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ENDOTHELIN-3 IS A NOVEL NEUROPEPTIDE: ISOLATION AND SEQUENCE DETERMINATION OF ENDOTHELIN-1 AND ENDOTHELIN-3 IN PORCINE BRAIN

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SUMMARY: The molecular forms of endothelin (ET) related peptides were investigated in porcine brain by using high performance liquid chromatography coupled with three specific radioimmunoassays. ET-1 and its oxidized form were isolated and sequenced as in the case of porcine spinal cord. A very small amount of big ET-1 (1-39) and its C-terminal fragment (big ET-1(22-39)) were also detected. Furthermore, immunoreactive (ir)-ET-3 was isolated and sequenced; its partial primary structure was identical to that of human (rat) ET-3. The concentrations of ir-ET-1 and ir-ET-3 in porcine brain were 140 fmol/g tissue and 5 fmol/g tissue, respectively. These results indicate that besides ET-1, ET-3 is a novel neuropeptide in the central nervous system.

Endothelin-1 (ET-1) is a novel vasoconstrictor peptide which was isolated and sequenced originally from the culture supernatant of porcine aortic endothelial cells. The sequence analysis of the porcine cDNA encoding ET-1 (1) and the analysis of the molecular forms of ET-1 in the culture supernatant of porcine aortic endothelial cells (2) have shown that ET-1 is produced from (pre)proET through a 39-residue intermediate form, big ET-1. In addition to the vasoconstrictor action, ET-1 has been demonstrated to have multiple biological actions in various organs; cardiac effects (3,4) including a positive inotropic and chronotropic actions, contraction of tracheal smooth muscle (5), stimulation of secretion of atrial natriuretic peptide (6), release of eicosanoids and endothelium derived relaxing factor from vascular beds (7), inhibition of renin release from isolated glomeruli (8), depolarizing action on the spinal neurons (9), stimulation of proliferation of vascular smooth muscle cells and fibroblasts (10,11). Tissue autoradiographic

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studies with [^{125}I-Tyr^{13}]ET-1 in the rat demonstrated the localization of specific, high affinity binding sites for ET-1 not only within vascular system but also in other tissues including intestine, heart, lung, kidney, adrenal glands and brain (12). Recently, the analysis of human ET genes identified three similar but distinct sequences of ET-like peptides and the three isopeptides were named ET-1, ET-2 and ET-3. Synthetic peptides according to these three sequences elicited the similar but distinct pharmacological activities (13). Nevertheless, it is still obscure in which tissue(s) they are expressed. However, immunoreactive (ir)-ET was not confined to vascular endothelial cells but rather widely distributed to non-vascular tissues, such as, kidney and lung (14,15). In addition, we previously demonstrated the presence of ET-1 and ir-ET-3 in porcine spinal cord (16).

To confirm the previous results that both ET-1 and ET-3 are present in the spinal cord, we investigated the molecular forms of ir-ET related peptides in porcine brain by using radioimmunoassays (RIAs) and high performance liquid chromatography (HPLC).

Material and Methods

Peptides and Antisera:
ET-1, ET-3 and Anti ET-1 serum (As-WB-4) were obtained from Peptide Institute Inc. (Osaka, Japan) and Anti ET-3 serum (As-ET-3) was purchased from Peninsula (California). [^{125}I-Tyr^{13}]ET-1 and [^{125}I-Tyr^{6}]ET-3 were purchased from Amersham Co.

Preparation of porcine brain extract:
Fresh porcine brains (ca. 50kg wet weight) were obtained from a local slaughter house and stored at -80°C until used. The brains were extracted with acetone/HCl as described previously (16).

Purification of the brain extracts:
The lyophylized tissue extracts were divided into four portions and each portion was loaded onto a Chemcosorb 5ODS-H column (20 x 250 mm, Chemco, Osaka). Elutions were carried out with a 100 min linear gradient of acetonitrile (0-50%) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 9.9 ml/min at 40°C. An aliquot of each fraction (9.9 ml) was subjected to RIAs with three antisera (As-ETC, As-ET-3, As-bETC). The two pooled fractions (peak C: Fr.52-56; and peaks D and E: fr.57-65 in Fig.1) containing ir-ET like peptides were separated by Toyopearl SP650S (22 x 200 mm, Tosoh, Tokyo) and Chemcosorb 5ODS-H (10 x 250 mm, Chemco) in a manner similar to our previous studies (2,16). Ir-ET like peptides were further purified by successive chromatographic systems (see Results).

