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CONVERSION OF PORCINE BIG ENDOTHELIN TO ENDOTHELIN BY AN EXTRACT FROM THE PORCINE AORTIC ENDOTHELIAL CELLS

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SUMMARY: Conversion of porcine big endothelin (big ET) to endothelin (ET) by an extract from cultured porcine aortic endothelial cells was investigated using a radioimmunoassay (RIA) specific for ET and reverse-phase high performance liquid chromatography (RP-HPLC). When big ET was incubated with the extract at an acid pH in the presence of E-64, a cysteine protease inhibitor, the amount of immunoreactive-ET (IR-ET) in the incubation mixture was greatly increased and the optimum pH for this increased reaction was 4.0. The extract-induced increase in IR-ET was completely inhibited by pepstatin-A. These immunoreactive alterations correlated with those in the vasoconstrictor activity. When the incubation mixture of big ET with the cell extract was applied to the RP-HPLC, the IR-ET eluted at the same retention time as seen with synthetic porcine ET. We suggest that a pepstatin-sensitive aspartic protease is involved in the conversion of big ET to ET in vascular endothelial cells.

Endothelin (ET), a highly potent vasoconstrictor peptide with 21-amino acid residues, has been identified in the culture supernatant of porcine aortic endothelial cells (EC) (1). In addition to the potent vasoconstrictor effect, various pharmacological actions of synthetic ET have been reported (2-5).

Sequence analysis of porcine cDNA encoding ET revealed the existence of a 203-residue prepro-form (1). From the deduced amino acid sequence, Yanagisawa et al. (1) proposed the possible biosynthetic pathway for the production of the 21-amino acid mature ET, i.e., the prepro-form is initially processed by dibasic pair specific proteolysis to produce a 39-amino acid intermediate form, termed big ET, and the big ET is converted to the mature form by an unusual proteolytic processing between Trp^{21} and Val^{22}. Most recently, the carboxy-terminal fragment of big ET (22-39) as well as ET and big ET, have been detected in the culture supernatant of EC (6,7), thereby suggesting that the mature ET is generated from big ET by a putative big ET converting enzyme in EC. Since the vasoconstrictor activity of big ET is much lower than that of ET (8,9), the conversion from big ET to ET appears to be essential for the pathophysiological significance of ET. In this study, we investigated the converting activity of the EC extract. Based on findings that the conversion

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of big ET to ET by the EC-extract occurs at an acid pH and is specifically inhibited by pepstatin-A, an aspartic protease may participate in the processing of big ET in vascular EC.

MATERIALS AND METHODS

Cell Culture and Preparation of the EC-Extract: Fresh porcine thoracic aortas were obtained from an abattoir. EC were isolated by gentle scraping of the intimal surface of the aortas with a scalpel blade. The cells were grown in 60-mm gelatin-coated petri dishes (Iwaki Glass, Osaka, Japan) at 37°C in a CO_2 incubator (95% air-5% CO_2) in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum and 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. EC were identified by the typical phase contrast "cobblestone" morphology and by immunofluorescence to Factor VIII related antigen. To obtain the EC-extract, the confluent cells after 4 to 7 passages were scraped with a Cell Lifter (COSTAR, MA). After washing with phosphate buffered saline, the cells were solubilized in 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM $MgCl_2$, 30 mM KCl and 0.5% Nonidet P-40, a nonionic detergent, then the cell suspension was centrifuged at 40,000 x g for 30 min. The resulting supernatant was used as the EC-extract.

