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Detection of immunoreactive endothelin in plasma of hemodialysis patients

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Two types of radioimmunoassay (RIA) methods for measuring endothelin (ET) in human plasma were developed. One was an extraction procedure using a Sep-Pak C_{18} cartridge, the other being a direct method. By the extraction method, plasma ET levels were lower than the detectable limit (7 pg/ml) in normal subjects and elevated in hemodialysis patients. The absolute values obtained via the direct method were 20-times higher than those from extraction. Gel-filtration experiments revealed that this discrepancy was mainly due to immunoreactive (IR-) endothelin-like substances of high molecular mass near 11.6 kDa (large IR-ET). Extraction of the peptide by the C_{18} cartridge could eliminate interference by large IR-ET and is important in the accurate measurement of ET concentrations in plasma.

Endothelin; Radioimmunoassay; Hemodialysis; Immunoreactivity; (Human plasma)

1. INTRODUCTION

Endothelin (ET), a novel 21-residue vasoconstrictor peptide, was recently discovered by Yanagisawa et al. [1]. ET is known to elevate the arterial blood pressure of rats and it has been suggested that disturbances in the control of ET production contribute to the pathogenesis of hypertension [1,2]. However, it remains unknown as to whether ET exists in human plasma. Here, we have developed highly sensitive and specific radioimmunoassay (RIA) systems for ET and surveyed immunoreactive (IR-) ET in human plasma. Two types of RIA were employed: one for extraction using a Sep-Pak C_{18} cartridge and the other for a direct procedure necessary for future studies using small animals.

2. MATERIALS AND METHODS

2.1. *Blood samples*

The studied population consisted of 42 subjects, including 10 normal subjects (6 men, 4 women; 34.7 ± 15.5 years, mean ± SD) and 32 patients with chronic renal failure (16 men, 16 women; 58.1 ± 13.0 years) undergoing hemodialysis (HD) 2-3 times per week for 3-5 h per session. Blood samples were collected from peripheral veins in the morning before breakfast after 30 min of recumbency in normal subjects. In HD patients blood samples were collected from the forearm subcutaneous A-V fistula before dialysis. The blood was transferred to a chilled tube containing EDTA and Trasylol, and centrifuged at 3000 rpm for 10 min at 4°C. The plasma was stored at −20°C until assayed.

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2.2. *Extraction procedure*

Extraction of ET was as follows: 3 ml of each plasma sample was acidified with 6 ml of 4% acetic acid and applied to a Sep-Pak C_{18} cartridge (Waters, Milford, MA). The adsorbed peptide was eluted with 2 ml of 60% acetonitrile in 0.5% ammonium acetate, dried down under compressed air, reconstituted in 0.5 ml assay buffer, and subjected to RIA. The assay buffer used for RIA was 0.1 M phosphate buffer (pH 7.7), containing 0.1% human serum albumin, 0.2% Triton X-100 and 0.1% NaN_3. ET was also extracted from 1 l of dialysate from a HD patient via the same procedure.

2.3. *Radioimmunoassay method*

Synthetic human ET and ET antisera were purchased from the Peptide Institute (Osaka). ET was radioiodinated using a modified chloramine-T method [3], labeled ET being purified on a Sephadex G-50 superfine column (1 × 25 cm) as described [4].

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First, 0.2 ml of sample (standard solution or reconstituted extract) was incubated with 0.1 ml diluted antibody (final dilution 1/10000) at 4°C for 48 h. For direct assays, 0.1 ml of sample plasma or charcoal-treated plasma, 0.1 ml assay buffer or standard solution and 0.1 ml antibody at the same dilution as used in the extraction were incubated. 0.1 ml ^{125}I-ET (approx. 3000 cpm) was then added, followed by a further incubation for 24 h at 4°C. Free and bound ligands were separated by a secondary antibody immunoprecipitation techniques as in [4].

2.4. *Gel filtration and high-performance liquid chromatography*

3 ml plasma from HD patients was run on a Sephadex G-50 superfine column (1.8 × 44 cm) at 0.3 ml/min. The eluent was 1 M acetic acid and fractions of 2 ml were collected, dried down and assayed as described. Reverse-phase high-performance liquid chromatography (HPLC) of the extracts from 60 ml pooled plasma of HD patients and from 1 l of dialysate was performed on a column of M-Bondapak C18 (3.9 × 300 mm, Waters). A linear gradient elution system was employed from 30 to 60% acetronitrile in 0.1% trifluoroacetic acid over 30 min. The flow rate was 1.0 ml/min, and fractions of 2 ml were collected, dried down, reconstituted and assayed as described.