RIAs for ET related peptides:
RIAs using As-ETC, As-WB-4, and As-bETC, were performed as described previously (2,16). RIA with As-ET-3 for ET-3 was similarly performed using ET-3 as standard and [^{125}I-Tyr^{6}]ET-3 as tracer. As-ET-3 crossreacted ET-1 with a relative sensitivity of less than 1% of ET-3.

Sequence analysis:
Amino acid sequence analysis were performed by a gas phase sequencer (Model 470A, Applied Biosystems). The resulting PTH-amino acids were analyzed by reverse phase HPLC (Model 120A, Applied Biosystems), linked directly to the gas phase sequencer.

Results and Discussion

Fig.1 shows the elution profiles of ir-ET related peptides in the brain extracts separated by preparative reverse phase HPLC. ET-1 (peak E) and [Met^7]-oxidized ET-1 (peak D) like peptides were detected as major ir-peaks by As-ETC which recognizes the C-terminal common part of ETs (Fig.1(a)). Furthermore, as minor ir-peaks, an ET-3 like peptide (peak C) and a C-terminal fragment like peptide (peak B) of big ET-1 were detected by As-ET-3 and As-bETC, respectively (Fig.1(b,c)), in addition to big ET-1 like peptides (around peaks D and E in (c)) and an unknown ir-peptide (peak A in (c)).

We further rechromatographed two ir-ET fractions, (1) peak C (fr: 52-56), (2) peaks D and E (fr: 57-65), respectively. Peak C was separated by a cation-exchange (Toyopearl SP650S) and a reverse phase (Chemcosorb 5-ODS-H) HPLCs, and monitored with the RIA using As-ET-3 (data not shown). The ir-ET-3 fractions thus obtained were further purified by four HPLCs (Fig.2 (a)-(d)). Fig.2(d) shows a single peak whose elution time was identical to that of synthetic ET-3. The amino acid sequence of the ir-ET-3 like peptide was determined by a gas phase sequencer as XTXFTYKDKEXVYYX?V?II? (X: blank signal because of cystine, and ?: not determined). The three unknown (?) amino acids were expected to be His^{16}, Asp^{18} and Trp^{21}, because His, Asp and Trp are known to give low yields of the corresponding PTH-amino acids in peptide sequencing and all the peptides belonging to ET/Sarafotoxin family (ET-1, -2, -3 and Sarafotoxins S6) have a common C-terminal sequence in three positions: His^{16}, Asp^{18} and Trp^{21}. Therefore, we concluded that the purified peptide is composed of 21 amino acid residues and identical to human/rat ET-3, taken together with the following two reasons: (i) the peptide was detected with As-

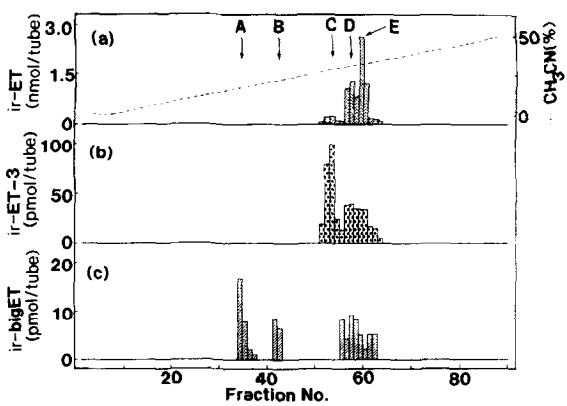

Fig.1 A reverse phase HPLC profile of the extract from the porcine brain on a Chemcosorb 5ODS-H (20 x 250 mm, Chemco), monitored by RIAs using three antisera; (a) As-ETC, (b) As-ET-3 and (c) As-bETC. Arrows denote the five separated immunoreactive peaks assayed with RIAs.

