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PRESENCE OF ENDOTHELIN-1 IN PORCINE SPINAL CORD: ISOLATION AND SEQUENCE DETERMINATION

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SUMMARY: We investigated the molecular forms of endothelin (ET) related peptides in porcine spinal cord by high performance liquid chromatography coupled with radioimmunoassays using three antisera raised against ET-1 and C-terminal fragments of ET-1 and big ET-1. ET-1 and its oxidized form were isolated as major immunoreactive peptides and sequenced. Furthermore, immunoreactivities like ET-3 and big ET-1(22-39) (contents: < 8% and < 1% of ET-1, respectively) were detected based on their chromatographic retention times and characteristics of immunoreactivity to the antisera. Big ET-1 was only scarecely detected. Immunohistochemical study showed the presence of ET-1-like immunoreactivity in motoneurons, dorsal horn neurons and dot- and fiber-like structures in the dorsal horn of lumbar spinal cord. These results indicate that ET-1 is present not only in endothelial cells but also in spinal cord, and that big ET-1 is converted into ET-1 in spinal cord by specific processing between Trp^{21}-Val^{22}. The data also indicate that ET-1 may act as a neuropeptide in the central nervous system.

Endothelin (ET) is a potent vasoconstrictor/pressor peptide isolated and sequenced from the culture supernatant of porcine aortic endothelial cells and is composed of 21 amino acid residues with two disulfide bonds. Based on the sequence analysis of porcine preproET cDNA, we proposed the biosynthetic pathway that ET is produced through an unusual proteolytic processing between Trp^{21}-Val^{22} of a 39-residue peptide big endothelin (big ET) (1). Recently, the three isopeptides of human ET have been identified by screening a genomic DNA library and named ET-1 (the 'classical' ET), ET-2 and ET-3, which elicited the pharmacological activities distinct from each other (2). From structure-activity relationship studies (3,4), we have shown that ET-1(1-21) is the most active form and that the conversion from big ET-1 to ET-1(1-21) is essential to generate the physiologically most active form.

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Tissue autoradiographic studies with [^{125}I]-labeled ET-1 in rats have demonstrated that the binding sites for ET-1 are distributed not only in vascular smooth muscle but also widely in various non-cardiovascular tissues including the central nervous system (5,6). Furthermore, we have recently shown that in the new born rat spinal cord, ET-1 produces ventral root depolarization mediated by the release of substance P (7). In addition, we have found that ET-like immunoreactivity was localized in neurons in the dorsal horn of spinal cord. In the present study, to elucidate whether ET-1 and other isopeptides exist in neural tissues, we have established sensitive and specific radioimmunoassay (RIA) for ET related peptides and investigated the molecular forms of the immunoreactive ET related peptides in porcine spinal cord by high performance liquid chromatography (HPLC) coupled with the RIA.

Materials and Methods

Peptides: ET-1, ET-3 and Anti-ET-1 antiserum (As-WA-3) were obtained from Peptide Institute Inc. (Osaka, Japan). Porcine big ET-1(1-39) were synthesized by the solid phase method reported previously (4). Human big ET-1(1-38), human big ET-1(22-38), porcine big ET-1(22-39) and [Arg^{14}]ET(15-21) were also synthesized and purified by similar procedures.

Tissue extraction and isolation: Fresh porcine spinal cord (ca. 50kg wet weight) was obtained from a local slaughter house and stored at -80°C until used. Spinal cord was extracted as described previously (8).

Purification of the spinal cord extracts: The lyophylized tissue extracts were divided into four portions and each portion was loaded onto a Chemcosorb I-10C18 column (20 x 250 mm, Chemco, Osaka). Elutions were carried out with a 100 min linear gradient of acetonitrile (0-50%) in 0.1% TFA at a flow rate of 9.9 ml/min at 40°C. An aliquot of each fraction (9.9 ml) was assayed by RIAs with three different antisera (see RIA procedures). The pooled fractions (peak D in Fig.2) containing immunoreactive (ir)-endothelin related peptides were purified to homogeneity by six successive HPLCs (reverse phase and cation-exchange) as follows; (i) a Toyopearl SP650S (22 x 200 mm, Tosoh, Tokyo), (ii) a Chemcosorb 5ODS-H (10 x 250 mm, Chemco), (iii) a TSK SP-5PW (7.5 x 75 mm, Tosoh), (iv) an Ultrasphere ODS (10 X 250 mm, Beckman), (v) a TSK SP-2SW (4.6 X 250 mm, Tosoh) and finally (vi) a Chemcosorb 5ODS-H (4.6 x 250 mm, Chemco). Aliquots of fractions from HPLCs were submitted to the RIAs with three different antisera.

