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ABUNDANCE OF ENDOTHELIN-3 IN RAT INTESTINE, PITUITARY GLAND AND BRAIN

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SUMMARY: We established a highly sensitive and specific sandwich-enzyme immunoassay (EIA) for endothelin-3 (ET-3), which showed no crossreactivity with endothelin-1 (ET-1), endothelin-2 (ET-2) and big-endothelin-1 (big-ET-1). We had previously established a sensitive sandwich-EIA for ET-1, which fully crossreacted with ET-2, but not with ET-3 or big-ET-1. These EIAs were used to examine the tissue distribution of immunoreactive (ir-) ET-3 and compare them with those of ir-ET-1 (including ir-ET-2) in Sprague-Dawley rats. High concentrations of ir-ET-3 were found in the intestine, lung, pituitary gland and brain (>100 pg/g wet tissue). ir-ET-1(ET-2) showed widespread distribution, with large amounts in the lung and colon (>1000 pg/g wet tissue). The pituitary gland was the only organ containing higher amounts of ir-ET-3 than ir-ET-1 (ET-2). In reverse phase-high performance liquid chromatography coupled with EIAs, the ir-ET-3 was exclusively eluted at the position of synthetic ET-3, indicating that the ir-ET-3 was identical to ET-3. The abundance of ET-3 in the intestine, pituitary gland and brain indicates that ET-3 is a new brain-gut peptide which may have a physiological function in nervous and endocrine systems.

Endothelin-1 (ET-1), a 21-residue peptide isolated from porcine aortic endothelial cells, has potent vasoconstrictive effects on a variety of blood vessels (1). Studies on the cloning and sequences of prepro-ET-1 cDNA have revealed the identity of human and porcine ET-1s (1, 2). Recently, other types of human ET-related genes were found and named endothelin-2 (ET-2) and endothelin-3 (ET-3) (3, 4). The synthetic ET-3 peptide, differing from ET-1 by replacement of six amino acids, was also found to possess sustained vasoconstrictive and pressor activities characteristic of ETs, although ET-3 was the least potent among them (3, 4). ET-2, the most potent pressor peptide (3), differs from ET-1 by two amino acid residues. Although such structural and pharmacological studies have been done using synthetic ET peptides, there have been no direct evidence concerning

ABBREVIATIONS: ET-1, -2, -3, endothelin-1, -2, -3; big-endothelin-1, big-ET-1; EIA, enzyme immunoassay; ir-, immunoreactive; PBS, phosphate-buffered saline; RP-HPLC, reverse phase-high performance liquid chromatography; TFA, trifluoroacetic acid.

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the expression of ET-2 and ET-3 genes. Regional distribution of immunoreactive (ir-) ET was reported in some porcine and rat tissues (5, 6), but the concentrations of ET-2 and ET-3 in those tissues were not separately determined using immunoassays specific for ET-2 or ET-3.

Recently, we established a sensitive sandwich enzyme immunoassay (EIA) for ET-1 (7) and measured the plasma ir-ET levels in normal human adults (7, 8) and patients of acute myocardial infarction (9). In the present study, we established a sensitive and specific sandwich EIA for ET-3. Using these EIAs, we examined the tissue concentrations of ir-ETs in Sprague-Dawley (SD) rats and found relatively high concentrations of ir-ET-3 in the intestine, pituitary gland and brain.