Reaction of the EC-Extract with Big ET or ET: Fifty μl of the EC-extract (derived from 2 x 10^5 cells) and 0.05 ml of enzyme inhibitor solution were mixed with 0.35 ml of 0.05 M citrate buffer (pH 3.0 to 7.0). After preincubation at 37°C for 30 min, 0.05 ml of porcine big ET or ET solution (final concentrations: 400 ng big ET/ml, 40 ng ET/ml) was added to the mixture and the preparation incubated at 37°C for 0.5 to 24 hr. The reaction was stopped by boiling for 10 min. The samples were neutralized with 1 M NaOH and centrifuged at 8,000 x g for 5 min. The resulting supernatant was diluted and served as samples for the radioimmunoassay (RIA), bioassay and reverse-phase high performance liquid chromatography (RP-HPLC). Porcine big ET (1-39) and ET (ET-1) were obtained from Peptide Institute Inc. (Osaka, Japan). Enzyme inhibitors used were E-64 (cysteine protease inhibitor), pepstatin-A (aspartic protease inhibitor), (p-amidinophenyl)methanesulfonyl fluoride hydrochloride (p-APMSF, serine protease inhibitor), chymostatin (chymotrypsin inhibitor) and phosphoramidon (metalloprotease inhibitor).

Radioimmunoassay (RIA): The RIA for ET was performed as described (10), with some modifications. Antiserum to porcine ET and [^{125}I]-ET were obtained from Peptide Institute Inc. (Osaka, Japan) and Amersham Japan Ltd, respectively. The standard buffer of RIA was 0.05 M sodium phosphate buffer, pH 7.4, containing 0.05 M NaCl, 0.1% bovine serum albumin, 0.1% Triton X-100, and 1 mM EDTA-2Na. A mixture of 0.1 ml each of standard porcine ET or sample, assay buffer and antiserum (final dilution of 1:12,000) was incubated at 4°C for 24 hr, followed by the addition of 0.1 ml of [^{125}-I]-ET (approximately 10,000 cpm). After 48 hr incubation at 4°C, 0.1 ml of goat anti-rabbit γ-globulin (Organon Teknika Corp.-Cappel Products, PA) and 0.3 ml of 16.5% polyethylene glycol were added to the mixture and the preparation was further incubated at 4°C for 4 hr. Bound and free ligands were separated by centrifugation at 3,000 rpm for 30 min. The bound radioactivity was counted in a gamma spectrometer (Model ARC-301, Aloka Co., Ltd., Tokyo, Japan).

Bioassay: The vasoconstrictor activity for the reaction mixture of the EC-extract and porcine big ET was examined using helically cut strips of rat thoracic aortas, as described (10).

Reverse-Phase High Performance Liquid Chromatography (RP-HPLC): Samples were passed through a Millipore filter (0.22 μm); 0.4 ml portions were then applied on a Cosmosil 5C18-300 (4.6 x 250 mm, Nakalai Tesque Ltd., Kyoto, Japan) using a Waters HPLC system (Model 600E). Elution was performed by using 0.02% trifluoroacetic acid (TFA) in water (solvent A) and 0.02% TFA in acetonitrile (solvent B). The gradient consisted of a linear one from 0 to 36 vol/vol% solvent B in 15 min, followed by isocratic elution at 36 vol/vol% solvent B for 30 min and a linear gradient from 36 to 90 vol/vol% solvent B in 15 min. The flow rate was 0.5 ml/min.

RESULTS AND DISCUSSION

A typical standard curve of porcine ET and the cross-reactivity with porcine big ET in the RIA are shown in Fig. 1. The lower limit of sensitivity of the assay was 3 pg/tube and half maximal displacement was 80 pg/tube. Porcine big ET below 10^3 pg/tube was not detected in this RIA, although extremely high concentrations of big ET were recognized (cross-reactivity: <0.1%). Using this RIA system, we first measured the amount of immunoreactive ET (IR-ET) in the reaction mixture of big ET and the EC-extract. The assay sample was diluted so that big ET would be negligible in the RIA. In addition, we determined the IR-ET content after incubation of synthetic porcine ET (ET-1) with the EC-extract to check degradation of ET by the EC-extract. As shown in Fig. 2A, when the incubation of ET with the EC-extract was carried out at pH 7.0, no appreciable alteration in IR-ET content was observed, in the presence or absence of enzyme inhibitors, although the IR-ET slightly decreased by incubation with the EC-extract. Thus, ET appears to be

