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Plasma Concentrations of Atrial Natriuretic Peptide in Various Diseases

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NOZUKI, M., MOURI, T., ITOI, K., TAKAHASHI, K., TOTSUNE, K., SAITO, T. and YOSHINAGA, K. *Plasma Concentrations of Atrial Natriuretic Peptide in Various Diseases*. Tohoku J. exp. Med., 1986, **148** (4), 439-447 —— Using a radioimmunoassay for atrial natriuretic peptide (ANP) we studied plasma concentrations of immunoreactive ANP in order to investigate the pathophysiological role of ANP in patients with various diseases. Plasma ANP levels were elevated in patients with congestive heart failure (394 ± 260 pg/ml, $n=8$) and chronic renal failure (219 ± 86 pg/ml, $n=11$). In patients undergoing hemodialysis plasma ANP levels were markedly high and decreased after hemodialysis from 433 ± 166 pg/ml to 204 ± 92 pg/ml ($n=11$). ANP was removed from blood to dialysate (21 ± 13 pg/ml of dialysate, $n=6$, dialysate flow: 500 ml/min). Plasma ANP level was conversely correlated with creatinine clearance ($r=-0.812$, $p<0.001$) in patients with renal diseases ($n=29$). In patients with atrial fibrillation, pace maker implantation, lung disease, chronic glomerulonephritis, nephrotic syndrome, essential hypertension, liver disease and cerebrovascular disease, plasma ANP levels were not significantly different from those in normal subjects (70 ± 32 pg/ml, $n=28$). These results suggest that ANP may be a circulating hormone playing pathophysiological roles in congestive heart failure and chronic renal failure. —— plasma atrial natriuretic peptide; radioimmunoassay

Mammalian atrial myocytes contain numerous storage granules referred to as specific atrial granules (Jamieson and Palade 1964). The number of these specific granules is affected by changes in water and electrolyte balance (de Bold 1979) and intravenous infusion of atrial extract induces a rapid, potent diuretic and natriuretic response in rats (de Bold et al. 1981). In addition, it has been shown that atrial extract posseses a potent vasodilating action (Currie et al. 1983). Since then the research has progressed rapidly and atrial natriuretic peptides (ANP) of the rat and human have been purified, sequenced and synthesized (de Bold 1982; Thibault et al. 1983; Kangawa and Matsuo 1984; Kangawa et al. 1984; Misano et al. 1984).

Atrial natriuretic factor-like immunoreactive material has been shown to be released into the circulation in response to volume load in the rat (Lang et al.

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1985). There are three reports of elevated plasma ANP levels in paroxysmal atrial tachycardia (Schiffrin et al. 1985; Tikkanen et al. 1985; Yamaji et al. 1985) and recently two reports have shown elevated plasma ANP levels in patients with congestive heart failure (Hartter et al. 1985; Tikkanen et al. 1985). However, it has not yet been elucidated whether ANP is released into the circulation in other diseases.

We have developed a radioimmunoassay for ANP and studied the values of plasma immunoreactive ANP in order to investigate the pathophysiological significance of ANP in various diseases including congestive heart failure, chronic renal failure, essential hypertension and others.

Subjects and Methods

Subjects

The studied population consisted of 115 subjects, including 28 normal subjects [18 men and 10 wemen, 19-58 years of age (28±9 years)], 8 patients with congestive heart failure [New York Heart Association functional (NYHA) class III ($n=4$), NYHA class IV ($n=4$)], 6 patients with atrial fibrillation whose heart rates were 50-100 beats/min, 2 patients with complete A-V block who were implanted with a pace maker, 11 patients with restrictive or obstructive lung disease, 11 patients with chronic renal failure (creatinine clearance <20 ml/min), 13 patients with chronic glomerulonephritis, 5 patients with nephrotic syndrome with edema, 6 essential hypertensives without treatment, 5 patients with chronic hepatitis or liver cirrhosis with ascites, 9 patients with cerebrovascular disease and 11 patients undergoing maintenance hemodialysis who were dialyzed for 4 to 5 hr thrice weekly.

After an overnight fast, blood samples were obtained from peripheral vein in the morning before breakfast after 30-60 min of recumbency except for hemodialysis patients. In hemodialysis patients blood samples were obtained from the forearm subcutaneous A-V fistula before and after hemodialysis. Blood and dialysate samples were also obtained simultaneously from the inlet and outlet sides of dialyzer immediately after the start of hemodialysis in other 6 patients undergoing maintenance hemodialysis.

Endogenous creatinine clearance was measured in 29 patients with renal diseases consisting of chronic renal failure, chronic glomerulonephritis and nephrotic syndrome described above.

