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Effect of phosphoramidon on big endothelin-2 conversion into endothelin-2 in human renal adenocarcinoma (ACHN) cells

Analysis of endothelin-2 biosynthetic pathway

Kazuyuki Yorimitsu[a], Osamu Shinmi[b,c], Mariko Nishiyama[b], Kayoko Moroi[b], Yoshiki Sugita[c], Toshihiro Saito[a], Yoshiaki Inagaki[a], Tomoh Masaki[d] and Sadao Kimura[a]

[a]*The Third Department of Internal Medicine and* [b]*Center for Neurobiology and Molecular Immunology, School of Medicine, Chiba University, Chiba 260, Japan,* [c]*Department of Biochemistry, Institute of Basic Medical Sciences, Tsukuba University, Tsukuba 305, Japan* and [d]*Department of Pharmacology, Kyoto University School of Medicine, Kyoto 606, Japan*

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The biosynthetic pathway of endothelin (ET)-2 was analyzed in cultured ACHN cells. In the supernatant, we detected three ET-2-related peptides, ET-2, big ET-2(1–38) and big ET-2(22–38). Phosphoramidon decreased the amount of ET-2 and increased that of big ET-2(1–38) dose-dependently. The amount of big ET-2(1–37) did not significantly change. These results suggest that big ET-2 is composed of 38 and not 37 amino acid residues, and that a putative ET-2-converting enzyme (ECE-2) should be classified as a phosphoramidon-sensitive neutral metalloprotease, bearing a resemblance to the putative ET-1-converting enzyme (ECE-1) in endothelial cells.

Endothelin-2; Big endothelin-2; Endothelin-converting enzyme; Phosphoramidon; Human renal adenocarcinoma (ACHN) cell

1. INTRODUCTION

Endothelin-2 (ET-2), originally predicted from its nucleotide sequence, belongs to the endothelin peptide family [1–3]. Pharmacological studies revealed that ET-2 has similar biological activities, including vasoconstrictor activity, to ET-1, although its physiological roles are not thoroughly clarified [4]. Later, massive production of ET-2 by ACHN, a human renal adenocarcinoma cell line, was pointed out by isolation, amino acid sequence analysis and Northern blot analysis [5,6]. With respect to the biosynthetic pathway of ET-1, we have proposed that ET-1 is produced from a 39 (porcine) or 38 (human) intermediate form, big ET-1, through unusual proteolytic cleavage at Trp^{21}-Val^{22} by an ET-converting enzyme (ECE) [1,2]. The biosynthetic pathway was confirmed by finding of the related peptides, ET-1, big ET-1(1–39) and big ET-1(22–39) in the culture supernatant [7]. We have shown that the physiological importance of the conversion of big ET-1 is to increase the vasoconstrictor activity by 140-fold on cleavage to ET-1 [8]. Furthermore, we and others have shown that the intracellular conversion from big ET-1 to ET-1 in endothelial cells is markedly inhibited by a metalloprotease inhibitor, phosphoramidon, suggesting that ET-1-converting enzyme (ECE-1) is a phosphoramidon-sensitive neutral metalloprotease [9,10].

In contrast to ET-1, the native biosynthetic pathway of ET-2 in ET-2-producting cells has not been reported yet, although Kosaka et al. analyzed the biosynthetic pathway using CHO-K1 cells transfected with an expression vector containing human prepro-ET-2 cDNA [11]. They suggested that ET-2 is produced from big ET-2(1–38) and not from big ET-2(1–37), although the latter has been proposed as the intermediate form from the nucleotide sequence of cDNA encoding ET-2 [5]. Therefore, we conducted our study to clarify the native biosynthetic pathway of ET-2 and the phosphoramidon sensitivity of ET-2-converting enzyme (ECE-2) using ACHN cells. This is the first demonstration reporting the characteristic of ECE-2.