MATERIALS AND METHODS

EIA for ET-3 : Synthetic peptides of human ET-1, ET-2, ET-3 and big-ET-1 were purchased from Peptide Institute (Osaka, Japan). Hybridoma, which produced monoclonal antibody against ET-3, was obtained by the fusion of myeloma cells (P_3U_1) with spleen cells of BALB/c mice immunized with synthetic ET-3-bovine thyroglobulin conjugates. The monoclonal antibody, AET-30, recognizes the N-terminal loop domain of ET-3 and does not react with an ET-3 C-terminal heptapeptide (15-21). The antibody against the C-terminal portion of ET-3, the sequence common to other ETs, was elicited in rabbits by immunizing ET-3(15-21)-bovine serum albumin conjugates. The Fab' fragment of this rabbit antibody was used as an enzyme-labeled detector antibody after being coupled with horseradish peroxidase (HRP). Sandwich-EIA for ET-3 was carried out as described previously (7). Briefly, AET-30-coated microtest plates were incubated at 4°C for 24 h with 100 µl of synthetic ET-3 or the test samples. After being washed with PBS, the plates were allowed to react at 4°C for 24 h with 100 µl of HRP-labeled anti-ET-3 (15-21) Fab'. After the plates were washed with PBS, the bound enzyme activity was measured using o-phenylenediamine as a chromogen (10).

EIA for ET-1 : ET-1 levels were measured by EIA for ET-1 as described previously in detail (7). The assay for ET-1 could detected as little as 0.2 pg/well of ET-1.

Tissues and extraction procedure : Male Sprague-Dawley (SD) rats weighing 390-410 g were bled from the abdominal aorta under anesthesia, and the tissues to be studied were removed, immediately frozen in liquid N_2 and stored at −30°C. ETs were extracted from rat tissues according to the method of Kitamura et al. (5) with minor modifications. Briefly, the tissues were homogenized with a polytron homogenizer for 60 sec in 10 volumes of 1 M acetic acid containing 10 µg/ml pepstatin (Peptide Institute, Japan) and immediately boiled for 10 min . The homogenates were centrifuged at 25,000 x g for 30 min at 4°C and the supernatants were stored at −30°C. About 0.6 g wet weight equivalent of the supernatant from each tissue was applied to a Sep-pak C18 cartridge (Waters, MA), and the adsorbed peptides were eluted with acetic acid:ethanol:water = 4:86:10, concentrated under a nitrogen gas stream (7), and subjected to sandwich EIAs for ET-3 and ET-1. Extraction efficiency of this method was examined by adding ^{125}I-ET-3 or ^{125}I-ET-1 (5 fmol, 2000 Ci/mmol, Amersham, Japan) to extracts of 0.6 g wet weight equivalent of cerebrum, lung, intestine and colon.

Reverse phase(RP) -HPLC : The tissue extracts were concentrated with a Sep-pak C18 cartridge as described above, and characterized by RP-HPLC on a TSK ODS-80TM (4.5 x 250 mm, TOSOH, Co., Japan) column. The solvents used were

A, 5% CH_3CN containing 0.05% TFA, and B, 60% CH_3CN containing 0.05% TFA. During the elution, the concentration of B was linearly elevated 0 - 40% over 5 min, 40 - 65% over 20 min and then 65 - 100% over 5 min at a flow rate of 1.0 ml/min. Each fraction (0.5 ml) was lyophilized and assayed for ir-ETs by the sandwich-EIAs.

RESULTS

Sandwich-EIAs

Sandwich-EIAs for ET-3 and ET-1 were conducted to find their reactivities with synthetic human ET-1, ET-2, ET-3 and big-ET-1 (Fig. 1). The EIA for ET-3 could detect as little as 0.2 pg/well of ET-3 without crossreactivity (less than 0.1%) with other ETs (Fig. 1A). On the other hand, the EIA for ET-1 fully crossreacted with ET-2, but not with ET-3 and big-ET-1 (crossreactivity : less than 0.2%) (Fig. 1B). These results indicate that the immunoreactivity of ETs can be divided into that of ET-3, and of a mixture of ET-1 and ET-2 [designated as ET-1 (ET-2)], and separately quantified by these EIAs.

