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REGIONAL DISTRIBUTION OF IMMUNOREACTIVE ENDOTHELIN IN PORCINE TISSUE: ABUNDANCE IN INNER MEDULLA OF KIDNEY

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SUMMARY: A specific and sensitive radioimmunoassay for endothelin has been developed. Half maximal inhibition of binding of radioiodinated endothelin was observed at 37 pg/tube and endothelin was detectable as low as 1 pg/tube. With this assay, the regional distribution of endothelin was determined in porcine tissue. The highest concentration of immunoreactive endothelin was observed in inner medulla of kidney (6.2 $\pm$ 1.1 pg/mg wet weight), while the concentration in kidney cortex was very low. Immunoreactive endothelin was also found in lung in relatively high concentration. The immunoreactive endothelin in porcine lung and inner medulla of kidney was further characterized by reverse phase high performance liquid chromatography combined with radioimmunoassay.

Endothelin (ET) is a potent vasoconstrictor peptide which has been recently identified in the culture supernatant of porcine aortic endothelial cells (1). ET consists of 21 amino acid residues with two intrachain disulfide bonds. The sequence is: H-Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp-OH. ET constricts a variety of blood vessels in many species and may be important in control of systemic blood pressure as well as local blood flow. The action of ET is thought to be associated with calcium entry via calcium channel (1). However the molecular form and distribution of ET in vivo has not yet been elucidated.

We have established a highly sensitive and specific radioimmunoassay (RIA) for ET. By using the RIA, we examined regional distribution of ET in porcine tissues and characterized immunoreactive (ir-) ET in lung and inner medulla of kidney.

ABBREVIATIONS: ET, endothelin; RIA, radioimmunoassay; ir-, immunoreactive; BSA, bovine serum alubumin; RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid.

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MATERIALS AND METHODS

Chemicals: Porcine ET and antiserum for porcine ET was purchased from Peptide Institute, Inc. (Osaka, Japan). Bovine serum albumin (BSA, Sigma) for RIA was pretreated with 5 mM N-ethylmaleimide for 24 hr at room temperature and was twice recrystallized.

Radioiodination: ET (5 μg) in 25 μl of 0.4 M sodium acetate buffer (pH 5.6) was introduced into a tube followed by the addition of Na ^{125}I (0.5 mCi/5 μl, Amersham). Lactoperoxidase (Boehringer Mannheim: 20 ng/10 μl of 0.1 M sodium acetate, pH 5.6) and H_2O_2 (100 ng/5 μl of water) were added into the reaction tube. After standing for 10 min at 30°C, H_2O_2 (100 ng/5 μl of water) was added to the tube, which was left for another 10 min at 30°C. Immediately after reaction, the mixture was submitted to reverse phase high performance liquid chromatography (RP-HPLC), where two major radioactive peaks were obtained. After removal of the first peak (monoiodinated ET sulfoxide), monoiodinated ET eluted in the second peak was purified and used as a tracer.

Procedures for RIA: The incubation buffer for RIA was 0.05 M sodium phosphate buffer (pH 7.4), containing 1% BSA, 0.1% Triton X-100, 0.08 M NaCl, 0.025 M EDTA 2Na, 0.05% NaN_3, and Trasylol 500 KIU/ml. A glass tube (10x75 mm) which had been siliconized was used for assay. All assay procedures were performed at 4°C. The standard ET or the unknown sample (100 μl) was incubated with anti-ET antiserum diluent and the tracer solution (18,000-20,000 cpm in 100 μl). After incubation for 48 hr, anti-rabbit IgG goat serum diluent (50 μl) was added. After standing for 40 hr, the tubes were centrifuged at 2,000 g x 30 min at 4°C and radioactivity of the precipitate was measured in an Aloka ARC-600 gamma counter.

Preparation of tissue sample: Porcine tissues were obtained at a local slaughter house. The tissues were homogenized with a polytron mixer for 60 sec in 10 volume of 1 M acetic acid and immediately boiled for 10 min to inactivate protease. After chilling, the homogenate was centrifuged at 25,000 g for 30 min and the supernatant was then stored. About 0.5 g wet weight equivalent of the supernatant from each tissue was loaded onto a Sep-Pak C-18 cartridge (Waters) which was preequilibrated with 1 M acetic acid, and the adsorbed materials were eluted with 3 ml of 60% acetonitrile in 0.1% trifluoroacetic acid (TFA). The eluate was evaporated in vacuum to dryness. The residual materials were dissolved in RIA buffer and the clear solution was submitted to RIA.