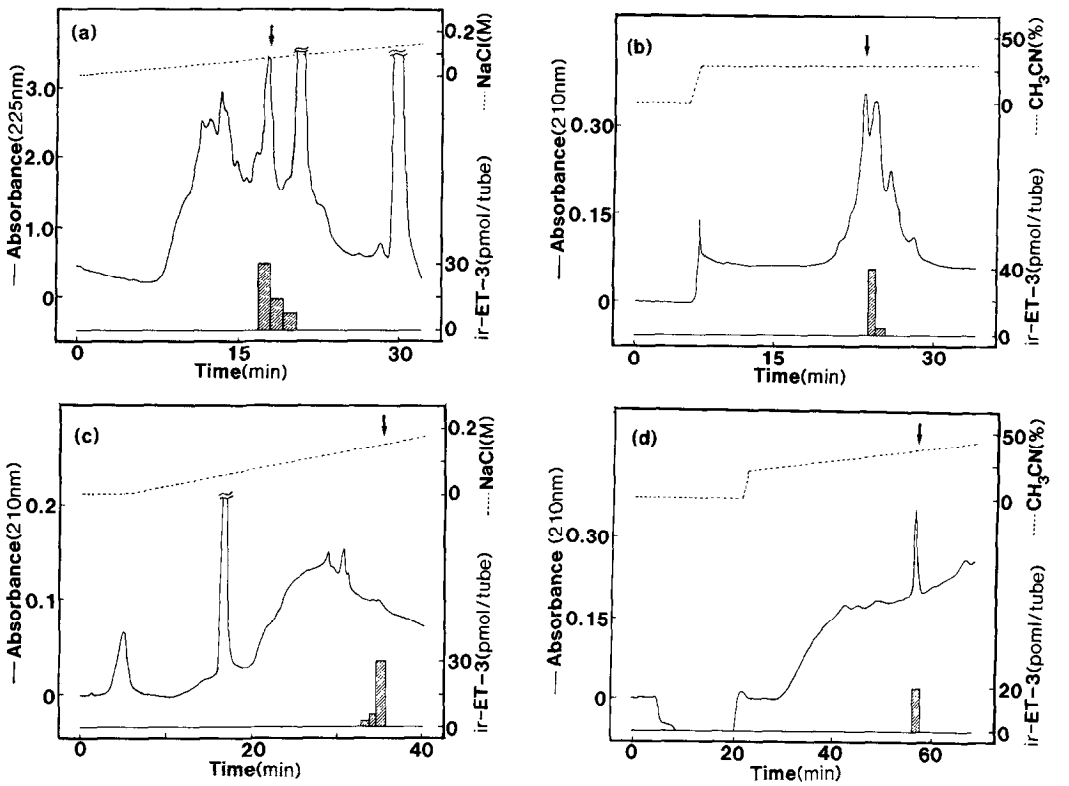

Fig.2 Successive purification of ET-3 by HPLC.
(a) Anion-exchange HPLC of ir-fractions after two chromatographic purification (Toyopearl SP650S and Chemcosorb 5ODS-H) of peak C in Fig.1.
Column: TSK-gel DEAE-5PW (7.5 x 75 mm, Tosoh)
Elutions were carried out by a 100 min linear gradient of NaCl (0-0.5M) in 5mM Tris-HCl (pH 7.4) and 10% CH_3CN at a flow rate of 2 ml/min.
(b) Reverse phase HPLC of ir-fractions shown with shaded bars in Fig.2(a).
Column: Cosmosil C18 (4.6 x 250 mm, Nacalai tesque)
Elutions were carried out by a 100 min linear gradient of acetonitrile (27.5-50%) in 0.1% TFA at a flow rate of 1 ml/min.
(c) Anion-exchange HPLC of ir-fractions shown with shaded bars in Fig.2(b).
Column: TSK-gel DEAE-2SW (4.6 x 250 mm, Tosoh)
Elutions were carried out by a 100 min linear gradient of NaCl (0-0.5M) in 5mM Tris-HCl (pH 7.4) and 10% CH_3CN at a flow rate of 1 ml/min.
(d) Reverse phase HPLC of an ir-fraction (fr.34-35min) shown with a shaded bar in Fig.2(c).
Column: Chemcosorb 5ODS-H (2.1 x 250 mm, Chemco)
Elutions were carried out by a 60 min linear gradient of acetonitrile (20-50%) in 0.1% TFA at a flow rate of 0.2 ml/min.
Immunoreactivities in fractions were monitored by RIAs using As-ET-3.
An arrow indicates the elution position of synthetic ET-3.