Tissue distribution of immunoreactive ET-3 and ET-1 (ET-2)

The establishment of highly specific and sensitive EIAs enable us to identify rat organs containing ET-3 and also those abundant in ET-1(ET-2). The efficiency of extraction with Sep-pak C18 cartridges was assessed by adding ^{125}I-ET-3 or ^{125}I-ET-1 to extracts of cerebrum, lung, intestine, and colon. The recoveries were 93.5 ± 3.1% and 99.7 ± 5.5% for ^{125}I-ET-3 and ^{125}I-ET-1,

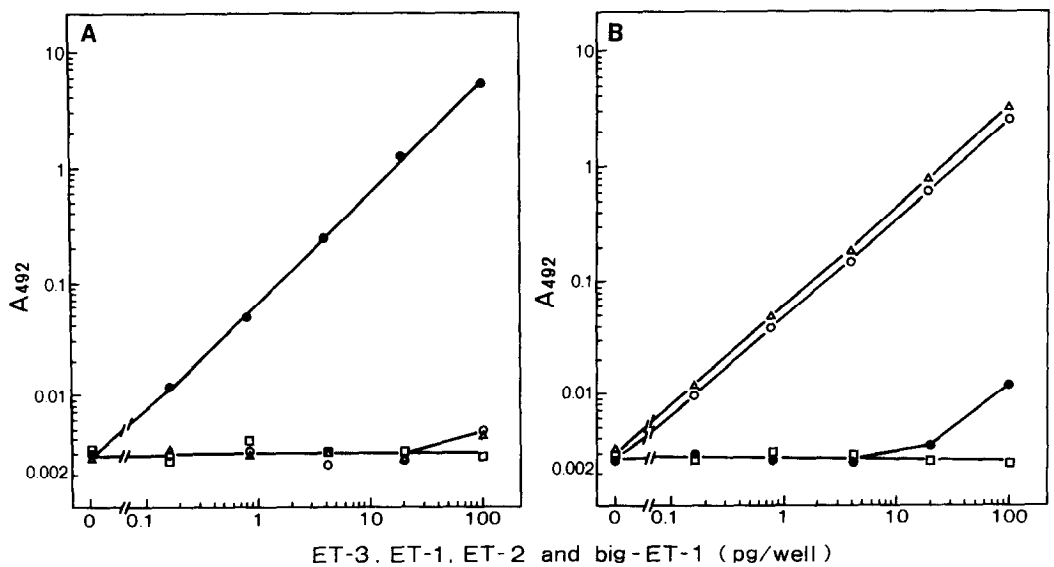

Fig. 1. Standard curves of synthetic human endothelins in sandwich-EIAs for (A) ET-3 and (B) ET-1. Synthetic human endothelins: ET-1 (○), ET-2 (△), ET-3 (●) and big-ET-1 (□).

Table 1

Distribution of ir-ET-3 and ir-ET-1 (ET-2) in rat organs

Organ	ir-ET-3	ir-ET-1(ET-2)	ir-ET-3/ir-ET-1(ET-2)
Cerebrum	100 ± 4.6	390 ± 35	0.25
Cerebellum	160 ± 30	250 ± 29	0.63
Medulla oblongata	40 ± 2.1	220 ± 7.1	0.18
Pituitary gland	190 ± 19	53 ± 5.8	3.58
Lung	250 ± 32	3400 ± 340	0.073
Heart	3.6 ± 0.43	340 ± 37	0.011
Aorta	< 2.2	180 ± 29	< 0.012
Liver	23 ± 2.1	190 ± 4.0	0.12
Spleen	2.4 ± 0.17	400 ± 18	0.006
Stomach	11 ± 1.5	540 ± 44	0.021
Pancreas	11 ± 1.3	390 ± 38	0.028
Intestine	460 ± 99	770 ± 30	0.61
Colon	57 ± 4.3	1100 ± 31	0.051
Kidney	27 ± 4.2	280 ± 37	0.096
Adrenal gland	12 ± 1.2	120 ± 16	0.10
Urinary bladder	2.2 ± 0.38	150 ± 35	0.014
Testis	7.7 ± 0.69	570 ± 47	0.014

Results are expressed as pg/g wet tissue.
Values are the mean ± S.E.M. of 5 samples.

respectively, indicating that both could be almost completely extracted by our methods. In addition, dilution curves for tissue extracts in EIAs were found to be parallel to the standard ones of both ET-3 and ET-1 (data not shown).

