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Presence of immunoreactive endothelin in human plasma

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A highly specific and sensitive radioimmunoassay has been established for measurement of human endothelin (hET) in human plasma. After extraction of plasma with an octyl-silica column, this assay allowed for detection of immunoreactive (IR) hET as low as 0.2 fmol/ml. In 16 healthy subjects, the mean concentration of plasma IR-hET was 0.6 fmol/ml. Reverse-phase HPLC coupled with radioimmunoassay revealed two major IR-hET components, one corresponding to authentic hET(1–21) and another with more hydrophilicity than hET(1–21). These data indicate that ET is a circulating vasoconstrictor hormone in man.

Endothelin; Radioimmunoassay; Reverse-phase HPLC; (Human plasma)

1. INTRODUCTION

A novel potent vasoconstrictor peptide, endothelin (ET), has recently been isolated and sequenced from the supernatant of cultured porcine aortic endothelial cells [1]. Porcine (p) ET consists of 21-amino acid residues containing two intramolecular disulfide linkages and derives from prepropET with 203-amino acid residues through an unusual proteolytic processing. Recent DNA cloning studies have shown that human (h) ET was identical with pET [2], but rat (r) ET was different by substitution of 6-amino acid residues [3]. However, there is no information available yet as to whether ET circulates in the blood. Therefore, the present study was designed to establish a highly sensitive and specific radioimmunoassay (RIA) for hET, to measure concentrations of immunoreactive (IR) hET in normal human plasma and characterize the circulating form of IR-hET by reverse-phase HPLC.

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2. MATERIALS AND METHODS

2.1. *Production of antibody for hET*

Synthetic hET (Peptide Institute, Inc., Osaka, Japan) was conjugated to bovine thyroglobulin (Sigma Chemical, St. Louis, MO) using carbodiimide as a coupling agent. The conjugate (100 μg) emulsified with complete Freund's adjuvant (Difco Labs, Detroit, MI) was biweekly injected intradermally at multiple sites on the back of Japanese white rabbits. After repeated immunizations, the antiserum suitable for RIA was obtained.

2.2. *hET RIA*

The assay buffer for RIA was 0.01 M phosphate buffer, pH 7.4, containing 0.14 M NaCl, 0.01 M EDTA dipotassium salt (K_2EDTA), 0.02 M glycine, 0.01 M ϵ-aminocaproic acid, 0.001 M sodium azide, 0.1 mg/ml heat inactivated human serum albumin (KabiVitrum, Stockholm, Sweden) and 0.1% Triton X-100. This assay buffer was used to dissolve all reagents. The incubation mixture consisted of 0.1 ml sample or standard, 0.1 ml assay buffer and 0.1 ml anti-hET-serum (final dilution: 1:7000). Incubation was carried out at 4°C for 24 h, followed by the late addition of 0.05 ml ^{125}I-hET (83 pmol/ml; spec. act. 74 TBq/mmol, Amersham International plc, England) and further incubation at 4°C for 48 h. The bound from free ligands were separated by adding 0.5 ml assay buffer containing 10 μl goat anti-rabbit γ-globulin (Daiichi-RI, Tokyo, Japan), 1 μl normal rabbit serum and 5% polyethylene glycol (M_r 7500), and centrifugation at 2000 × g for 30 min. The radioactivity in the precipitate was counted in a γ-spectrometer. The standard curve was constructed using B/T vs fmol of hET standard. The amount of ET in the unknown

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samples was extrapolated from the standard curve linearized by a logit-log transformation.

2.3. *Plasma extraction*

10-ml blood samples from normal volunteers were withdrawn into ice-chilled tubes containing K_2EDTA, and plasma was immediately separated by centrifugation at 4°C. A 2-ml aliquot of plasma acidified with trifluoroacetic acid (TFA) was applied to Spe-C_8 cartridge (J.T. Baker Chemical Co., Phillipsburg, NJ) prewashed sequentially with methanol, distilled water and 0.09% TFA. The materials adsorbed to the cartridge were eluted with 2 ml 60% acetonitrile/0.09% TFA and evaporated by a centrifugal concentrator. The dried residues were reconstituted in the assay buffer and subjected to RIA. For chromatographic analysis of plasma IR-hET, 100 ml aliquot of pooled normal human plasma was similarly separated and subjected to reverse-phase HPLC.

2.4. *Reverse-phase HPLC*

Reverse-phase HPLC was performed using an octadecyl-silica column (0.45 × 25 cm, JASCO, Tokyo, Japan) eluted with a linear gradient of acetonitrile from 15 to 60% in 0.09% TFA for 1 h with a flow rate of 1 ml/min; 1-ml fractions were collected and assayed for IR-hET. The recovery of standard hET was 96% during HPLC analysis.

