC profiles of IR-ET in culture medium of VSMCs. VSMCs were incubated with (●) or without (○) phosphoramidon (10^{-4} M), in the presence of synthetic big ET-1 for 24 h. Arrow indicates the elution position of ET-1.

studies are underway to clarify the precise mechanisms and sites for the conversion of big ET-1 in ECs.

We also measured the amount of IR-ET and IR-CTF accumulating in culture medium of VSMCs. Cultured VSMCs released IR-ET and IR-CTF in a time-dependent manner (0.10 ± 0.01 pmol of IR-ET/10^6 cells/24 h and 0.21 ± 0.02 pmol of IR-CTF/10^6 cells/24 h, respectively), although the amounts released were considerably lower than those seen with cultured ECs. In contrast to the case of ECs, the IR-ET release from cultured VSMCs was not influenced by phosphoramidon. As shown in Fig. 3, when cultured VSMCs were incubated with synthetic big ET-1 (70 pmol/2 × 10^6 cells), the amount of IR-ET in the culture supernatant was markedly increased, in a time-dependent manner. During incubation for 24 h with big ET-1, the observed increase in IR-ET was 0.81 ± 0.08 pmol/10^6 cells. Unlike the endogenous release of IR-ET, phosphoramidon suppressed (85–95% inhibition) the increases in IR-ET observed by the exogenous application of big ET-1. Analysis of the culture supernatant of VSMCs incubated with exogenous big ET-1 by RP-HPLC revealed one major IR-ET component corresponding to the elution position of synthetic ET-1. In the case of the culture supernatant in the presence of phosphoramidon, a notable decrease in ET-1-like materials was observed (Fig. 4). These results strongly suggest that exogenously added big ET-1 is converted to the mature ET-1 by phosphoramidon-sensitive enzyme, similar to events seen with ECs. On the other hand, the IR-ET release from cultured VSMCs in the absence of big ET-1 was not influenced by phosphoramidon, thereby suggesting that endogenous ET-1 generation in cultured VSMCs is mediated by phosphoramidon-insensitive enzyme(s). Most recently, we noted that the membrane fraction of VSMCs contains 2 types of metalloproteinases, which convert big ET-1 to the mature ET-1 [10]; one is phosphoramidon-sensitive and the other is phosphoramidon- insensitive. However, since the amount of IR-ET released endogenously from cultured VSMCs was minute (about 3% of that from cultured ECs), the physiological significance of the insensitivity to phosphoramidon remains to be explored.

Resink et al. [12] stated that cultured VSMCs can express ETmRNA and secrete ET-1-like materials. They proposed an autocrine regulatory mechanism of the action for ET-1 in addition to the known paracrine function of ET-1 in the vasculature. In the present study, we found that both cultured ECs and VSMCs can generate mature ET-1 from exogenously applied big ET-1, via the action of phosphoramidon-sensitive metalloproteinase, which is also involved in the endogenous ET-1 generation in ECs [8]. We [13] and others [14,15] noted that the big ET-1-induced hypertensive action (probably by conversion to ET-1) is effectively inhibited by phosphoramidon. Moreover, we [16] obtained evidence that both the pressor response and the increase in IR-ET observed during perfusion of big ET-1 in isolated rat mesenteric artery were markedly suppressed by phosphoramidon. Taken together, phosphoramidon-sensitive metalloproteinase(s) in both ECs and VSMCs seems to be responsible for the local generation of ET-1, putatively related to pathophysiological regulation of the cardiovascular system.

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