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IMMUNOREACTIVE ENDOTHELIN IN RAT KIDNEY INNER MEDULLA: MARKED DECREASE IN SPONTANEOUSLY HYPERTENSIVE RATS

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SUMMARY: Using a specific and sensitive radioimmunoassay for endothelin, the regional distribution and molecular form of endothelin was investigated in rat tissue. The highest concentration was observed in the inner medulla of the kidney (8.7±2.2 pg/mg wet weight). On two kinds of reverse phase high performance liquid chromatography, immunoreactive endothelin in the inner medulla of the kidney was separated into two peaks at positions where authentic porcine/human and putative rat/human endothelin eluted. Furthermore, the concentration of immunoreactive endothelin in the inner medulla of the kidney was remarkably decreased in spontaneously hypertensive rats (SHR) compared with normotensive control Wistar Kyoto rats (WKY), but no difference was observed in lung immunoreactive endothelin.

Endothelin (ET) is a potent vasoconstrictor peptide that has been identified in the culture supernatant of porcine aortic endothelial cells (1). ET consists of 21 amino acid residues with two intrachain disulfide bonds. Sequence analysis of cloned human ET cDNA showed that the amino acid sequence of human ET is identical to that of porcine ET (2). Subsequent studies have indicated that the nucleotide sequence of the rat gene predicts a 21 residue peptide with 6 replacements of amino acid residues when compared with porcine/human ET (3). Very recently, three distinct human endothelin-related genes were cloned, and one of the gene related ETs in humans, was found to be identical with rat ET (4). The sequences of the ETs are:

ET-1 (porcine/human ET) : CSCSSLMDKECVYFCHLDIIW
ET-2 (putative human ET) : CSCSSWLDKECVYFCHLDIIW
ET-3 (putative rat/human ET) : CTCFTYKDKECVYYCHLDIIW

ABBREVIATIONS: ET, endothelin; RIA, radioimmunoassay; ir-, immunoreactive; BSA, bovine serum albumin; RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto rats.

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ETs constrict a variety of blood vessels in many species and may be important in controlling systemic blood pressure as well as local blood flow. However, it has never been determined if ET-2 or ET-3 exists in vivo. Recently, we have developed a specific and sensitive radioimmunoassay (RIA) for ET (5). Using this RIA, the present study revealed that the inner medulla of rat kidney contains an abundant amount of immunoreactive (ir-) ET, and the major ir-ET consists of two types of ET: ET-1 and ET-3. Furthermore, we found that this ir-ET is significantly decreased in spontaneously hypertensive rats (SHR) as compared to the normotensive control Wistar Kyoto rats (WKY).

MATERIALS AND METHODS

Chemicals: ET-1, ET-2 and ET-3 and antiserum (Lot No. 884-381207) for porcine ET were 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 then recrystallized twice.

RIA for ET: The RIA for ET used in this study was performed as described (5), with some modifications. 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 siliconized glass tube (10x75 mm) was used for the assay. All assay procedures were performed at 4°C. The standard ET or unknown sample (100 µl) was incubated with anti-ET antiserum diluent (50 µl) at a final dilution of 1:10,000. After standing for 15 hr, the tracer solution (18,000-20,000 cpm in 50 µl), prepared as described (5), was added. After incubation for 24 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: To examine the regional distribution of ir-ET in rat tissue, male Wistar rats (200-250 g) were purchased from Kyudou Co. (Kumamoto, Japan). SHR and WKY were kindly donated by Dr. K. Okamoto (Kinki University School of Medicine, Osaka, Japan) and maintained by selective mating in our laboratory. Tissues were homogenized with a polytron mixer for 60 sec in 10 volumes of 1 M acetic acid containing 0.01% Triton X-100 and immediately heated at 100°C for 10 min to inactivate proteases. After chilling, the homogenate was centrifuged at 25,000 g for 30 min, and the supernatant was then stored. About 0.25 g wet weight equivalent of the supernatant from each tissue was loaded onto a Sep-Pak C-18 cartridge (Waters), which had been 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 under a vacuum to dryness. The residual materials were dissolved in RIA buffer, and the clear solution was subjected to RIA.

