The Radioimmunoassay for Human Plasma Atrial Natriuretic Peptide—Its Application to Uremic Patients

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A highly sensitive radioimmunoassay for α-human atrial natriuretic peptide (α-hANP) was established and applied to measure the human plasma α-hANP levels. In our assay system, anti-α-hANP antiserum was raised in albino rabbits by intradermally injecting synthetic α-hANP which was conjugated with bovine serum albumin. The final antiserum dilution was 1:50,000. Sensitivity was 2 pg/tube and the 50% intercept was at 28 pg/tube. The plasma α-hANP was extracted using a Sep-Pak C-18 cartridge. According to this procedure, the mean recovery was 73.8 ± 3.4% (mean ± SE). The averaged plasma levels of immunoreactive α-hANP (io-hANP) in normal subjects were 24.8 ± 2.1 pg/tube. In patients with chronic renal failure undergoing hemodialysis, the averaged plasma io-hANP levels were 56.4 ± 5.0 pg/ml before hemodialysis. Plasma io-hANP levels were significantly higher in the patients with chronic renal failure than in the normal subjects. After hemodialysis, plasma io-hANP levels decreased significantly (32.2 ± 2.8 pg/ml). These results suggest that the alteration in extracellular fluid volume (ECFV) may affect the plasma levels of io-hANP in patients with chronic renal failure under hemodialysis; i.e., an increase in ECFV elevates and a decrease in ECFV lowers the circulating levels of α-hANP.

Key Words: Atrial natriuretic peptide, Uremia, RIA, Hemodialysis

Recently, α-human atrial natriuretic peptide (α-hANP) has been thought to be involved in the water and sodium metabolism as well as blood pressure regulation. Increased plasma α-hANP levels were reported in a patient with paroxysmal atrial tachycardia. Stretching of the atria by volume overload has suggested that blood volume change is one important regulating factor for the α-hANP release from the atria. It is well known that expanded blood volume is frequently observed in renal failure. Thus, to investigate the pathophysiological role of α-hANP, the determination of its level in plasma seems essential. In the present study, a very sensitive radioimmunoassay system for plasma α-hANP was established, and plasma immunoreactive α-hANP (io-hANP) levels were measured before and after hemodialysis in order to investigate the role of α-hANP in patients undergoing hemodialysis.

MATERIALS AND METHODS

Radioimmunoassay of α-hANP: In the preparation of antiserum, synthetic α-hANP (28 amino acid, Peptide Institute, Inc., Osaka) was conjugated to bovine serum albumin by the modification methods of Goodfriens et al. Conjugated α-hANP was injected intradermally into 3 albino rabbits after emulsification with an equal volume of complete Freund's adjuvant. Each injected solution contained 0.5 to 0.6 mg of

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conjugated α-hANP. Blood was drawn 3 months after the first immunization.

Synthetic α-hANP was labeled with $^{125}$I by a modification of the chrolamine-T method described by Greenwood et al. The reaction mixture was applied to high performance liquid chromatography (HPLC), and the labeled α-hANP was purified by this column system.

The cross-reactivity to the rat ANP (Peptide Institute, Inc., Osaka), the 5-25 rat ANP (Peninsula Labo., Inc., CA) and the 5-28 hANP (Peptide Institute, Inc., Isaka) were all determined in α-hANP antiserum.

Phosphate-buffered saline (PBS; pH 7.0) containing 0.1% egg albumin, 30 mM EDTA and 3 mM 1,10-phenanthroline was used as the assay buffer. The assay buffer was added to the standard α-hANP (1-125 pg) or sample to yield 200 μl. Then, 100 μl of antiserum and an equal volume of non-immune rabbit serum (1:100) were added. Tubes were incubated at 4°C for 24 hours. After incubation, the radio-iodinated α-hANP (4,000–5,000 cpm) in 100 μl assay buffer was added to the tubes. Forty-eight hours after the addition of the labeled α-hANP, a sufficient amount of anti-rabbit gamma-globulin antiserum was added. After the tubes were centrifuged at 3,000 rpm for 20 min at 4°C, the supernatant was carefully decanted, and the radioactivity in the precipitates was counted by an Aloka autogamma spectrometer. The tubes containing the assay buffer and normal rabbit serum were assigned as the control tube. The counts of the precipitate were expressed as the percent of the control tubes.

Clinical study: Plasma samples were collected into plastic syringes, then EDTA (1 mg/ml) and aprotinin (500 KIU/ml) were added. Plasma was quickly separated by centrifugation at 4°C and subjected to extraction. Extraction was performed by a Sep-Pak C-18 cartridge (Waters Inc.). The adsorbed peptide was eluted with acetonitrile and 0.5% ammonium acetate solution. The elution were evaporated under air stream, dissolved in the assay buffer and subjected to radioimmunoassay.

Plasma α-hANP levels were determined in 12 normal subjects (aged 49.2 ± 1.8 years, mean ± SE) and 18 patients with chronic renal failure (aged 51.8 ± 2.8 years). All of the patients were maintained on 4–5 hours of hemodialysis twice or three times a week. The renal function in these patients was extremely lowered, since creatinine clearance was less than 1.0 ml/min in all patients. The patients remained in a lying position for 30 min before the blood sampling. Blood samples were drawn immediately before and after hemodialysis for the measurement of plasma α-hANP levels, hematocrit, blood urea nitrogen (BUN), serum creatinine and electrolytes. In addition, changes in body weight and blood pressure were observed following the hemodialysis.