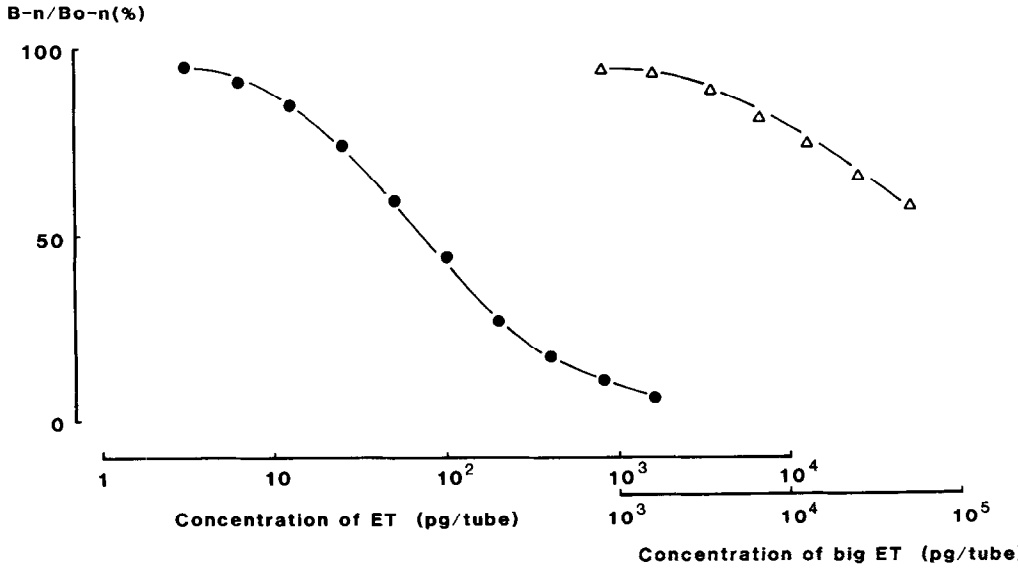

Figure 1. A typical standard curve of porcine ET (●) and a cross-reactivity with porcine big ET (△) in RIA.

