DETERMINATION OF ATRIAL NATRIURETIC POLYPEPTIDE (ANP) IN HUMAN PLASMA

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ABSTRACT
A technique to measure immunoreactive human atrial natriuretic polypeptide (IR-hANP) in plasma was developed using immuno-affinity chromatography for extraction. In normal subjects, the plasma IR-hANP level was 25 ± 15 pg/ml (mean ± SD; ranged from 9 to 65 pg/ml, n = 40). The level in patients with angina pectoris without overt heart failure was 19 ± 8.7 pg/ml (mean ± SD; ranged from 8 to 32 pg/ml, n = 14). The level was markedly elevated in patients with heart failure (340 ± 230 pg/ml, mean ± SD; ranged from 110 to 920 pg/ml, n = 18), and with chronic renal failure (160 ± 94 pg/ml, mean ± SD; ranged from 65 to 310 pg/ml, n = 6). Sephadex G-50 gel filtration studies revealed that plasma IR-hANP was composed of at least 3 peaks; a macromolecular form, α-hANP and its fragment. There was no significant difference in molecular size heterogeneity of plasma IR-hANP between normal subjects and patients with heart failure or with chronic renal failure.

Three polypeptide species called α-, β- and γ-human atrial natriuretic polypeptide (hANP) were isolated from human atrium (4, 5). These polypeptides seem to be involved in the regulation of extracellular fluid volume.

In the present work, we investigated the plasma immunoreactive hANP (IR-hANP) level by a newly developed method using immuno-affinity chromatography, and the molecular size heterogeneity of plasma IR-hANP in normal subjects and patients with heart or renal diseases.

MATERIALS AND METHODS

Materials
The following materials were obtained from the sources indicated. α-hANP, α-hANP[1-11], α-hANP[7-28], α-hANP[13-28] and α-hANP[18-28] were from Peninsula Laboratories, (Belmont, CA, U.S.A.); [Met(O)2]-α-hANP, α-hANP[4-28], α-hANP[5-28], α-hANP[5-27] and α-rat ANP (α-rANP) were from Peptide Research Foundation (Osaka, Japan); activated CH-Sepharose 4B and Sephadex G-50 superfine were from Pharmacia Fine Chemicals AB (Uppsala, Sweden); Na125I and 125I-human albumin were from New England Nuclear (Boston, MA, U.S.A.); porcine thyroglobulin was from Sigma Chemicals (St. Louis, MO, U.S.A.); Trasylol was from Bayer (Leverkusen, F.R.G.); bovine serum albumin (Cohn Fraction V) was from Dai-ichi Pure Chemicals (Osaka, Japan); 125I-α-hANP with specific activity of about 2,000 Ci/mmol was from Amersham International (Buckinghamshire, U.K.).
Subjects

Forty normal subjects (aged 19-86 years), 14 patients (aged 50-72 years) with angina pectoris without overt heart failure, 18 patients (aged 34-80 years) with heart failure but untreated, and 6 patients (aged 37-76 years) with chronic renal failure before hemodialysis were investigated. The heart failure was caused by dilated cardiomyopathy in 6 patients, by mitral stenosis in 4, by tricuspid regurgitation in 3, by myocardial infarction in 3, by aortic regurgitation in 1, and by ischemic heart disease in 1. The renal failure resulted from chronic glomerulonephritis in 4 patients, from diabetic nephropathy in 1, and from polycystic kidney in 1.

Preparation of Plasma and Tissue Samples

Fasting blood samples were taken from cubital vein into chilled heparinized tubes containing Trasylol at a final concentration of 500 KIU/ml blood, and centrifuged at 1,500 g for 15 min at 4°C. After centrifugation, plasma samples were stored at -20°C until extraction.

The human atrial tissue obtained at autopsy from a patient with lung cancer was extracted by the boiling-water method (13). Chromatography of this tissue extract on a Sephadex G-50 superfine column revealed two immuno-reactive peaks. The first peak was eluted just behind the void volume, and the second at the position corresponding to that of α-hANP. The fractions corresponding to the first peak were collected and IR-hANP in these fractions was used as a macromolecular form of IR-hANP in this study. The amount of the macromolecular form was expressed as weight equivalent of synthetic α-hANP.

Production of Antisera and Radioimmunoassay

Synthetic α-hANP was conjugated to porcine thyroglobulin by carbodiimide condensation (2). The conjugate was emulsified with an equal volume of complete Freund's adjuvant and used for immunizing ten New Zealand White rabbits. One of the antisera, MCR-314, was used in this study. The assay was performed in 0.1 M phosphate buffer, pH 7.4, containing 0.1 M NaCl, 0.06% (v/v) monooethanolamine, 1% bovine serum albumin (Cohn Fraction V) and Trasylol (250 KIU/ml). The α-hANP antiserum was used at a final dilution of 1:42,000. Synthetic α-hANP was used as the assay standard and the results were expressed as weight equivalent of α-hANP. The total incubation volume was 0.7 ml, and the incubation was carried out for 72 h at 4°C. The double antibody method was used for separating the bound from free 125I-α-hANP. The specificity of this radioimmunoassay (RIA) was examined with synthetic α-hANP and its fragments. Cross-reactions with other polypeptides including adrenocorticotropic hormone, arginine vasopressin, calcitonin, somatostatin, substance P, secretin, vasoactive intestinal polypeptide, glucagon, growth hormone-releasing factor, C-peptide, corticotropin-releasing factor and gastrin-releasing peptide were also examined.