Table 1 shows the distribution of ir-ET-3 and ir-ET-1(ET-2) in 17 organs of SD rats. High concentrations of ir-ET-3 were found in the intestine, lung, pituitary gland and brain (cerebellum and cerebrum) (>100 pg/g wet tissue). Moderate concentrations of ir-ET-3 were detected in the colon, medulla oblongata, kidney and liver (>20 pg/g wet tissue). Parts of the circulatory systems such as the heart and aorta were found to contain low amounts of ir-ET-3. On the other hand, widespread distribution of ir-ET-1(ET-2) was observed in rat organs with the largest amounts in the lung (approximately 3400 pg/g wet tissue). Relatively high concentrations of ir-ET-1 (ET-2) were detected in the colon, intestine, testis and stomach (>500 pg/ g wet tissue). Other organs except for

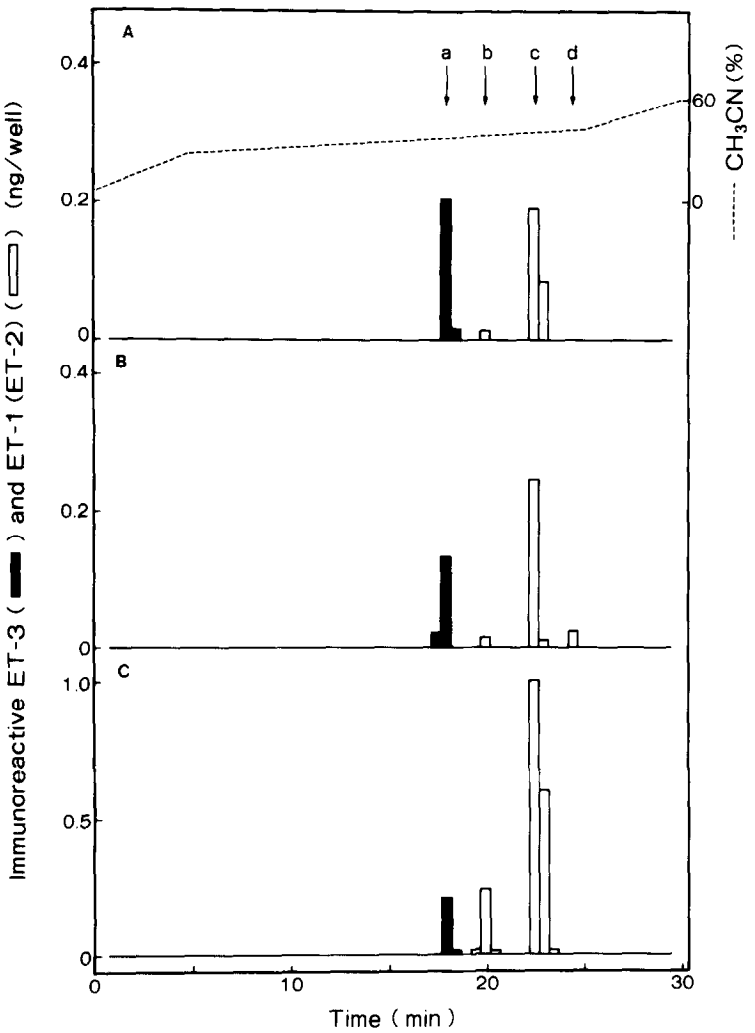

Fig. 2. RP-HPLC profiles of the extracts of (A) cerebellum (4.8g), (B) intestine (1.1g) and (C) lung (3.8g) on a TSK ODS-80TM column. Each fraction was lyophilized and subjected to sandwich-EIAs for ET-3 (solid bars) and ET-1 (open bars). Arrows indicate the eluted positions of (a) ET-3, (b) Met sulfoxide form of ET-1, (C) ET-1, and (d) ET-2.

the pituitary gland also contained more than 100 pg/g wet tissue of ir-ET-1(ET-2). The pituitary gland was the only organ which contained a higher concentration of ir-ET-3 than ir-ET-1(ET-2). Also relatively rich in ir-ET-3 were the cerebellum and intestine.