Preparation of antisera and radioimmunoassay procedures: [Arg^{14}]ET(15-21) was conjugated with KLH (key hole limpet hemocyanin) by the MBS (maleimdobenzoyl-n-hydroxysuccinimide ester) method (9). Human big ET-1(22-38) was conjugated to KLH using the glutaraldehyde coupling procedure. [Arg^{14}]ET(15-21) or hbig ET-1(22-38) conjugate was emulsified with an equal volume of Freund's complete adjuvant and used for immunizing New Zealand White rabbits, by subcutaneous injection at multiple sites in the interscapulo-vertebral region. They were boosted every two weeks. ET-1 and pbig ET-1(22-39) were radioiodinated by the chroramine T method (10). The labeled peptides were purified by reverse phase HPLC. The incubation buffer for RIA was 0.05M sodium phosphate buffer (pH 7.4) containing 0.1% BSA, 0.1% Triton X-100, 0.15M NaCl, 0.025M EDTA-2Na, 0.05% NaN_3 and 500 KIU/ml Trasylol. All assay procedures were performed at 4°C. The standard peptide (ET-1, ET-3, pbig ET-1(1-39), pbig ET-1(22-39) or hbig ET-1(1-38)) or the unknown sample (100 µl) was preincubated with diluted antiserum (As-WA-3, As-ETC, or As-bETC) (200 µl) for 12 h. Then, the tracer solution (15,000-20,000 cpm in 50 µl) was added. After the incubation for 24 h, anti-rabbit IgG goat serum diluent (500 µl) was added. After kept standing

for 30 min, the tubes were centrifuged at 3,000rpm for 30 min and radioactivity of the precipitate was measured in an Aloka gamma counter.
Sequence analysis: Amino acid sequence analysis was 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 with the gas-phase sequencer.
Immunohistochemistry: A male miniature-pig (body weight 3.5kg) was perfused intracardally with a 4% paraformaldehyde solution buffered with 0.1M phosphate buffer (PB) (pH 7.2). The lumbar spinal cord was transversely sectioned at 20 μm with a cryostat. After 20 min-preincubation with 2% normal goat serum, the sections were immersed for 48 h at 4°C to As-WA-3 diluted 1:500 in PBS and followed by the ordinary avidin-biotinyl enzyme complex method. Specificity of the immunostaining was examined by the absorption test.

Results and Discussion

Typical standard curves for RIA with three antisera (As-WA-3, As-ETC and As-bETC) are shown in Fig.1. As-WA-3 recognized ET-1(1-21) and pbig ET-1(1-39) but not ET-3. As-ETC recognized ET-1 and ET-3 but not pbig ET-1(1-39), which reacted to ET-1 with a relative potency of 52% that of ET-3, when [^{125}I]ET-1 was used as a radioactive ligand. As-bETC also recognized both pbig ET-1(1-39) and pbig ET-1(22-39) with a relative potency of 10% that of hbig ET-1(1-38).

Fig.2 shows elution patterns of ir-ET related peptides in the spinal cord extracts separated by reverse phase HPLC. Three major peaks (B:fr.67-68,