Affinity Column

A 100 μl aliquot of rabbit anti-ANP antiserum was coupled to activated CH-Sepharose 4B (1 g) according to the manufacturer's instructions (9), and a portion of the gel was packed in a small column (0.7 x 1.2 cm). The plasma sample (3 ml) was applied to the column and washed with an assay buffer and distilled water. ANP was eluted from the column with 1 M acetic acid (4 ml). The eluate was lyophilized, reconstituted in the assay buffer and assayed for α-hANP. Before another plasma sample was applied, the column was washed 6 times with 5 ml of 1 M acetic acid and once with 5 ml of the assay buffer.

Accuracy and Reusability of Affinity Column

ANP-free plasma was prepared by immuno-affinity chromatography. In order to determine the recovery rate, synthetic α-hANP (25 or 5,000 pg) was added to ANP-free plasma (3 ml), and the plasma was extracted by the method described above. These recovery experiments were performed using the five different affinity columns at varying concentrations.

The reusability of affinity columns was examined using three affinity columns. ANP-free plasma (3 ml) containing α-hANP (1,700 pg/ml, high dose) was extracted by these columns, and the concentrations of α-hANP in the extracts were determined by RIA. After washing (vide supra), ANP-free plasma (3 ml) containing α-hANP (8.3 pg/ml, low dose) was extracted by these columns.
and the concentrations of α-hANP in the extracts were determined by RIA. Three successive applications of pooled plasma (3 ml) were carried out similarly without determination of ANP in the extracts. Then, ANP-free plasma (3 ml) containing a high dose of α-hANP and subsequently ANP-free plasma (3 ml) containing a low dose of α-hANP were extracted. These applications in the same order were repeated up to 22 times and the concentrations of α-hANP in the extracts were determined by RIA.

The recovery experiments were also performed using the macromolecular form of IR-hANP, whose amounts were 25 and 5,000 pg equivalent of synthetic α-hANP.

**Gel Filtration Studies**

Extracted samples were chromatographed on a Sephadex G-50 superfine column (1.0 x 50 cm) which had been equilibrated, and eluted with 1 M acetic acid. The samples were supplemented with 125I-human albumin and Na125I and eluted at a rate of 6 ml/h from the column by the fraction collector-pump control system (13). Fractions of 0.8 ml each were collected, lyophilized and reconstituted in the assay buffer. The column was also calibrated with α-hANP and α-hANP[5-28].

**Statistics**

The data obtained were evaluated by the Wilcoxon test.

**RESULTS**

**RIA of ANP**

The amounts of synthetic α-hANP that inhibited the binding of labeled antigen by 10% and by 50% in this RIA system were 3.0 and 19 pg/tube, respectively. The coefficient of variations in inter- and intraassay at 25 pg/tube were 7.3% (n=10) and 3.4% (n=10), respectively. Cross-reactions with ANP fragments are shown in Table 1; the results suggest that the antisemur used in this study recognized mainly the central portion of α-hANP. Other polypeptides including adrenocorticotropic hormone, arginine vasopressin, calcitonin, somatostatin, substance P, secretin, vasoactive intestinal polypeptide, glucagon, growth hormone-releasing factor, C-peptide, corticotropin-releasing factor and gastrin-releasing peptide did not show any significant cross-reactivity in doses up to 1 μg/tube.

**Table 1 Relative Immunoreactivities of α-hANP and Its Fragments**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Immunoreactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-hANP</td>
<td>100</td>
</tr>
<tr>
<td>[Met(O)125]-α-hANP</td>
<td>93</td>
</tr>
<tr>
<td>α-hANP[1-11]</td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td>α-hANP[4-28]</td>
<td>130</td>
</tr>
<tr>
<td>α-hANP[5-28]</td>
<td>150</td>
</tr>
<tr>
<td>α-hANP[5-27]</td>
<td>130</td>
</tr>
<tr>
<td>α-hANP[7-28]</td>
<td>160</td>
</tr>
<tr>
<td>α-hANP[13-28]</td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td>α-hANP[18-28]</td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td>α-ANP</td>
<td>16</td>
</tr>
</tbody>
</table>

The amounts of synthetic peptides that inhibited the binding of labeled antigen by 50% in a molar ratio were determined; activity with α-hANP was taken as 100%.