ETC which specifically recognizes the C-terminal [Trp^{21}] of ETs (16), (ii) the retention times of the peptide were completely identical to those of synthetic ET-3(1-21) in both a reverse phase and an anion-exchange HPLCs.

The combined fractions, peaks D and E (Fr.57-65), were further separated by a cation exchange (TSK-gel SP650S) and a reverse phase (Chemcosorb 5ODS-H) HPLCs in a manner similar to the case of peak C (data not

shown). The ir-ET-1 like fractions were further purified by four successive HPLCs using TSK-gel DEAE-5PW (i) and Cosmosil 5C18 (ii)-(iv) (Fig.3(a)-(d)). Steps (ii) and (iv) were eluted with acetonitrile in 0.1% TFA but step (iii) was eluted with the same solvent containing an ionpair reagent, sodium pentanesulfonate to add a cation-exchange mode. The elution time of the peak was corresponded with that of ET-1 and the sequence was found to be that of

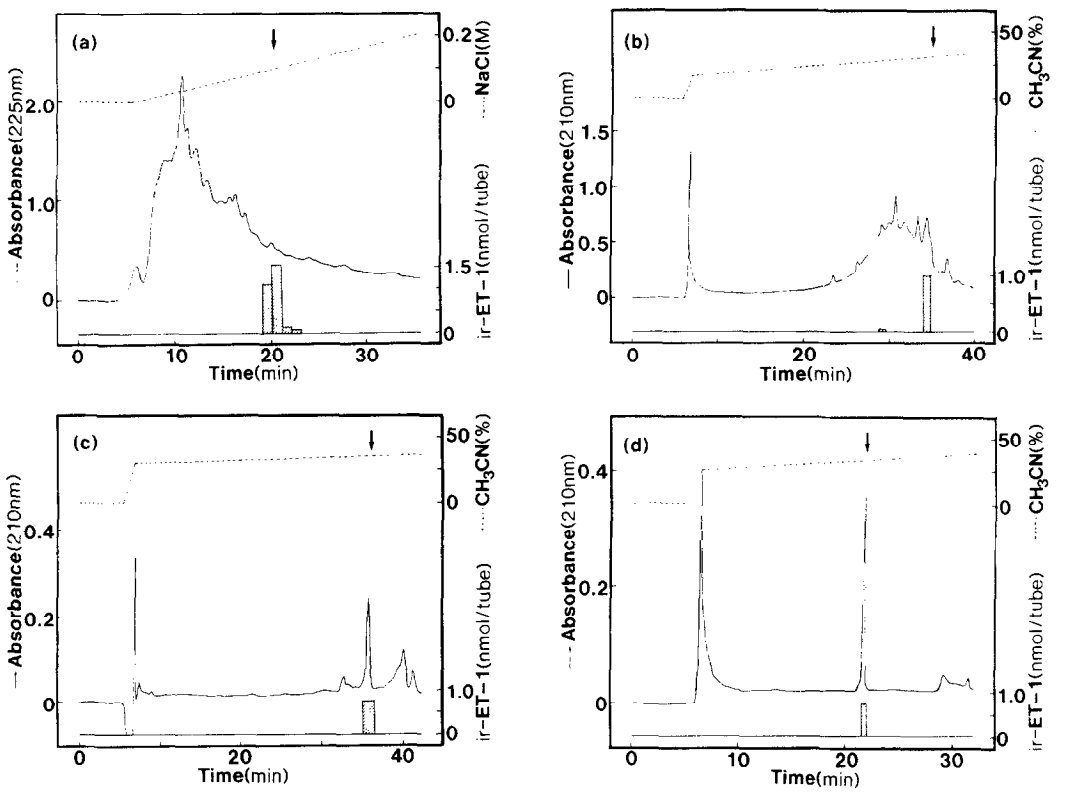

Fig.3 Successive purification of ET-1 by HPLC.
(a) Anion-exchage HPLC of the major ir-fractions after two chromatographic purification (Toyopearl SP650S and Chemcosorb 5ODS-H) of peaks D and E in Fig.1.
Column: TSK-gel DEAE-5PW (7.5 x 75 mm, Tosoh)
Elutions were carried out by a 75 min linear gradient of NaCl (0-0.5M) in 5mM Tris-HCl (pH 7.4) and 10% CH_3CN at a flow rate of 2ml/min.
(b) Reverse phase HPLC of ir-fractions (fr.19-21 min) in Fig.3(a).
Column: Cosmosil C18 (4.6 x 250 mm, Nacalai tesque)
Elutions were carried out by a 100 min linear gradient of acetonitrile (25-50%) in 0.1% TFA at a flow rate of 1 ml/min.
(c) Reverse phase HPLC of an ir-fraction shown with a shaded bar in Fig.3(b).
Column: Cosmosil C18 (4.6 x 250 mm, Nacalai tesque)
Elutions were carried out by a 168 min linear gradient of acetonitrile (29-50%) in 0.1% TFA and 5mM sodium pentanesulfonate at a flow rate of 1 ml/min.
(d) Reverse phase HPLC of an ir-fraction shown with a shaded bar in Fig.3(c).
Column: Cosmosil C18 (4.6 x 250 mm, Nacalai tesque)