2. MATERIALS AND METHODS

2.1. *Peptides and antisera*

Human ET-1, ET-2, ET-3 and big ET-2(1–37) were obtained from Peptide Institute Inc. (Osaka, Japan). Human big ET-2(22–37) was obtained from Peninsula Laboratories Inc. (Belmont, CA). Human

Correspondence address: S. Kimura, Division of Functional Neurobiology, Center for Neurobiology and Molecular Immunology, School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260, Japan. Fax: (81) (43) 222-7853.

Abbreviations: ET, endothelin; ECE, endothelin-converting enzyme; Trp, tryptophan; Val, valine; As-ETC, anti-ET-2(15–21) antiserum; As-bET2C, anti-big ET-2(31–38) antiserum; As-ETL, anti-ET-2(1–21) antiserum; AcCN, acetonitril; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; RIA, radioimmunoassay; ir, immunoreactive.

big ET-2(1–38), big ET-2(22–38) and big ET-2(31–38) were synthesized by an automated peptide synthesizer (Applied Biosystems, Model 430A). Anti-ET-2(15–21) antiserum (As-ETC) was obtained from IBL (Tochigi, Japan). Anti-big ET-2(31–38) (As-bET2C) and anti-ET-2-(1–21) (As-ETL) antisera were produced with a similar method reported previously [12]. Briefly, big ET-2(31–38) or ET-2(1–21) was conjugated with keyhole limpet hemocyanin using the glutaraldehyde coupling procedure. As-bET2C or As-ETL was obtained by immunizing New Zealand white rabbits 5 times with the conjugates every 2 weeks. ACHN, a human renal adenocarcinoma cell line, was obtained from American Type Culture Collection.

2.2. *Analysis of culture supernatant*

ACHN cells were cultured to confluence on 90 mm diam. dishes in minimum essential medium supplemented with Earle's salts, glutamic acid, non-essential amino acids (MEM, Gibco, Grand Island, NY), 10% fetal calf serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a CO_2 incubator (95% air–5% CO_2). Then, the culture medium was changed to a serum-free medium and further incubated for 24 h. The supernatants were collected and loaded onto a Sep-Pak C18 cartridge (Waters, Milford, MA). ET-2-related peptides were eluted with 50% acetonitril (AcCN) in 0.1% trifluoroacetic acid (TFA) and separated by a reverse-phase column (Cosmosil 5C18-AR, 4.6 × 250 mm, Nacalai tesque, Kyoto, Japan) with a 37.5 min linear gradient of AcCN (12.5–50%) on 0.1% TFA at a flow rate of 1 ml/min at 40°C. Aliquots of fractions were assayed by radioimmunoassay (RIA) with three antisera (As-ETC, As-ETL, As-bET2C).

2.3. *Effect of phosphoramidon on conversion from big ET-2 to ET-2*

ACHN cells were cultured to confluence in 90 mm diam. dishes as described above. Then, the medium was changed to a serum-free medium with phosphoramidon (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M) and further incubated for 24 h. The supernatants from three dishes at each concentration were combined and loaded onto a Sep-Pak C18 cartridge and the eluates were separated on a reverse-phase column (Cosmosil 5C18-AR, 4.6 × 250 mm) with the same conditions as described above. Aliquots of fractions were submitted to RIA with the three antisera.

2.4. *RIAs for ET related peptides*

RIAs were performed similarly as described previously [12]. ET-1 and big ET-2(22–38) were radioiodinated by the chloramin T method and the monoiodinated peptides were purified by reverse-phase HPLC. Labeled ET-1 was used as a radioactive ligand for measuring ET-2 using cold ET-2 as standard. EC_{50}s of the antisera to ET-2-related peptides were as follows: EC_{50}s of As-bET2C to big ET-2(1–38) and big ET-2(22–38) were 295.3 and 198.0 fmol/tube, respectively; those of As-ETC to ET-1, ET-2, ET-3 were 310.3, 379.5, 255.7 fmol/tube, respectively; and those of As-ETL to ET-2, big ET-2(1–38), big ET-2(1–37) were 36.8, 66.2, 37.0 fmol/tube, respectively.