Statistical analysis was performed with the Student's t-test for paired or unpaired data. Correlations were calculated using the linear regression analysis.

RESULTS

Radioimmunoassay of α-hANP: The high titered antiserum was obtained in one of the immunized rabbits which can be used at a final dilution of 1:50,000. In radio-iodination, 3 peaks were obtained after the HPLC procedure. The first peak represents the 90% binding to excess anti-α-hANP antiserum, but the other two showed lower binding than first. The first peak was used as $^{125}$I-labeled α-hANP in this radioimmunoassay system. The specific activity of this tracer was 2,100 Ci/mmol. A typical standard curve is shown.

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in Fig. 1. A good dilution curve was obtained in the range of 1 pg to 125 pg of α-ANP. Sensitivity was 2 pg/tube with a 95% confidence limit. The 50% intercept was at 28 pg/tube. A 100% cross-reactivity was observed in the 1-28 rat ANP and the 5-25 rat ANP, whereas the 7-28 α-ANP showed less cross-reactivity (56%). However, the 18-28 α-ANP showed no cross-reactivity at all. The extracted plasma sample showed a good parallel relationship to the α-ANP standard curve. On the other hand, the non-extracted plasma sample showed no parallel dilution curve to the α-ANP standard curve. When known amounts of α-ANP was added to the plasma, the calculated recovery was 73.8 ± 3.4% (mean ± SE). Intra-assay and inter-assay coefficients of variation was 4.5% and 5.6%, respectively.

**Clinical study**: Plasma α-ANP levels in normal subjects were 24.8 ± 2.1 pg/ml. The values for plasma α-ANP, hematocrit, mean arterial pressure and body weight before and after hemodialysis, in each of the patients are shown in Table 1. After hemodialysis, the blood pressure and body weight were reduced, while the hematocrit rose significantly. Plasma α-ANP levels before hemodialysis were 56.4 ± 5.0 pg/ml, being significantly higher than in normal subjects (Table 1). After hemodialysis, plasma α-ANP levels decreased significantly (32.2 ± 2.8 pg/ml) (Table 1). Significant positive and negative correlations were found between the % change of body weight and that of plasma α-ANP, and between basal α-ANP and the % change of α-ANP, respectively.

**DISCUSSION**

Recently, various methods for determination of plasma α-ANP level have been reported. Kuribayashi et al. described high levels of plasma α-ANP, about 100 pg/ml in normal subjects. While lower plasma α-ANP levels were stated by Hartter et al. and Tikkanen et al. These differences appear whether the extraction method was used or not. In our radioimmunoassay system, a non-extracted plasma sample did not show a parallel relationship, and revealed a very high plasma level of α-ANP. However, using the extraction method by Sep-Pak C-18 cartridge, a good parallel relation has been found between the dilution curve of plasma extract and the standard curve, suggesting that

Table 1. The values of plasma atrial natriuretic peptide (ANP), hematocrit (Ht), mean blood pressure (m-BP) and body weight (BW) before and after hemodialysis in patients undergoing chronic hemodialysis.

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<th>Age</th>
<th>Sex</th>
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<th>Ht %</th>
<th>m-BP mmHg</th>
<th>BW kg</th>
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| Mean     | 51.8 | 76.9 | 56.4 | 23.1 | 94.4 | 51.1 |
| SEM      | 2.8  | 12.1 | 5.0  | 1.4  | 3.8  | 2.5  |

*Significantly lower or higher than before hemodialysis (P < 0.01).
RIA for Plasma hANP

due to the extraction procedure is necessary in the measurement of the plasma α-hANP. In our α-hANP radioimmunoassay, the sensitivity was 2 pg/tube, and the 50% intercept was at 28 pg/tube, which is one of the most sensitive methods when compared to the techniques previously reported.3,9–13 Plasma α-hANP levels in normal subjects were 24.8 ± 2.1 pg/ml, in good agreement with the data reported by Schiffrin et al.3 and Hartter et al.12

In patients with severe right heart failure12 and supraventricular tachycardia,3,14 elevation of plasma α-hANP levels were reported in some cases. In patients with heart failure, α-hANP appears to be released as a consequence of increased blood volume. Our results in patients with chronic renal failure indicate that volume expansion elicits the release of α-hANP into the circulation. However, it cannot be excluded that elevation of plasma α-hANP levels may be due to the blood pressure elevation in some cases or the reduction of metabolism. In our study, high plasma α-hANP before hemodialysis was then decreased to a remarkable degree after hemodialysis. It was suggested that the marked decline of increased plasma α-hANP levels after hemodialysis was caused by the reduction of plasma volume and a fall in blood pressure. It seems likely that the diminished plasma volume may greatly contribute to this mechanism because of the significant correlation between the % change of body weight and that of plasma α-hANP. However, the possibility of the dialysis of α-hANP from plasma cannot be excluded. Further studies will be necessary to clarify the mechanism of marked decrease of plasma α-hANP after hemodialysis.

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REFERENCES