Characterization of ir-ET in the inner medulla of rat kidney: To identify and characterize the ir-ET in the inner medulla of rat kidney, inner medulla of the kidneys from twelve rats (1.85 g) were extracted with five volumes of the above extraction buffer and immediately heated at 100°C for 10 min. After removal of precipitates, the supernatant was loaded on three Sep-Pak C_{18} cartridges. The fraction eluting at 60% CH_3CN in 0.1% TFA was concentrated, and then submitted to Sephadex G-50 gel filtration. ET immunoreactive fractions were further separated by reverse phase high performance liquid chromatography (RP-HPLC) on a Cosmosil 300 ODS (4.6x300 mm, Nakarai Chemicals) column with a linear gradient elution of CH_3CN (10-

60%) in 0.1% TFA solution. ET-immunoreactive fractions were also analyzed by a different RP-HPLC on a TSK-gel ODS-120T column (4.0x150 mm, Toyosoda) using a linear gradient of CH_3CN (10-60%) in 10 mM NH_4COOH (pH 4). Each fraction was monitored by RIA for ET. Oxidation of the peptide or sample was performed in 1 M HCOOH containing 0.05% H_2O_2 at 30°C to convert Met residues into Met sulfoxides.

Statistical analysis: The data for the SHR were compared to those of WKY rats by Student's t-test. Data are presented as means ± standard deviation.

RESULTS AND DISCUSSION

Regional distribution of ir-ET in rat tissue: The results of RIA shown in Fig. 1 indicate that half-maximum inhibition of the binding of radioiodinated ET by ET-1 was at 26pg/tube, which was improved compared to the RIA described before (5) because of the late addition of ^{125}I-ET. The RIA used here recognized ET-3 with 40% crossreactivity as shown in Fig. 1. This figure also shows that the dilution of the RIA sample from rat kidney inner medulla and lung yielded competition curves that were roughly parallel with the standard curves of ET-1 and ET-3. Prior to the measurement of ir-ET, the efficiency of extraction, purification, and reliability of this RIA procedure were validated by experiments with rat kidney inner medulla and the lung as previously described (5).

Table I summarizes the regional distribution of ir-ET in porcine tissue. The inner medulla of the kidney contained ir-ET at the level of 8.7 ± 2.2 pg/mg wet tissue, the highest of all the rat tissue examined. In contrast, the concentration of ir-ET in the kidney cortex was very low. Recently, it was shown that the inner medulla of rat kidney contained an abundant number of ET receptors (6). Therefore, ET in the inner medulla

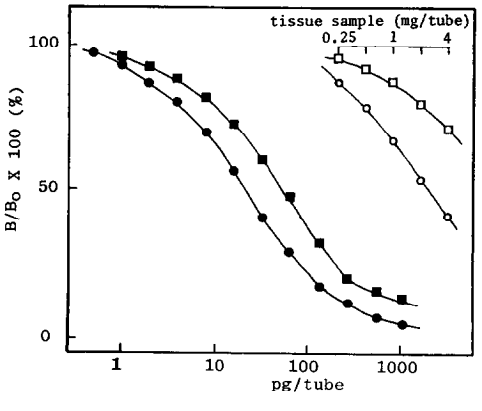

Fig. 1. Standard curve of radioimmunoassay for ET-1 (●-●) and ET-3 (■-■). Inhibition of ^{125}I-ET binding to the antiserum by serial dilution of the sample from rat kidney inner medulla (○-○) and lung (□-□), respectively. The dilution curves are roughly parallel to those of standard ET.

Table I

Distribution of ir-ET in rat tissue

Region	Ir-ET
Brain Cortex	0.65 ± 0.089
Hypothalamus	0.72 ± 0.093
Heart Atrium	0.91 ± 0.12
Ventricle	0.94 ± 0.14
Lung	3.4 ± 0.82
Liver	0.22 ± 0.027
Spleen	0.97 ± 0.15
Kidney Cortex	0.58 ± 0.071
Inner medulla	8.7 ± 2.2
Duodenum	0.39 ± 0.037

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

would work in situ if it is produced there. Since the inner medulla contains collecting ducts and Henle's loop, ET may be a regulator of urine formation as well as a vasoconstrictor. Ir-ET was also found in rat lung in a comparable concentration, whereas the concentration of ir-ET in other tissues was not high. The regional distribution of ir-ET in rat tissue determined in this work is similar to the results for porcine tissue (5).