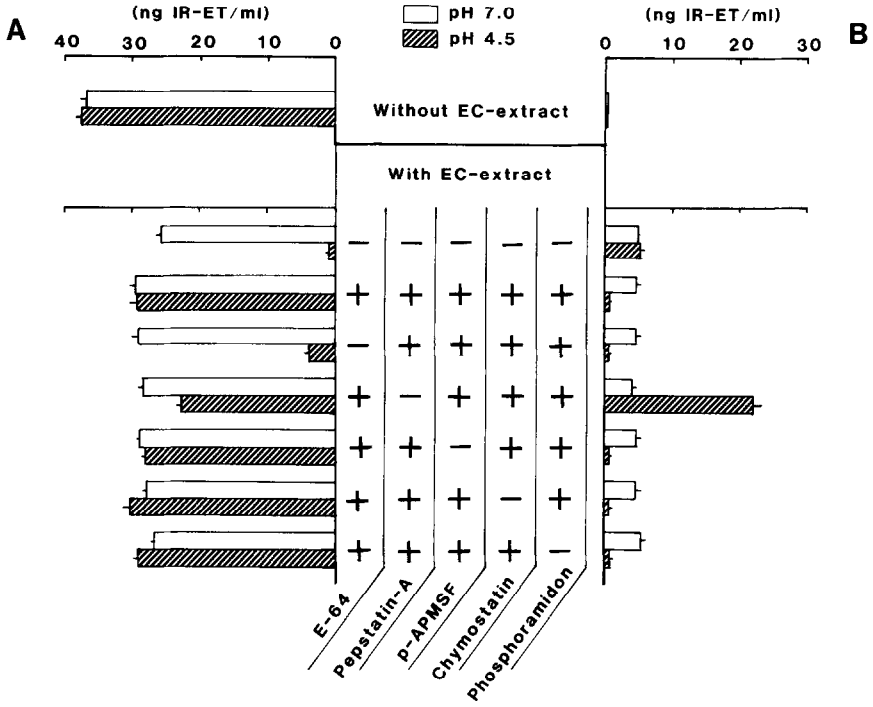

Figure 2. Changes in IR-ET content in the reaction mixture of the EC-extract with ET (A) or big ET (B). The incubation was carried out at 37°C for 6 hr in the absence or presence of enzyme inhibitors (E-64, 10^{-4} M; pepstatin-A, 10^{-7} M; p-APMSF, 10^{-4} M; chymostatin, 2×10^{-6} M; phosphoramidon, 10^{-5} M). The EC-extract alone had no ET immunoreactivity. Each column and bar represents the mean ± S.E. from four separate experiments.

relatively stable at neutral pH; this may be due to the structural characteristics of ET, as suggested (4). At pH 7.0, the IR-ET content in the reaction mixture of big ET and the EC-extract increased slightly but there was no difference in the presence or absence of enzyme inhibitors (Fig. 2B). On the other hand, when big ET was incubated with the EC-extract at pH 4.5, a slight increase in IR-ET was also observed, without enzyme inhibitors. In contrast to the case seen at pH 7.0, the increasing effect at pH 4.5 was abolished by the addition of enzyme inhibitors. To determine the enzyme inhibitor responsible for this abolishment, these inhibitors were removed from the reaction system one by one and it became increasingly evident that the removal of pepstatin-A greatly increased the IR-ET content in the reaction mixture, thereby suggesting that a pepstatin-A-sensitive enzyme participates in the increase in IR-ET. Removal of any of the other enzyme inhibitors did not alter the IR-ET content (Fig. 2B). In addition, a markedly pronounced increase in the IR-ET content by the pepstatin-A removal, compared with the case in the absence of all inhibitors, suggested that other inhibitors could protect the generated IR-ET from degradation during incubation. In fact, when

synthetic porcine ET was incubated with the EC-extract at pH 4.5, the IR-ET in the reaction mixture disappeared almost completely and this response was suppressed markedly by the addition of enzyme inhibitors. Since a striking decrease in IR-ET was observed in the absence of E-64, an enzyme sensitive to E-64, like cysteine protease, seems to mainly contribute to the degradation of IR-ET generated during incubation. Removal of pepstatin-A also decreased the IR-ET, although the extent was much less than seen with E-64 removal (Fig. 2A). Hence, the amount of IR-ET generated during incubation of big ET with