RESULTS AND DISCUSSION

RIA for ET: The antiserum to ET recognized the peptide with high affinity at a final dilution of 1:10,000. As shown in Fig.1, half-maximum inhibition of radioiodinated ligand binding by ET was observed at 37 pg/tube. From 1 to 256 pg/tube of ET was measurable by this RIA system. The intra- and inter-assay coefficients of variance were less than 3% and 7%, respectively. This antiserum recognized the C-terminus of ET (2) and had 100% crossreactivity with the Met sulfoxide form of ET. Putative rat ET, whose structure was deduced from the neucleotide sequence of cloned cDNA and had the same sequence as porcine ET near the carboxy terminus (3), showed nearly 100% crossreactivity in this RIA system. Recently, a study of structure-activity relationships of ET revealed that the C-terminus of ET is especially important for the potent vasoconstrictor

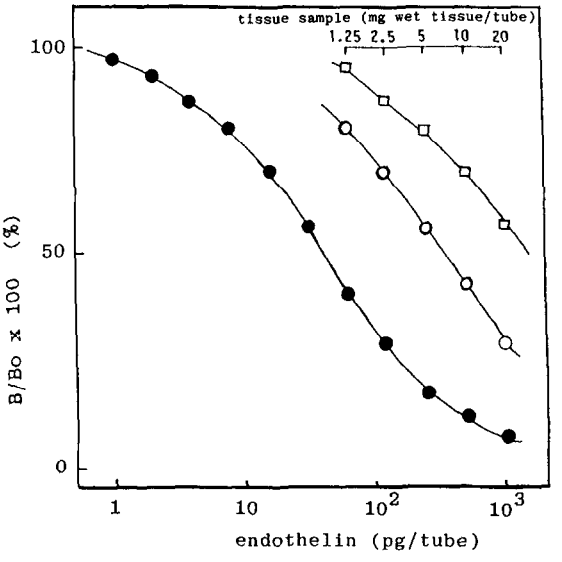

Fig.1. Standard curve of radioimmunoassay for ET(●-●). Inhibition of ^{125}I-ET binding to the antiserum by serial dilution of each sample from porcine inner medulla of kidney (○-○) and lung (□-□) respectively. The dilution curves are roughly parallel to those of standard ET.

activity characteristics of ET (4). Therefore the ir-ET determined by this RIA probably corresponds to the biological activity of ET.

Distribution of ir-ET in porcine tissue: The high specificity and sensitivity of the RIA for ET prepared above enabled us to determine its regional distribution in porcine tissue. However the acid extract from each tissue had to be treated with Sep-Pak C-18 cartridge (Waters), since tissue concentrations of ir-ET in pig were found to be low.

Table I

Distribution of immunoreactive endothelin in porcine tissue

Region	Immunoreactive endothelin
Brain Cortex	0.58 ± 0.084
Hypothalamus	0.69 ± 0.085
Heart Atrium	0.94 ± 0.092
Ventricle	0.91 ± 0.086
Lung	2.19 ± 0.37
Liver	0.25 ± 0.036
Spleen	0.57 ± 0.10
Aorta	0.34 ± 0.077
Kidney Cortex	0.36 ± 0.051
Outer medulla	0.31 ± 0.049
Inner medulla	6.2 ± 1.1
Adrenal gland	0.24 ± 0.035
Duodenum	0.34 ± 0.039

Results are expressed as pg/mg wet tissue.
All values are mean ± standard deviation for six samples.

Prior to the measurements of ir-ET, the efficiency of extraction and purification, and reliability of RIA procedures were validated by the following experiments with porcine inner medulla of kidney and lung;

1) ^{125}I-ET, which had been added to the tissue before homogenization, was found to be completely extractable within experimental error.

2) ET in the acid extracts was reproducibly found to be more than 90% recovered when it was purified with Sep-Pak C-18 cartridge.

3) Dilution of RIA sample, prepared as described in Methods, yielded competition curves that were parallel to the standard curves of ET as shown in Fig 1.

4) An appropriate amount of cold synthetic ET, which had been added to the RIA sample, proved to be precisely determined by the present RIA.