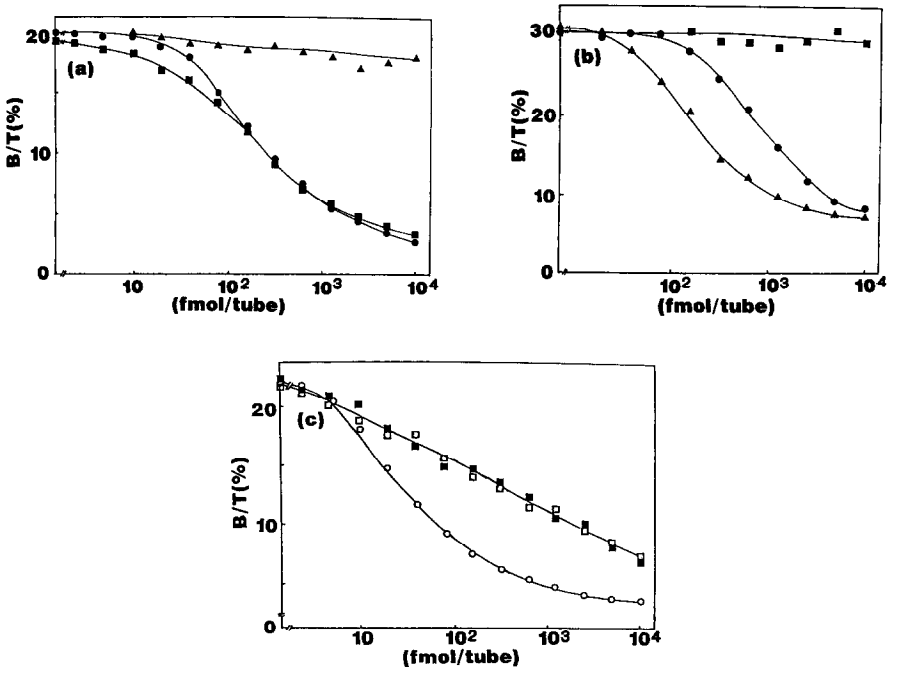

Fig.1: Typical standard curves of ET-1 (●—●), ET-3 (▲—▲), pbig ET-1 (1-39) (■—■), pbig ET-1(22-39) (□—□), and hbig ET-1(1-38) (○—○) in the RIAs with the antiserum As-WA-3 (a), antiserum As-ETC (b) and antiserum As-bETC (c).

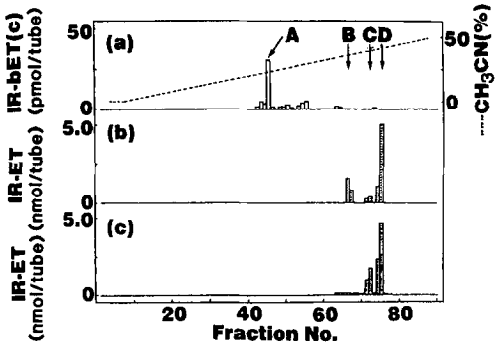

Fig.2: A reverse phase HPLC profile of the extract from the spinal cord on a Chemcosorb I-10C18 column (20 x 250 mm, Chemco), monitored by three antisera; (a) antiserum: As-bETC, (b) antiserum: As-ETC and (c) antiserum: As-WA-3. Arrows denote the four separated immunoactive peaks assayed with RIAs.

C:fr.72-73, D:fr.75-76) and a minor peak (A:fr.46) of immunoreactive materials were detected using three antisera.

The peak D was re-chromatographed by three successive HPLCs: cation exchange HPLC (Toyopearl SP650S), reverse phase HPLC (Chemcosorb 5ODS-H) and cation exchange HPLC (TSK SP-5PW) as described in methods (data not shown). The immunoreactive peak obtained was further purified by three HPLCs (Fig.3(a),(b),(c)). Fig.3(c) shows the single peak, whose elution time corresponded with that of synthetic ET-1. The amino acid sequence of the peak D peptide was determined by a gas phase sequencer as ET-1(1-21) (XSXSSLMDKEXVYFXHLDIIW) (X could not be determined because of cystine).

The peak B was separated by cation-exchange HPLC and reverse phase HPLC. In the cation exchange HPLC, the elution time was the same as that of the synthetic ET-3 (data not shown). Fig.3(d) shows the reverse phase HPLC pattern of the immunoreactive peak obtained and the elution time also corresponded with that of synthetic ET-3. As-WA-3 detected little immunoreactivity around peak B, however As-ETC recognized the peak B as an ir-ET related peptide (Fig.2). As shown in Fig.1(a) and (b), As-WA-3 shows no crossreactivity with ET-3 but As-ETC recognizes ET-3. Although whether the amino acid sequence of porcine ET-3 is identical to that of the predicted human ET-3 is not yet known, these results indicate that the peak B is ET-3 or a very similar peptide.