3. RESULTS

As shown in fig.1, the minimum detectable quantity of hET was 0.2 fmol/tube (99% confidence), and the 50% intercept was 6 fmol/tube. The intra- and interassay coefficients of variation (n = 5) were 10 and 13%, respectively. The antibody crossreacted equally with pET and rET, 27% with hET(16–21), and less than 0.1% with

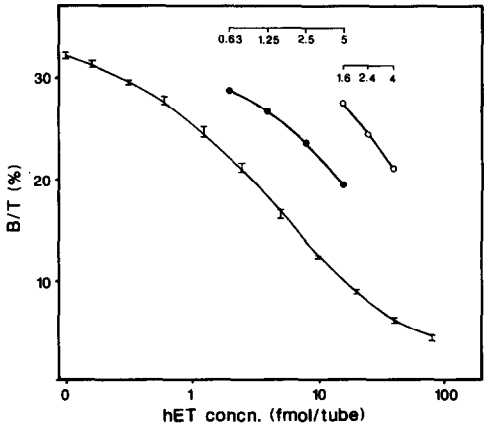

Fig.1. Radioimmunoassay (RIA) for hET. Standard curve for hET (bars: mean ± SD, n = 3) and dilution curves of normal human plasma extracts (○,●) are shown. Serial dilutions of original plasma volume are denoted above the curves.

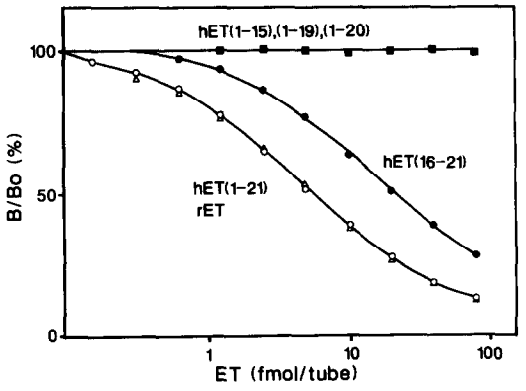

Fig.2. Crossreactivities of various ET analogs in hET RIA. Serial dilution curves of hET(1–21) (○), rET(1–21) (△), hET(16–21) (●), hET(1–20) (■), hET(1–19) (■), and hET(1–15) (■) are shown. Total binding was 31% of ^{125}I-hET added; nonspecific binding was 2.9 ± 0.6% of total binding (n = 10). Each point is the mean of two experiments.

hET(1–20), hET(1–19) and hET(1–15) on a molar basis (fig.2), whereas it did not show any crossreactivities with α-human atrial natriuretic peptide, porcine brain natriuretic peptide, angiotensin II or arginine-vasopressin. The dilution curves generated from human plasma extracts were parallel to that of standard hET (fig.1). The recovery of unlabeled hET added to human plasma was 61 ± 3% throughout the extraction procedure. The mean concentrations of IR-hET in 16 normal

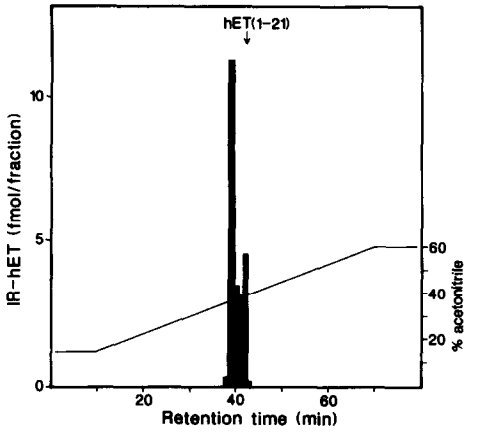

Fig.3. Reverse-phase HPLC profile of IR-hET in human plasma extracts. Closed columns indicate the concentrations of IR-hET in each fraction. The elution position of standard hET(1–21) is shown by the arrow. A linear gradient of acetonitrile from 15 to 60% is denoted by the solid line.

subjects (8 males and 8 females, aged 28.9 ± 5.4 yr old) were 0.6 ± 0.2 fmol/ml (mean ± SD).

An elution profile of pooled human plasma extracts on reverse-phase HPLC is shown in fig.3. Two major components with IR-hET were observed: one component eluted in the position of standard hET(1–21); while the other component, representing about two-thirds of the total IR-hET, eluted earlier than hET standard.

4. DISCUSSION

In the present study, we have developed a sensitive RIA for hET with a minimum detectable dose of 0.2 fmol, thus enabling us to measure IR-hET in normal human plasma. The antibody used in the present RIA recognizes the common carboxyl-terminal residues shared by hET and rET. Using a variety of synthetic hET analogs, the principal antigenic determinant of the antibody was shown to be C-terminal Trp^{21} residue. This single amino acid residue has recently been shown to be essential for its biological activity [4] as well as receptor-binding activity (unpublished). Therefore, IR-hET as determined by the present RIA should reflect bioactive hET and related peptides. By extracting plasma with octyl-silica, the present study has demonstrated for the first time the presence of IR-hET in normal human plasma, of which concentrations were very low (0.6 fmol/ml).

Reverse-phase HPLC revealed that IR-hET in human plasma consisted of at least two major forms. The second IR-hET component appears to have a retention time identical with that of authentic hET(1–21). On the other hand, the first component representing about two-thirds of the total IR-hET eluted earlier than hET(1–21), suggesting its weaker hydrophobicity than hET(1–21). It has been suggested that hET(1–21) is generated from 'big-ET', an intermediate form with 39-amino acid residues, through an unusual proteolytic processing [1]. Big-ET has a C-terminal extension with more hydrophilic residues. Therefore, it is possible that the major IR-hET component in human plasma may be a big-hET and/or related peptides. The precise mechanism of posttranslational processing of hET in vivo remains to be determined.

In conclusion, our study demonstrates the presence of IR-hET in human plasma, suggesting that it has a potential role as a circulating hormone. Determination of plasma levels of IR-hET in normal and pathological conditions should provide important clues to understanding its physiological and pathophysiological role(s) in man.

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