Results are expressed as means ± SD.

3. RESULTS

3.1. *Characteristics of the radioimmunoassay*

Fig.1 shows a standard curve for the extraction method, derived from 10 consecutive assays, and serial 2-fold dilution curves of a plasma extract and plasma of 3 HD patients. The standard curve for the direct method was the same as that from extraction (not shown). The minimum detectable limit of ET was 5.0 pg per tube ($P<0.001$, $n = 10$, t-test). Intra- and inter-assay coefficients of variation were 7.6% ($n = 8$) and 8.1% ($n = 8$), respectively. Recoveries ranged from 48 to 75% (58 ± 8%, $n = 8$). Cross-reactivities were 0.002% with rat ET, and zero with other peptides tested, including human α-atrium natriuretic peptide, porcine brain natriuretic peptide and β_2-microglobulin.

The dilution curve of the plasma extract paralleled the standard curve, however those for plasma of 3 HD patients did not.

3.2. *Gel filtration and HPLC*

Fig.2 illustrates that results, obtained by gel filtration, a single major immunoreactive peak, peak 1, being observed. Peak 1 eluted at a position of little before that of β_2-microglobulin. Serially diluted fractions from peak 1 (nos 31–40) inhibited radioligand binding in parallel with standard solutions of synthetic endothelin (fig.1).

Fig.3A and B illustrates the HPLC results for a plasma extract from hemodialysis patients and a dialysate extract, respectively. Two major immunoreactive peaks were observed and the later peak co-migrated with synthetic ET.

3.3. *Studies in normal subjects and hemodialysis patients*

Using the extraction method, plasma IR-ET was undetectable in normal subjects (fig.4A). In contrast, plasma IR-ET was detectable in 24 of 32 HD patients (10.9 ± 3.4 pg/ml, $n = 24$).

Employing the direct method, IR-ET was detectable in 1 of 10 normal subjects and in 31 of 32

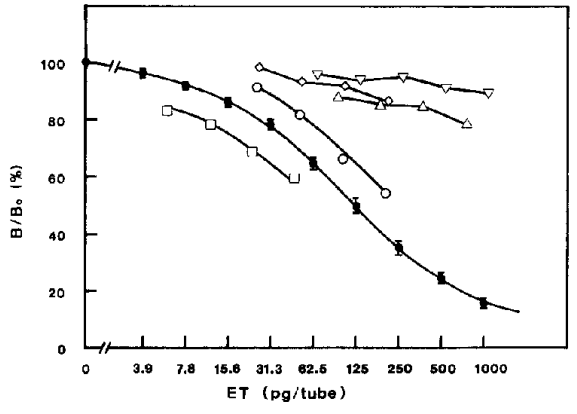

Fig.1. Standard curve for the extraction method (●) and serial 2-fold dilution curves of a plasma extract (○), fractions 31–40 from gel filtration (□) and plasma of 3 hemodialysis patients (△, ▽, ◇).

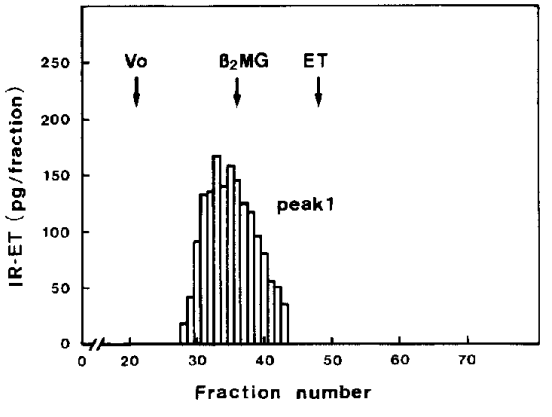

Fig.2. Gel-filtration profile of immunoreactive endothelin (IR-ET) in plasma of a hemodialysis patient. Arrows indicate the elution positions of β_2-microglobulin (β_2MG) and synthetic ET (ET), respectively.