Characterization of immunoreactive ET-3 and ET-1(ET-2) in cerebellum, intestine and lung

ir-ET-3 and ir-ET-1(ET-2) in the cerebellum, intestine and lung were further characterized by RP-HPLC (Fig. 2). With all the tissues, ir-ET-3 exclusively emerged at the elution time of synthetic ET-3, indicating that ir-ET-3 detected by the EIA was identical to ET-3. On the other hand, more than 97%, 85% and 84% of ir-ET-1(ET-2) in the cerebellum, intestine and lung appeared at the

elution time of synthetic ET-1, indicating that the major ir-ET-1 (ET-2) detected by the EIA was ET-1 itself. A minor peak of ir-ET-1(ET-2) observed at 20 min may represent an oxidative product of ET-1. Immunoreactivity at the eluted position of synthetic ET-2 was observed in the intestine, which accounted for 10% of the total ir-ET-1(ET-2), but showed only trace amounts in the cerebellum and the lung. The extracts of colon, kidney and cerebrum showed similar elution patterns and only the colon contained a significant amount of ir-ET-1(ET-2) eluted at the position of synthetic ET-2 (data not shown).

DISCUSSION

This study clearly demonstrated the presence of ir-ET-3 in various rat organs especially in the intestine, lung, pituitary gland and brain. The immunoreactivity of ET-3 was specifically detected by a sandwich-EIA consisting of two antibodies directed against the N- and C-terminal portions of ET-3. Furthermore, RP-HPLC analysis showed that the ir-ET-3 detected by the EIA was identical to synthetic ET-3. These results indicate that the gene of ET-3 expressed in vivo in rat organs.

A comparative distribution study showed that more than 100 pg/g wet tissue of ir-ET-3 was observed only in the intestine, lung, pituitary gland and brain, whereas this amount of ir-ET-1(ET-2) was found in all the organs except in the pituitary gland. These results suggest that the expression of ET-3 gene is restricted to minor cellular subpopulations. The result agreed with the observation that ir-ET-1 but not ir-ET-3 was produced in various endothelial and nonendothelial cells such as normal kidney cells (11) and some tumor cells with epithelial-like morphology (12) in vitro (unpublished observations). ET-3 is found to be distinct from ET-1 and ET-2 in structure (3), pharmacological activity (3), and tissue distribution as shown by our study, suggesting that ET-3 has a physiological function different from that of ET-1 and ET-2.

ir-ET-1 was found at high levels in the lung, which agrees with the recent finding of ir-ET in porcine and rat tissues (5, 6). These results may be partially explained by the abundance of ET-1 binding sites in the lung (13). In addition, ir-ET-1 (ET-2) at the elution position of synthetic ET-2 in RP-HPLC may indicate the storage and/or production of ET-2 in the intestine and colon; however, further work is necessary to examine this speculation using an immunoassay selective for ET-2.

It has been well known that there are several kinds of peptides which distribute both in the central nervous systems and gastrointestinal tract, so-called brain-gut peptides such as gastrin, vasoactive intestinal polypeptide, cholecystokinin, neuropeptide Y etc. (14). The abundance of ir-ET-3 in the intestine, pituitary gland and brain found in the present study leads us to propose

ET-3 as a new brain-gut peptide and suggests a possible role for ET-3 in nervous and endocrine systems.

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