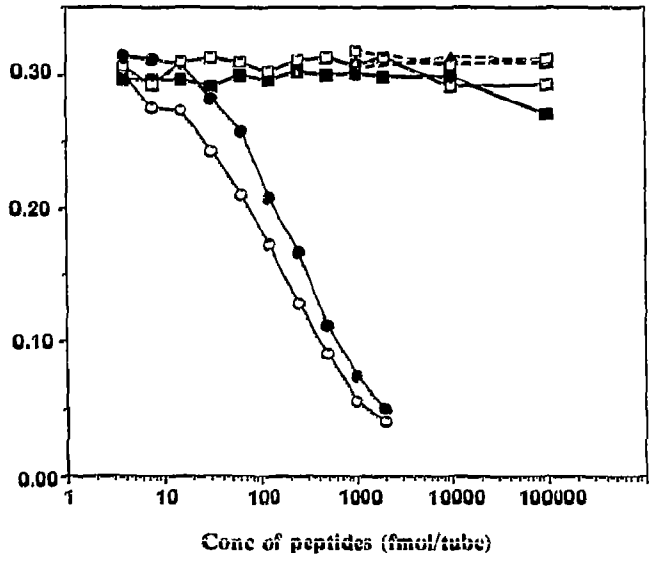

Fig. 1. Typical standard curves of big ET-2(1–38) (-●-), big ET-2(1–37) (-■-), big ET-2(22–38) (-○-), big ET-2(22–37) (-□-), ET-1 (-▲-), ET-2 (-△-) and ET-3 (-□-) in the RIA with the antiserum As-bET2C.

3. RESULTS AND DISCUSSION

3.1. *Cross-reactivities of antisera*

Typical standard curves for RIA with As-bET2C are shown in Fig. 1. As-bET2C recognized big ET-2(1–38) and big ET-2(22–38), but not big ET-2(1–37), big ET-2(22–37), ET-1, ET-2 and ET-3 (the cross-reactivity was less than 0.1%) The recognition site for As-ETL was considered to exist in the loop portion of ET-2, because As-ETL had almost equal EC_{50} values for ET-2, big

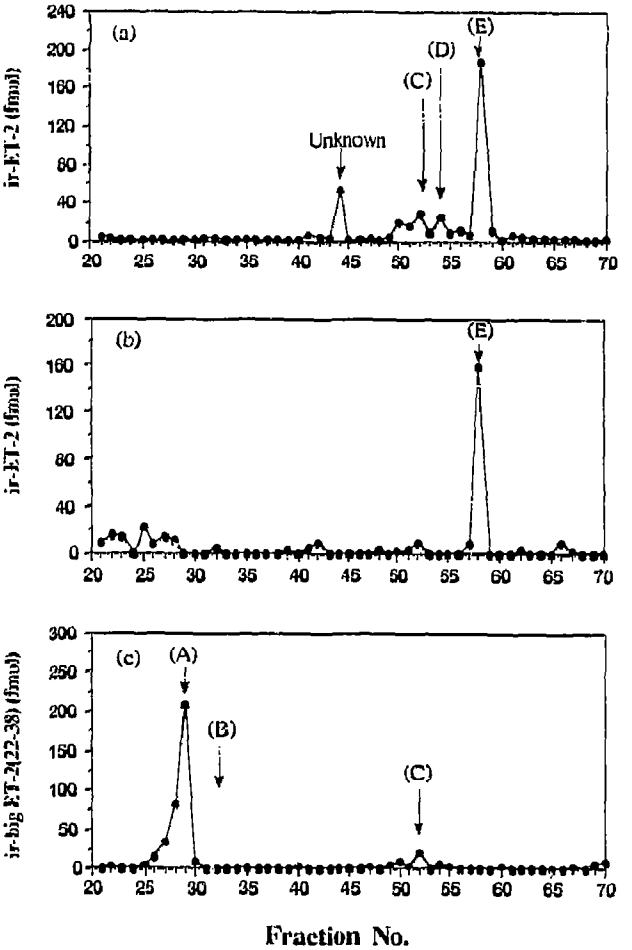

Fig. 2. Analysis of ir-ET-2-related peptides in the culture supernatant of ACHN cells. The supernatant was separated by reverse-phase HPLC as described in section 2. Ir-ET-2-related peptides were monitored by As-ETL (a), As-ETC (b) and As-bET2C (c). Arrows indicate the elution positions of synthetic standards of big ET 2(22–38) (A), big ET-2(22–37) (B), big ET-2(1–38) (C), big ET-2(1–37) (D) and ET-2(1–21) (E).