Characterization of ir-ET in rat inner medulla of the kidney: The ir-ET in the inner medulla of rat kidney was further characterized by Sephadex G-50 gel filtration and two kinds of RP-HPLC coupled with the RIA for ET. As shown in Fig. 2, on Sephadex G-50 gel filtration, about 85% of the medulla ir-ET emerged at the same elution position as authentic ET-3 and ET-1, but about 15% of the ir-ET eluted at an earlier time, corresponding to a molecular weight of about 6000 Da. Judging from the molecular weight, the earlier eluted ir-ET may be a dimer of the ETs.

The fractions from the Sephadex G-50 column containing the major ir-ET were collected and applied to RP-HPLC on a Cosmosil ODS 300 column (4.6 x 300 mm), which was eluted by a linear gradient of CH_3CN (10-60%) in 0.1% TFA. As shown in Fig. 3A, the ir-ET separated into major and minor peaks: Minor ir-ET emerged at the position of authentic ET-3 and the major ir-ET eluted later at the same position as authentic ET-1.

To confirm that elution positions determined above are identical with those of each authentic peptide, ir-ET in the same sample was reanalyzed with a different RP-HPLC on a TSK-gel ODS 120T column (4.6 x 150 mm) using a linear gradient of CH_3CN (10-60%) in 10 mM NH_4COOH (pH4). As shown in Fig. 3B, two peaks of ir-ET emerged at positions almost identical to those of authentic ET-3 and ET-1. These data strongly indicate that ET-3, which

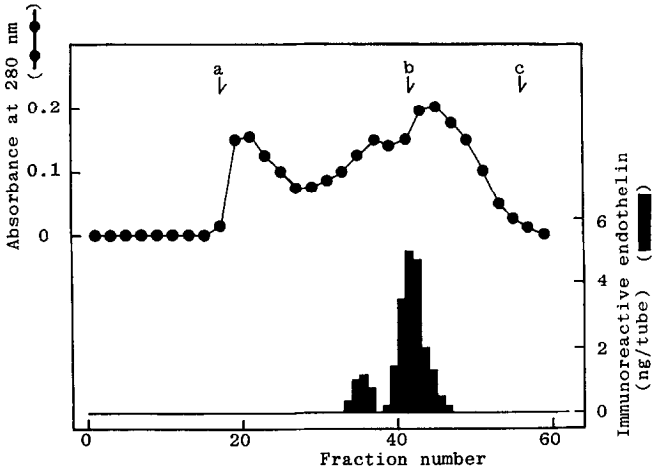

Fig. 2. Sephadex G-50 gel filtration of the tissue extract from rat kidney inner medulla monitored by absorbance at 280 nm as well as RIA for ET.
Column size : 1.8 x 140 cm, Fraction size: 8.0 ml/tube, and Elution buffer: 1 M acetic acid containing 0.01% Triton X-100.
Arrows indicate (a) Void volume and the elution positions of (b) ET-1 and ET-3 (c) NaCl.

has been deduced from the nucleotide sequence of cloned ET from the rat and human genomic library (3,4), is expressed from DNA and exists in vivo. Further purification and characterization of this ir-ET is now in progress.