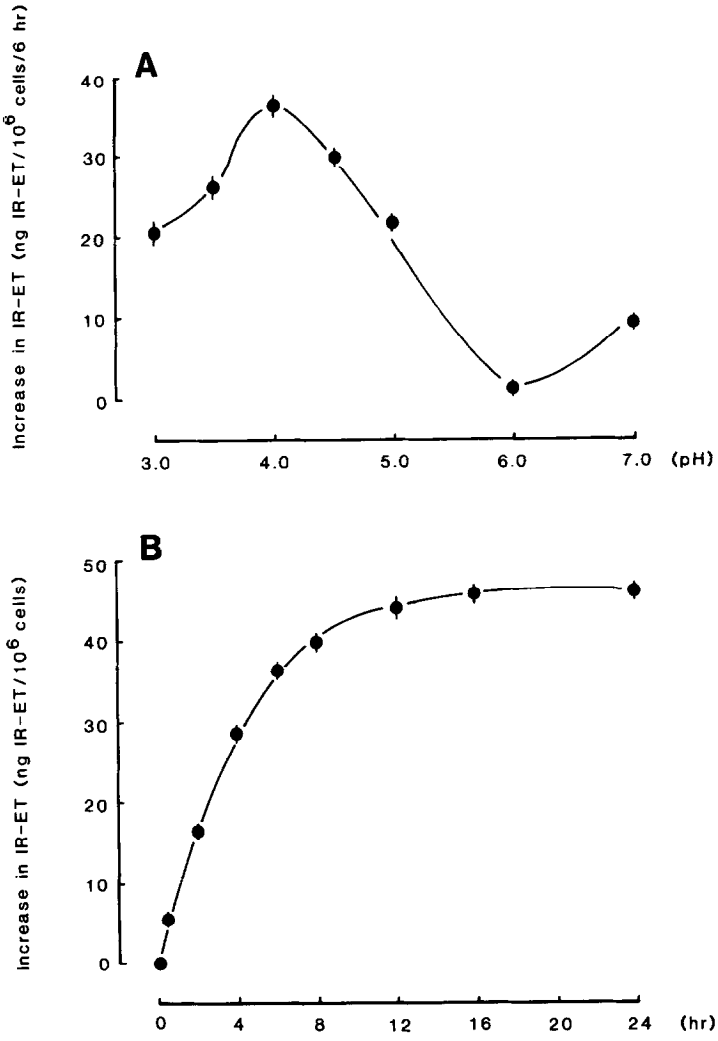

Figure 3. Effects of pH on the increase in IR-ET during incubation of big ET with the EC-extract in the presence of enzyme inhibitors, except pepstatin-A (A) and the time-course of the increased response (B). In experiments examining the pH sensitivity, the incubation was carried out for 6 hr. The time-course was examined at pH 4.0. Each point and bar represents the mean ± S.E. from four separate experiments.

the EC-extract in the presence of enzyme inhibitors, except for pepstatin-A, may be underestimated. If IR-ET generated by incubation of big ET with the EC-extract at pH 4.5 is the same form as porcine ET (ET-1), we can calculate that 9-10% of the total big ET is converted into ET in the case of pepstatin-A removal. When the sample after incubation without pepstatin-A was serially diluted, the dilution curve clearly revealed a parallel displacement with the standard curve in the RIA (data not shown).

Figure 3 depicts the pH sensitivity (A) and the time-course (B) of the increased response of IR-ET during incubation of big ET with the EC-extract in the presence of enzyme inhibitors, except pepstatin-A. The optimum pH of the response was 4.0. Although a notable increase in IR-ET was not observed at pH 6.0, the increased response of IR-ET was observed at pH 7.0, as described above. When the incubation was carried out at pH 4.0 for 0.5 to 24 hr, the IR-ET increased linearly during 6 hr after the start of incubation, and thereafter the response reached a plateau.

We examined the biological activity of the reaction mixture of big ET and the EC-extract using isolated rat aortas (Fig. 4). The mixture without the