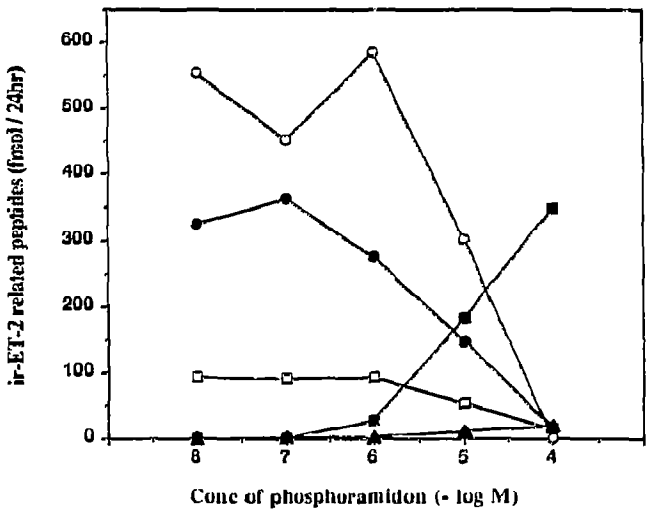

Fig. 3. Dose dependence of the effects of phosphoramidon on big ET-2 conversion. ET-2(1–21) (-●-), big ET-2(1–38) (-■-), big ET-2(22–38) (-○-), big ET-2(1–37) (-▲-) and unknown (-□-).

ET-2(1–37) and big ET-2(1–38). As-ETC recognized the C-terminal Trp moiety of ET-1, ET-2 and ET-3, and cross-reacted with big ET-2(1–38), -(1–37), -(22–38) and -(22–37) less than 0.1% (data not shown).

3.2. *ET-2 related peptides in culture supernatant of ACHN cells*

Fig. 2 showed the elution pattern of immunoreactive (ir)-ET-2-related peptides after the HPLC. Two major ir-peaks (Fraction 58 and 29) and three minor ir-peaks (Fraction 52, 54 and 44) were detected. Except Fraction 44, the retention times of Fraction 58, 29, 52 and 54 corresponded to those of synthetic peptides, ET-2(1–21), big ET-2(22–38), big ET-2(1–38) and big ET-2(1–37), respectively, agreeing with the cross-reactivities of the three antisera mentioned above. As an example, Fraction 58 (ET-2) was recognized with As-ETL and As-ETC. No ir-peak could be seen with Fraction 58 with As-bET2C, because As-bET2C cross-reacted with ET-2 less than 0.1% (Fig. 1). ACHN cells produced almost equal quantities of big ET-2(22–38) and ET-2(1–21) simultaneously into the culture media. Fraction 44 did not correspond chromatographically to any one of the ET-2-related peptides mentioned above, and seemed to be a fragment of ET-2 or big ET-2 with an N-terminal loop but not with a free COOH group of C-terminal Trp.