Elutions were carried out by a 50 min linear gradient of acetonitrile (25-50%) in 0.1% TFA at a flow rate of 1 ml/min.
Immunoreactivities in fractions were monitored by RIAs using As-WB-4.
An arrow indicates the elution position of synthetic ET-1.

ET-1. The ir-[Met^7]-oxidized ET like fractions were also purified to a single peak by similar procedures of ET-1 purification (data not shown). The sequence of the ir-peptide was found to be [Met^7(O)]ET-1. These results are consistent with those in our previous work in porcine spinal cord (16).

The results demonstrated that both ET-1 and ET-3 are present in the brain as well as in the spinal cord. From Fig.1, the concentrations of ir-ET-1 and ir-ET-3 in porcine brain were estimated to be 140 fmol/g tissue and 5 fmol/g tissue, respectively. The amount of ir-ET-3 in porcine brain is lower than that in the spinal cord (9 fmol/g tissue), while the content of ir-ET-1 is slightly higher than that in the spinal cord (120 fmol/g tissue) (16). The observation that the concentration of ir-ET-1 is over ten times higher than that of ir-ET-3 in the brain, agrees with the case of the spinal cord.

The processing pathway of ET-1(1-21) has been proposed to be produced via big ET-1(1-39) in vascular endothelial cells (1,2). In porcine brain as well as in the spinal cord, ir-big ET-1(1-39) (around peaks D and E) and ir-big ET-1(22-39) (peak B) were detected in amount less than 1% of ir-ET-1 (Fig.1). These results indicate that big ET-1(1-39) is mostly processed to a mature form, ET-1(1-21), in ET-1 producing cells in the CNS. Although the presence of proET-3 is unknown at present, ET-3(1-21) seems to be a major form in porcine brain as can be detected by RIAs using As-ET-3. It is not clear at present whether ET-1 and ET-3 are localized similarly or differently within the brain and spinal cord. However, we have found the localizations of ir-ET-1 in limited brain regions (cerebellum, hypothalamus and hippocampus) as well as in the spinal cord (10,16 and T. Y., et al. unpublished results).