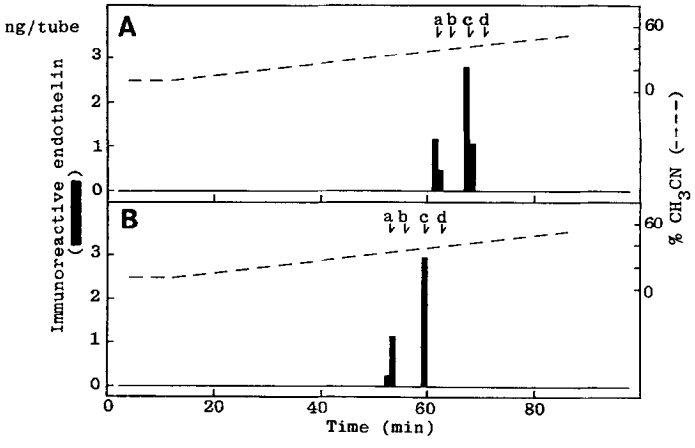

Fig. 3. Reverse phase HPLC of ir-ET in rat kidney inner medulla monitored by RIA for ET.
Sample: (A) One third of the major ir-ET fraction in Fig. 2.
(B) Same as (A).
Column: (A) Cosmosil ODS-300 (4.6 x 250 mm, Nakarai Chemicals).
(B) TSK ODS SIL 120-T (4.6 x 150 mm, Toyosoda).
Solvent system: (A) H_2O:CH_3CN:10%TFA = (I) 90:10:1, (II) 40:60:1 (v/v). Linear gradient elution from (I) to (II) for 120 min.
(B) H_2O:CH_3CN:100mMNH_4COOH(pH 4) = (I) 80:10:1, (II) 30:60:1 (v/v). Linear gradient elution from (I) to (II) for 120 min.
Flow rate: 1 ml/tube. Arrows indicate the elution positions of (a) ET-3, (b) ET-1 sulfoxide, (c) ET-1 and (d) ET-2.

The later eluted ir-ET emerged at almost the same position as ET-1 on two kinds of RP-HPLC. Furthermore, the later eluted ir-ET, which had been oxidized by H_2O_2, eluted at the same position as Met sulfoxide ET-1 (data not shown), indicating that the later eluted ir-ET contained a Met residue that is not contained in ET-3 but is found in ET-1. Although the ratio of the earlier eluted ir-ET to the later eluted one was observed to vary among the sample preparations, the later eluted ir-ET is 2-4 times larger than the earlier eluted one. If the earlier and later eluted peptides are identical to ET-3 and ET-1, respectively, the quantities of these two peptides in the inner medulla of rat kidney are estimated to be comparable because the RIA used in this work recognized ET-3 with 40% crossreactivity. Recently, it has been suggested that rat genomic DNA also has three chromosomal loci that encode endothelin or similar peptides (4). According to the data presented here, we think that the major ir-ET in the rat kidney inner medulla is composed of two types of ET, one is ET-3 and the other is identical to or very similar to ET-1.

Comparison of ir-ET in lung and inner medulla of the kidney between SHR and WKY: ET is a potent vasoconstrictor peptide, so it may be related to the pathophysiology of hypertension. To elucidate the possible roles of ET in blood pressure regulation, the concentrations of ir-ET in the kidney inner medulla and lung from SHR were compared with those of WKY.

The systolic blood pressure of SHR and WKY was 188 ± 15 mmHg and 114 ± 10 mmHg, respectively. Table II summarizes the concentration of ir-ET in the kidney inner medulla and lung from SHR and WKY. SHR and WKY had similar lung ir-ET concentrations, but the ir-ET concentration in the kidney inner medulla was markedly decreased in SHR compared with that in WKY.

The pathophysiological significance of the decreased ir-ET in the inner medulla of the kidney from SHR remains obscure. One possibility is that the marked decrease of ir-ET in SHR inner medulla is due to a compensatory reaction to hypertension, because ET is a vasoconstrictor

Table II

Comparison of systolic blood pressure (SBP) and ir-ET between WKY and SHR

	SBP (mmHg)	Ir-ET in lung	Ir-ET in inner medulla of the kidney
WKY	114 ± 9.9	3.54 ± 1.13	10.6 ± 2.4
SHR	188 ± 15[a]	3.32 ± 0.82[b]	2.16 ± 0.56[a]

The concentration of Ir-ET is expressed as pg/mg wet tissue.
All values are the mean ± standard deviation for eight samples.
a : $P < 0.001$, compared to WKY; b : not significant.

peptide. If we compare the renal and pulmonary circulation between SHR and WKY, the inner medulla of the kidney may be perfused at a higher pressure in SHR than in WKY, whereas the pulmonary pressure is considered to be equal between WKY and SHR, if neither have heart failure as was true in the present study. Therefore, the results presented here are reasonable and the compensatory decrease of ir-ET in hypertension is not inconsistent with reported results, i.e., the expression of the preproendothelin gene is significantly down-regulated when endothelial cells are cultured in the presence of a medium flow (1). However, the possibility of a hereditary lack of ir-ET in SHR cannot be ruled out at present. We are now investigating whether our results are due to such a hereditary lack of ir-ET in SHR inner medulla of the kidney or due to a compensatory reaction to hypertension.

The physiological function of ir-ET in the inner medulla of rat kidney is unknown, but its marked decrease in SHR inner medulla indicates that this peptide plays a profound role in the pathophysiology of hypertension.

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