3.3. *Effect of phosphoramidon on ET-2 production in ACHN cells*

The ir-patterns of ET-2-related peptides in the supernatant of ACHN cells incubated with phosphoramidon were observed to be similar to those in Fig. 2. Amounts of ET-2(1–21) and big ET-2(22–38) were decreased, and that of big ET-2(1–38) was increased by phosphoramidon in a dose-dependent manner (Fig. 3). This metalloprotease inhibitor reversed the ratio of ET-2/big ET-2(1–38) at a concentration of 10^{-6}–10^{-4} M. The amount of ir-big ET-2(1–37), which has been proposed as an intermediate form from the nucleotide sequence of cDNA encoding ET-2, did not change significantly in the presence of phosphoramidon. These results clearly suggest that the intermediate form, big ET-2, is composed of 38 amino acid residues with a C-terminal arginine, and that phosphoramidon inhibits the activity of a putative ECE-2 which produces mature ET-2 from big ET-2(1–38). Although the physiological role of ET-2 has not been throughly clarified, it should be noted that ECE-2 possesses the same enzymatic characteristic as ECE-1 with regard to phosphoramidon sensitivity [10]. Recently, as with the physiologically relevant ECE-1 in endothelial cells, a phosphoramidon-sensitive neutral metalloprotease has been proposed [13]. Furthermore, it was suggested that ECE-1 can convert also big ET-3 to ET-3 [14]. It is of great interest to reveal whether or not these enzymes (ECE-1 and ECE-2) have the same characteristics with respect to substrate specificity to big ETs, and whether or not these enzymes are identical. These studies are now in progress.

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REFERENCES

[1] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature 322, 411–415.
[2] Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K. and Masaki, T. (1989) Proc. Natl. Acad. Sci. USA 86, 2863–2867.
[3] Itoh, Y., Yanagisawa, M., Ohkubo, S., Kimura, C., Kosaka, T., Inoue, A., Ishida, N., Mitsui, Y., Onda, H., Fujino, M. and Masaki, T. (1988) FEBS Lett. 231, 440–444.
[4] Doherty, A.M. (1992) J. Med. Chem. 35, 1493–1508.
[5] Ohkubo, S., Ogi, K., Hosoya, M., Matsumoto, H., Suzuki, N., Kimura, C., Onda, H. and Fujino, M. (1990) FEBS Lett. 274, 136–140.
[6] Kosaka, T., Ishibashi, Y., Matsumoto, H., Kubo, K., Kitada, C., Suzuki, N., Yasuhara, T., Onda, H. and Fujino, M. (1991) in: Peptide Chemistry 1990 (Shimonishi, Y. ed.), pp. 135–138, Protein Research Foundation, Osaka, Japan.
[7] Sawamura, T., Kimura, S., Shinmi, O., Sugita, Y., Yanagisawa, M. and Masaki, T. (1989) Biochem. Biophys. Res. Commun. 162, 1287–1294.
[8] Kimura, S., Kasuya, Y., Sawamura, T., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. and Masaki, T. (1989) J. Cardiovasc. Pharmacol. 13 (Suppl. 5), S5–S8.
[9] Ikegawa, R., Matsumura, Y., Tsukahara, Y., Takaoka, M. and Morimoto, S. (1990) Biochem. Biophys. Res. Commun. 171, 669–675.
[10] Sawamura, T., Kasuya, Y., Matsushita, Y., Suzuki, N., Shinmi, O., Kishi, N., Sugita, Y., Yanagisawa, M., Goto, K., Masaki, T.

and Kimura, S. (1991) Biochem. Biophys. Res. Commun. 174, 779–784.
[11] Kosaka, T., Ishibashi, Y., Suzuki, N., Matsumoto, H., Ohkubo, S., Kitada, C., Onda, H. and Fujino, M. (1992) in: Peptide Chemistry 1991 (Suzuki, A. ed.), pp. 19–24. Protein Research Foundation, Osaka, Japan.
[12] Shinmi, O., Kimura, S., Yoshizawa, T., Sawamura, T., Uchiyama, Y., Sugita, Y., Kanazawa, I., Yanagisawa, M., Goto, K. and Masaki, T. (1989) Biochem. Biophys. Res. Commun. 162, 340–346.
[13] Okada, K., Miyazaki, Y., Takada, J., Matsuyama, K., Yamaki, T. and Yano, M. (1990) Biochem. Biophys. Res. Commun. 171, 1192–1198.
[14] Matsumura, Y., Tsukahara, Y., Kuninobu, K., Takaoka, M. and Morimoto, S. (1992) FEBS Lett. 305, 86–90.