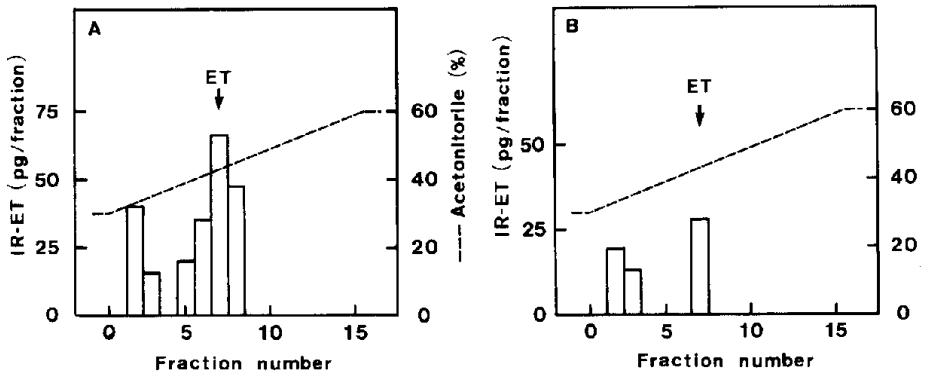

Fig.3. Analysis of immunoreactive (IR-) endothelin (ET) in a plasma extract (A) and a dialysate extract (B) by reverse-phase HPLC. Arrows indicate the elution position of synthetic ET.

HD patients (217 ± 104 pg/ml, n = 31) as shown in fig.4B.

4. DISCUSSION

Here, we have found that at least two types of IR-ET exist in plasma of HD patients. One type is detectable with the extraction procedure. It seems likely that this substance is identical to ET on the basic of the finding that it eluted at the same position as synthetic ET in HPLC (fig.3). The other substance gave a single major peak in gel filtration (peak 1) with a molecular mass greater than 11.6 kDa of β_2-microglobulin (fig.2). The IR-ET-like substance, tentatively designated large IR-ET, was also detectable via the direct method and had plasma levels 20-fold those of ET (fig.4). The fact that the dilution curve of large IR-ET paralleled the standard curve suggests that large IR-ET is a precursor of ET. Large IR-ET is probably on intermediate produced from preproendothelin [2] through unknown processing.

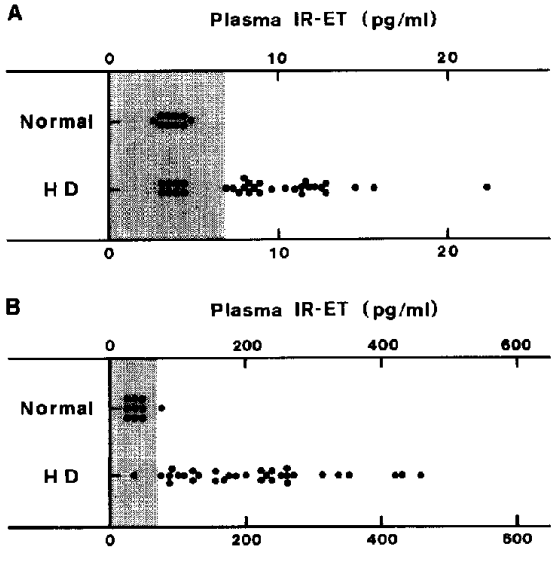

Fig.4. Immunoreactive endothelin (IR-ET) levels in plasma of normal subjects and hemodialysis patients (HD) measured by the extraction (A) and direct (B) method. Values below the limit of sensitivity are plotted within the stippled areas.

The findings that the dilution curves of plasma from HD patients were not parallel with the standard curve may indicate the existence of unknown substances in plasma of HD patients which accelerate radioligand binding in the RIA system. From these results, we tentatively conclude that the extraction of endothelin with the Sep-Pak C_{18} cartridge is particularly important for accurate measurement of ET concentrations in plasma.

Our extraction method with a detection limit of 5 pg/tube was as highly sensitive as an RIA system, but not enough to detect ET in plasma of normal subjects. ET levels of normal subjects were estimated to be lower than 7 pg/ml. In contrast, plasma ET levels were elevated in HD patients (fig.4), but the physiological meaning of this elevation remains obscure in the present study. The elevated level of ET in HD patients might suggest impaired metabolism and excretion of ET, in keeping with the cases of many other peptide hormones and neuropeptides, by the decreased renal functions [5].

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