The peak C was also purified to a single peak (data not shown), whose elution time corresponded with that of [Met^{7}(O)]ET-1. The sequence of peak C was found to be oxidized ET-1 ([Met^{7}(O)]ET-1), which is probably an artifact product during purification.

The peak A was expected to be a C-terminal peptide (pbig ET(22-39)) of pbig ET-1(1-39) or its related peptides, because the peak A was eluted around the elution time of synthetic pbig ET(22-39) by reverse phase HPLC. Big ET-1

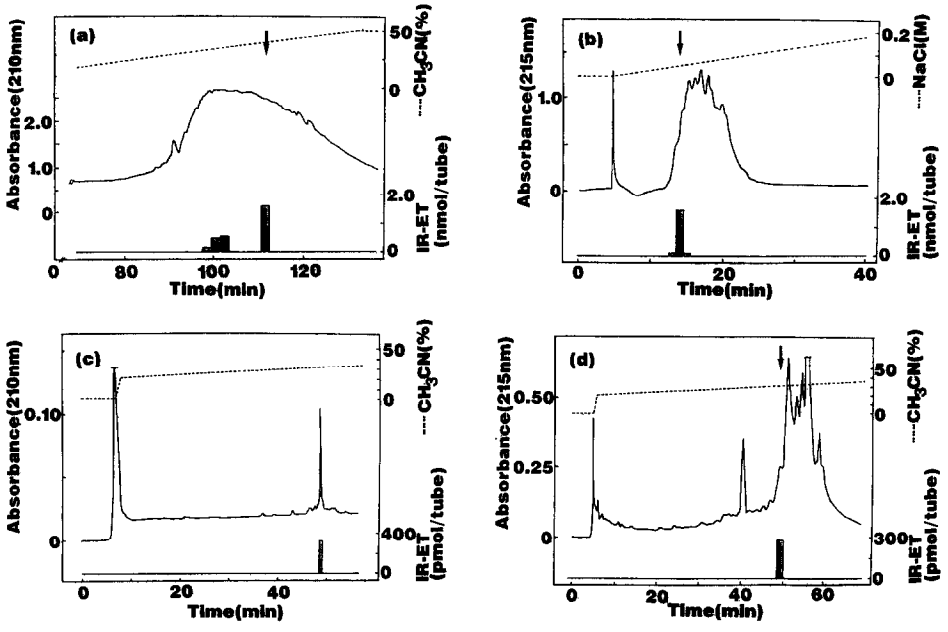

Fig.3: Successive HPLC patterns of ir-ET related peptides.
(a) Reverse phase HPLC of peak D in Fig.2 after three chromatographic purification steps (i, ii, iii described in methods).
Column: Ultrasphere ODS (10 x 250 mm, Beckman).
Elutions were carried out with a 120 min linear gradient of acetonitrile in 0.1% TFA at a flow rate of 3 ml/min.
(b) Cation-exchange HPLC of ir-peak (fr.112-114 min) in Fig.3(a).
Column: TSK-gel SP-2SW (4.6 X 250 mm, Tosoh).
Elutions were carried out by a 100 min linear gradient of NaCl (0-0.5M) in 10 mM CH_3COONa (pH 5.0) and 10% CH_3CN at a flow rate of 1 ml/min.
(c) Reverse phase HPLC of ir-ET related peptides in Fig.3(b).
Column: Chemcosorb 5ODS-H (4.6 x 250 mm, Chemco).
Elutions were carried out a 120 min linear gradient of acetonitrile (20-50%) in 0.1% TFA at a flow rate of 1 ml/min.
(d) Reverse phase HPLC of peak B in Fig.2.
Column: UQ C18 (7.6 x 250 mm, Gasukuro).
Elutions were carried out a 120 min linear gradient of acetonitrile (20-50%) in 0.1% TFA at a flow rate of 3 ml/min.
An arrow denotes the elution time of synthetic ET-1 (a, b) or synthetic ET-3 (c). Immunoreactivities in fractions were monitored by RIAs using As-WA-3 (a, b, c) and As-ETC (d).

(1-39) was only scarecely detected (Fig.2(a)), which was expected to be eluted just before ET-1.