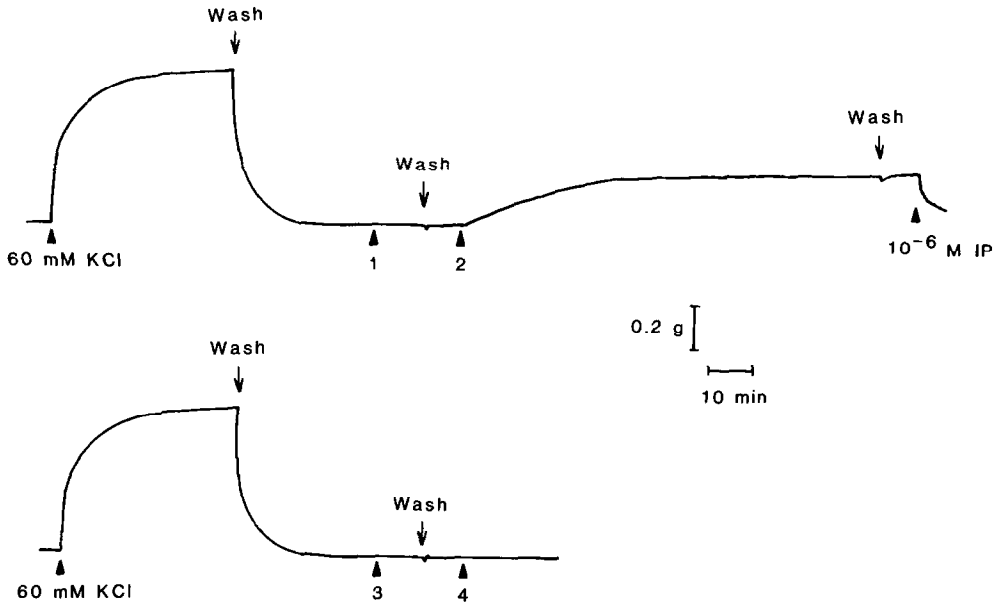

Figure 4. Typical contractile responses of rat aortic strips to the reaction mixture of big ET and the EC-extract. The incubation of big ET with the EC-extract was carried out at pH 4.0 for 6 hr. At the time noted by the arrows, the reaction mixtures (0.2 ml each) were added to the organ bath containing 20 ml of Krebs-Ringer bicarbonate solution (1, without the EC-extract; 2, with the EC-extract in the absence of pepstatin-A; 3, with the EC-extract in the absence of E-64; 4, with the EC-extract in the presence of pepstatin-A). IP, isoproterenol.

EC-extract had no vasoconstrictor activity (a final concentration of big ET: about 10^{-9} M). It has been reported that this concentration of porcine big ET has no vasoconstrictor activity (8,9). The reaction mixture of big ET and the EC-extract in the presence of enzyme inhibitors, except E-64, was also without effect. In contrast, when the mixture in the presence of enzyme inhibitors, except pepstatin-A, was added to the organ bath, a slow-onset and long-lasting vasoconstriction occurred (a final concentration of IR-ET based on the molecular weight of porcine ET: about 1.5×10^{-10} M), and washout led to little change. This magnitude of vasoconstriction was almost equal to that seen with the same concentration of synthetic porcine ET. As the sample containing pepstatin-A has no vasoconstrictor activity, the IR-ET generated by incubation of big ET with the EC-extract appears to have the same biological activity as porcine ET (ET-1). We most recently reported that the relationship between the immunoreactivity and the biological activity in culture medium from porcine aortic EC is similar to that obtained with synthetic porcine ET (10).

To confirm the correspondence between the IR-ET generated by the EC-extract and porcine ET, we examined the elution profile of the reaction mixture on RP-HPLC coupled with RIA. As shown in Fig. 5, when the reaction mixture in the presence of enzyme inhibitors, except pepstatin-A, was applied to RP-HPLC, ET immunoreactivity consisted of one major and one minor