Table I summarizes the regional distribution of ir-ET in porcine tissue. Inner medulla of kidney contained 6.2 ± 1.1 pg/mg wet tissue of

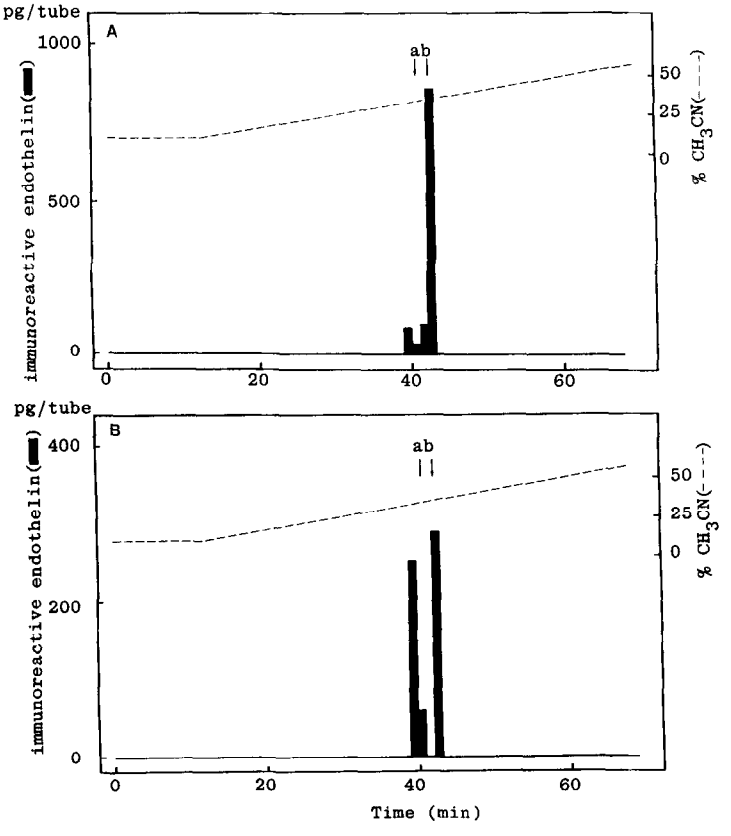

Fig.2. Reverse phase HPLC of porcine tissue extract monitored by RIA for ET.
Sample: (A) Lung (400 mg eq.). (B) Inner medulla of kidney (100 mg eq.). Column: Cosmosil ODS-300 (4.6 x 250 mm, Nakarai Chemicals. LTD.). Solvent system: H_2O:CH_3CN:10%TFA = (I) 90:10:1, (II) 40:60:1 (v/v). Linear gradient from (I) to (II) for 60 min. Flow rate: 1 ml/min. Arrows indicate elution position of (a) ET sulfoxide and (b) ET.

ir-ET. This concentration was highest of all the porcine tissue examined, while the concentration of ir-ET in cortex and outer medulla of kidney was very low. Since the inner medulla of the kidney contains collecting duct, tubules and Henle's loop, ET may be a regulator of urine formation as well as vasoconstrictor when it acts in situ. We are now testing whether ir-ET is released from the kidney and whether there is immunocytochemical localization of ET in inner medulla of kidney. Ir-ET was also found in porcine lung in comparable concentrations, however the concentration of ir-ET in other tissue was less than 1 pg/mg wet tissue. The low concentration of ir-ET in aorta was not expected because it has been reported that preproendothelin mRNA is expressed in porcine aortic intima in situ (1). This discrepancy may be explained by the possibility that the ET related peptide in the aorta is another molecular form whose carboxy terminus cannot be recognized by the present RIA.

Characterization of ir-ET in lung and inner medulla of kidney: The ir-endothelin in lung and inner medulla of kidney was further characterized by RP-HPLC coupled with the RIA for ET. More than 90% of ir-ET in lung emerged at an identical elution time with authentic endothelin as shown in Fig. 2A, suggesting that ir-ET in lung is ET itself. However ir-ET in inner medulla of kidney was separated into two major peaks as shown in Fig. 2B. About 50% of ir-ET emerged at an elution position identical to that of authentic ET, but the rest of the ir-ET eluted 3 min earlier. The Met sulfoxide form of ET eluted between the two peaks of ir-ET. The physiological function of the earlier eluted ir-ET is unknown, but the RIA used in this work recognized the carboxy terminal region of ET whose sequence is especially important for the potent vasoconstrictor activity (5). Therefore the earlier eluted ir-ET probably had the carboxy terminal region of ET and also had the biological action of ET. This is now undergoing further investigation.

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