We could not obtain an evidence whether ET-2 exists in porcine brain. If any, its amount may be very small because the ir-peak was small which was eluted slightly after ET-1 (the tailing part of peak E in Fig.1) on a reverse phase column. Regarding peak A, we could not analyze it further because of its limited amount. Judging from its immunoreactivity to As-bETC, it is likely that peak A is another form of the C-terminal fragment of big ET-1 or a C-terminal fragment of big ET-3.

Recently, Kosaka et.al. have reported that ir-ETs are actually present in kidney cells; especially an ET-2 like peptide in addition to an ET-1 like peptide was detected in the supernatant from COS-7 cells (14). Kitamura et.al. also have reported that ir-ETs are present in the porcine tissues; especially in lung and inner medulla of kidney (15). Taken together with these results, it appears that ET family peptides are expressed in various tissues both within and outside vascular tissues.

In this report and in the previous study (16), we clearly demonstrated the presence of both ET-1 and ET-3 in the porcine CNS. The similar but distinct pharmacological activities of ET-1 and ET-3 were reported by in vivo

and in vitro experiments (13). Binding assay using [^{125}I]ET-1, -2, -3 on chick cardiac membranes indicated that at least two distinct types of ET (probably ET-1 type and ET-3 type) receptors coexisted on chick cardiac membranes (17). These results suggest that ET-1 and ET-3 might act on their own receptors and exert distinct physiological roles as neuropeptides in the CNS. Further studies including the clarification of their detailed distributions are required to understand the physiological roles of ET family peptides in the CNS.

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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. Sawamura, T., Kimura, S., Shinmi, O., Sugita, Y., Yanagisawa, M. and Masaki, T. (1989) Biochem. Biophys. Res. Commun. in press.
3. Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K. and Masaki, T. (1988) Am. J. Physiol. 255, H970-H973.
4. Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K. and Masaki, T. (1988) Pflugers Arch. 413, 108-110.
5. Uchida, Y., Ninomiya, H., Saotome, M., Nomura, A., Ohtsuka, M., Yanagisawa, M., Goto, K., Masaki, T. and Hasegawa, S. (1988) Eur. J. Pharmacol. 154, 227-228.
6. Fukuda, Y., Hirata, Y., Yoshimi, H., Kojima, T., Kobayashi, Y., Yanagisawa, M. and Masaki, T. (1988) Biochem. Biophys. Res. Commun. 155, 167-172.
7. De Nucci, G., Thomas, R., D'Orleans-Juste, P., Antunes, E., Walder, C., Warner, T.D. and Vane, J. (1988) Proc. Natl. Acad. Sci. USA 85, 9797-9800.
8. Rakugi, H., Nakamura, M., Saito, H., Higaki, J. and Ogihara, T. (1988) Biochem. Biophys. Res. Commun. 155, 1244-1247.
9. Yoshizawa, T., Kimura, S., Kanazawa, I., Uchiyama, Y., Yanagisawa, M. and Masaki, T. (1989) Neurosci. Lett. 102, 179-184.
10. Komuro, I., Kurihara, H., Sugiyama, T., Takaku, F. and Yazaki, Y. (1988) FEBS Lett. 238, 249-252.
11. Takuwa, N., Takuwa, Y., Yanagisawa, M., Yamashita, K. and Masaki, T. (1989) J. Biol. Chem. 264, 7856-7861.
12. Koseki, C., Imai, M., Hirata, Y., Yanagisawa, M. and Masaki, T. (1989) Am. J. Physiol. 256, R858-R866, 1989.
13. Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K. and Masaki, T. (1989) Proc. Natl. Acad. Sci. USA 86, 2863-2867.
14. Kosaka,T., Suzuki, N., Matsumoto, H., Itoh, Y., Yasuhara, T., Onda, H. and Fujino, M. (1989) FEBS. Lett. 249, 42-46.
15. Kitamura, K., Tanaka, T., Kato, J., Eto, T. and Tanaka, K. (1989) Biochem. Biophys. Res. Commun. 161, 348-352.
16. 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.
17. Watanabe, H., Miyazaki, H., Kondoh, M., Masuda, Y., Kimura, S., Yanagisawa, M., Masaki, T. and Murakami, K. (1989) Biochem. Biophys. Res. Commun. 161, 1252-1259.