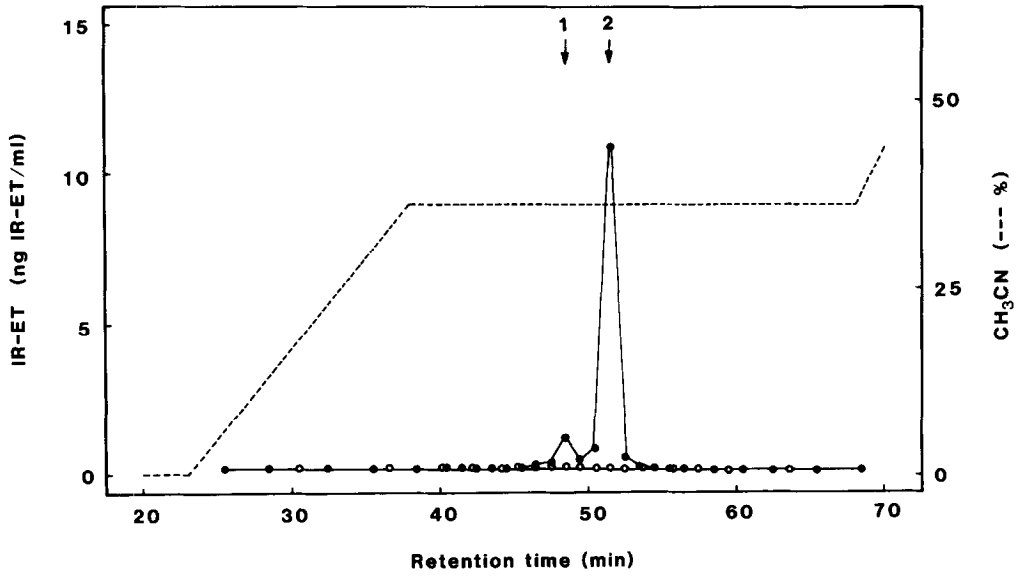

Figure 5. RP-HPLC profiles of the reaction mixture of big ET with (●) or without (○) the EC-extract. The incubation of big ET with the EC-extract was carried out at pH 4.0 for 6 hr, in the presence of enzyme inhibitors, except pepstatin-A. The EC-extract alone had no ET immunoreactivity. Arrows indicate the elution positions of big ET (1) and ET (2).

component. The position of the major peak corresponded to that of synthetic porcine ET, thereby suggesting that most of IR-ET generated during incubation of big ET with the EC-extract is 21-residue ET produced by cleavage between Trp^{21} and Val^{22}. The position of the minor peak was identical with that of synthetic porcine big ET. However, since we found no ET immunoreactivity after application of big ET alone on RP-HPLC, the immunoreactivity detected as the minor peak is presumably derived from the Met sulfoxide form of ET. Using the same type of column, other workers found that the sulfoxide form eluted slightly earlier than ET (11).

In this study, when big ET was incubated with the EC-extract at an acid pH, only 10% of the total big ET was converted into ET. It is unclear whether this value indicates a steady state level of the conversion or whether a phenomenon such as product inhibition is involved in the reaction.

We did not determine whether the small increase in IR-ET occurring at neutral pH was due to the conversion of big ET to ET, since an enzyme inhibitor preventing specifically the increased response of IR-ET was not available. Further studies are required to clarify whether or not big ET is converted to ET by the EC-extract at neutral pH.

In conclusion, we detected big ET converting activity in the extract of EC. The conversion occurred at acid pH, and was prevented specifically by pepstatin-A, an aspartic protease inhibitor. Thus, an aspartic protease may participate in the processing of big ET in vascular EC.

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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 332, 411-415
2. Yanagisawa, M. and Masaki, T. (1989) Biochem. Pharmacol. 38, 1877-1883
3. Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K. and Masaki, T. (1988) Am. J. Physiol. 255, H970-H973
4. Thiemermann, C., Lidbury, P., Thomas, R. and Vane, J. (1988) Eur. J. Pharmacol. 158, 181-182
5. Matsumura, Y., Nakase, K., Ikegawa, R., Hayashi, K., Ohyama, T. and Morimoto, S. (1989) Life Sci. 44, 149-157
6. Emori, T., Hirata, Y., Ohta, K., Shichiri, M., Shimokado, K. and Marumo, F. (1989) Biochem. Biophys. Res. Commun. 162, 217-223
7. Sawamura, T., Kimura, S., Shinmi, O., Sugita, Y., Yanagisawa, M. and Masaki, T. (1989) Biochem. Biophys. Res. Commun. 162, 1287-1294
8. Kashiwabara, T., Inagaki, Y., Ohta, H., Iwamatsu, A., Nomizu, M., Morita, A. and Nishikori, K. (1989) FEBS Lett. 247, 73-76
9. 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-S7
10. Matsumura, Y., Ikegawa, R., Ohyama, T., Hayashi, K. and Morimoto, S. (1989) Biochem. Biophys. Res. Commun. 160, 602-608
11. Kitamura, K., Tanaka, T., Kato, J., Eto, T. and Tanaka, K. (1989) Biochem. Biophys. Res. Commun. 161, 348-352