The above results unequivocally demonstrated that both ET-1 and ET-3 are present in the porcine spinal cord. From Fig.2, the concentrations of ir-ET-1 and ir-ET-3 in porcine spinal cord were estimated to be 0.120 pmol/g tissue and 0.040 pmol/g tissue respectively. The amount of ir-ET-3 was corrected to be 0.009 pmol/g tissue by using ET-3 as a standard of RIA (Fig.1(b)). Therefore, ir-ET-1 exists at a concentration of about 13 times higher than that of ir-ET-3 in porcine spinal cord.

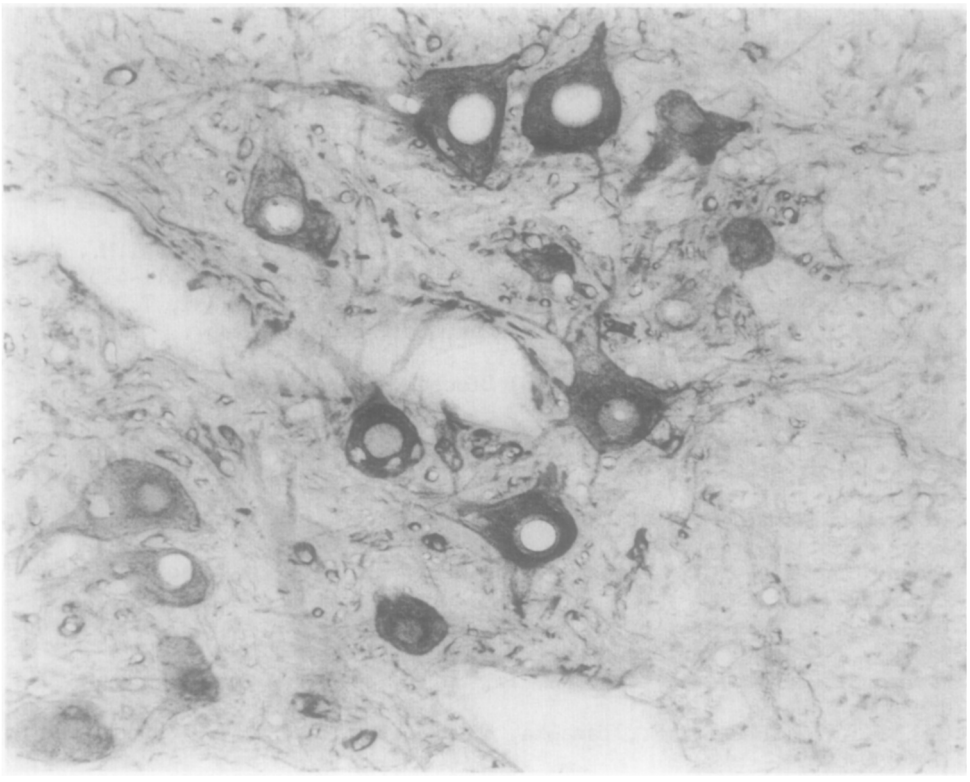

Fig.4: ET-like immunoreactivity in the lumbar motoneurons of the porcine spinal cord. Original magnification: x50.

We further examined the presence of ET-1 in porcine spinal cord by immunohistochemistry with As-WA-3. ET-1-like immunoreactivity was localized in dot- and fiber-like structures and neurons in the dorsal horn (7). Moreover, motoneurons were also densely immunostained (Fig. 4). Although the biosynthetic pathway of ET-1 in endothelial cells is unclear at present, we have recently isolated and sequenced a C-terminal peptide of big ET-1(pbig ET-1(22-39)) and big ET-1(1-39) in addition to ET-1(1-21) from the culture supernatant of porcine aortic endothelial cells, and found that the amount of big ET-1(1-39) was much less compared to that of ET-1(1-21) (T. Sawamura et al., manuscript in preparation). In porcine spinal cord, since big ET-1 was only scarcely detected, big ET-1(1-39) may be mostly converted to ET-1(1-21) by the ET converting enzyme. Combining the present results with the reports of T. Yoshizawa et.al.(7) and A. Giaid et.al.(manuscript submitted), the presence of ET-1 in the central nervous system was established chemically, physiologically and anatomically. The presence of ET-3 was also suggested in spinal cord. Further study is needed to establish the presence and localization of ET-3 in the spinal cord, which might have its own physiological role distinct from ET-1.

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