**Accuracy and Reusability of Affinity Column**

The minimum detectable amount was 3 pg/ml when 3 ml of plasma were used as starting material. When synthetic α-hANP (25 or 5,000 pg) in ANP-free plasma (3 ml) was extracted, the recovery rate was 100 ± 6.1% and 98 ± 3.3% (mean ± SD), respectively. In respect to the macromolecular form of IR-hANP, the recovery rate was 78 ± 4.7% and 75 ± 5.7% (mean ± SD), respectively, when examined at the amounts of 25 and 5,000 pg equivalent of synthetic α-hANP.

As far as the reusability study is concerned, the α-hANP values after repeated use of the column were similar (Fig. 1).

**Plasma IR-hANP Levels**

As shown in Fig. 2, the plasma IR-hANP level in normal subjects was 25 ± 15 pg/ml (mean ± SD; ranged from 9 to 65 pg/ml). In patients with angina pectoris without overt heart failure, the level was 19 ± 8.7 pg/ml (mean ± SD; ranged from 8 to 32 pg/ml). The plasma IR-hANP level in patients with heart failure was 340 ± 230 pg/ml (mean ± SD; ranged from 110 to 920 pg/ml). There was no significant difference in the plasma IR-hANP level in heart failures caused by various disorders. In patients with chronic renal failure, the level was 160 ± 94 pg/ml (mean
or chronic renal failure ($P < 0.05$), as compared with normal subjects.

**Gel Filtration Studies**

Gel filtration patterns of IR-hANP in three plasma extracts prepared from normal subjects are presented in Fig. 3. The first peak was eluted behind the void volume. The second peak was eluted at the position between the first peak and $\alpha$-hANP. The third peak was eluted at the position corresponding to that of $\alpha$-hANP. Another peak was eluted just behind the elution position of $\alpha$-hANP.

Gel filtration patterns of three plasma extracts prepared from two patients with heart failure caused by either dilated cardiomyopathy or ischemic heart disease, and one patient with chronic renal failure caused by polycystic kidney are shown in Fig. 4. All peaks observed in normal subjects were found, with the exception of the second peak.

**DISCUSSION**

Immuno-affinity chromatographic technique employed in the present study was found to be suitable for extraction of plasma IR-hANP. After extraction, plasma IR-hANP was detectable by the present RIA and the level in nor-
Fig. 3 Sephadex G-50 gel filtration patterns of three plasma extracts from normal subjects, using 106 ml (a), 112 ml (b) and 130 ml (c) of plasma, respectively.

Fig. 4 Sephadex G-50 gel filtration patterns of three plasma extracts from two patients with heart failure (a: dilated cardiomyopathy, b: ischemic heart disease) and one with chronic renal failure (c: polycystic kidney), using 3.5 ml (a), 3.0 ml (b) and 6.8 ml (c) of plasma, respectively.

Normal subjects ranged from 9 to 65 pg/ml, whereas the plasma IR-hANP level in patients with heart failure but untreated was markedly elevated. The present results agree with those reported by other investigators (8, 12).

However, the plasma IR-hANP level was found to be within a normal range in patients with angina pectoris without overt heart failure. The results suggest that volume overload
caused by heart failure is a stimulus for secreting IR-hANP into the circulation, as was observed by Lang et al. in rats (6). In patients with chronic renal failure, delayed degradation of ANP might be one reason for the elevation of plasma IR-hANP. It has also been reported (10) that expansion of blood volume, which is frequently observed in these patients, may cause the elevation of plasma IR-hANP.

Gel filtration studies of plasma extracts prepared from normal subjects revealed the presence of four hANP immunoreactivities. One component is a macromolecular form of IR-hANP and considered to be a γ-hANP-like molecule. The immunoreactivity, eluted between the macromolecular form and α-hANP, may be a β-hANP-like component. The third peak is compatible with α-hANP. In addition, the immunoreactivity with a smaller molecular size was eluted at the position corresponding to that of α-hANP[5-28]. The present study clearly demonstrated that a macromolecular form of IR-hANP, γ-hANP-like component, was always present in plasma extracts, although other studies (1, 3, 7, 11) failed to detect the presence of such a macromolecular component. However, predominant forms of plasma IR-hANP were α-hANP and its fragment in agreement with previous studies (1, 3, 7, 11).

Although the plasma IR-hANP level was elevated in patients with heart failure or with chronic renal failure, there was no significant difference in molecular size heterogeneity of plasma IR-hANP between normal subjects and these patients, except for the second peak, which was not observed in the patients. Thus far examined, the predominant forms of plasma IR-hANP were lower molecular weight peptides. The method for determination of IR-hANP developed in the present study will provide a useful tool to clarify the regulation and physio-pathological roles of hANP in health and disease.

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REFERENCES


geneity of immunoreactive gastrin-releasing peptide in fetal and adult lungs and primary lung tumors. Cancer Res. 43, 3932-3939