Radioimmunoassay method of ANP

Antibody to ANP was raised in three rabbits against 2.8 mg of synthetic human ANP (1-28) (Peninsula Laboratories, Inc., Belmont, CA., USA) coupled with 51.3 mg of bovine thyroglobulin (Sigma Chemical Co., St. Louis, MO., USA) by glutaraldehyde. All three rabbits produced high titer antisera (T. Mouri et al., unpublished). Antiserum from one of the rabbits was used for the present study.

Human ANP (1-28) was iodinated with I^{125} by a modified chloramine-T method (Hunter and Greenwood 1962), omitting the addition of sodium metabisulphite. To 5 μg of human ANP, 1 mCi of NaI^{125} and 50 μg of chloramine-T were added. After agitation for 40 seconds, 0.2 ml of 0.5 M acetic acid containing 10% human serum albumin was added to the reaction mixture. I^{125} labelled human ANP was purified on a Sephadex G-50, superfine column with the eluent of 0.5 M acetic acid containing 0.1% human serum albumin.

The buffer used for radioimmunoassay was 0.1 M phosphate buffer, pH 7.7, containing 0.1% human serum albumin, 0.2% Triton X-100 and 0.1% sodium azide. Unlabelled human ANP (1-28) was used for standards. First, 0.1 ml of standard or sample and 0.1 ml of antiserum (final dilution 1 : 9000) were added to polystyrene tubes and incubated at 4°C

for 48 hr. Then 0.1 ml of I^{125} human ANP (approximately 5000 cpm) was added to each tube. After additional incubation for 24 hr at 4°C, the separation of free and bound peptides was performed by adding 0.1 ml of 4% anti-rabbit IgG (Miles-Yeda, Ltd., Kiryat Weizmann, Israel) and 0.4 ml of 8% polyetyleneglycol. After overnight incubation at 4°C, the tubes were centrifuged at 3000 rpm for 30 min at 4°C. The supernatant was decanted and the precipitates were counted in a gamma counter.

Heparinized blood was collected in tubes containing aprotinin (2500 U/ml blood) and EDTA (1 mg/ml blood) which were kept in ice. Plasma was separated by centrifugation at 4°C and stored at −20°C until assayed. Extraction procedure of ANP was as follows; One ml of each plasma sample was applied on a Sep-Pak C_{18} cartridge and adsorbed peptides were eluted with 2 ml of 60% acetonitrile containing 0.2% ammonium acetate according to the advice of Dr. K. Naruse (personal communication). The elutes were dried under an air stream in a 40°C water bath. The dried residues were dissolved in 0.4 ml of the phosphate buffer and subjected to radioimmunoassay. ANP from 20 ml of dialysate was extracted by the same procedure.

Statistical analyses

Values were given in terms of mean±S.D. and statistical significance of the difference in plasma ANP levels was analysed with Student's *t*-test for group comparison. Linear regression analysis was done by means of the method of least squares.

Results

Typical standard curve, dilution curve of a plasma extract and cross-reactivity are shown in Fig. 1. The sensitivity of the assay was 2.5 pg/tube with 95% confidence limit. The serial two fold dilution curve was in parallel with the standard curve. The cross-reactivity was 100% with human ANP (7-28), human Met $(O)^{12}$-ANP (1-28) and rat ANP (1-28), and no cross-reactivity was observed with other peptides demonstrated in Fig. 1. The coefficients of variation of withinassay and betweenassay were 6.4% ($n=9$) and 10.5% ($n=6$), respectively. The recoveries, as determined by addition of 200 pg of human ANP to 1 ml of

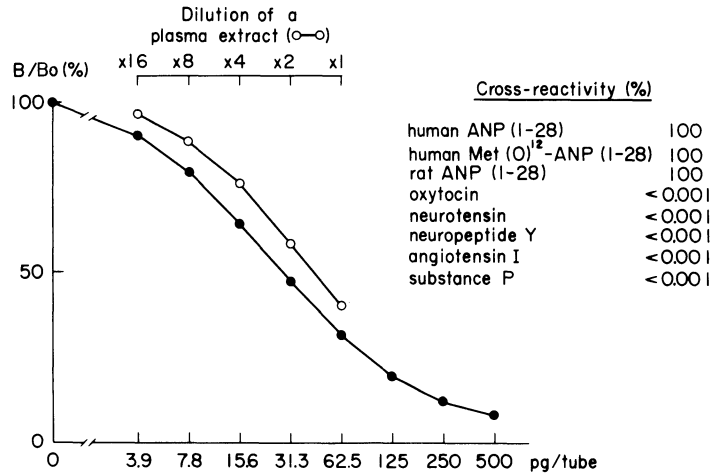

Fig. 1. Typical standard curve (●——●), serial two fold dilution curve of a plasma extract (○——○) and cross-reactivity.

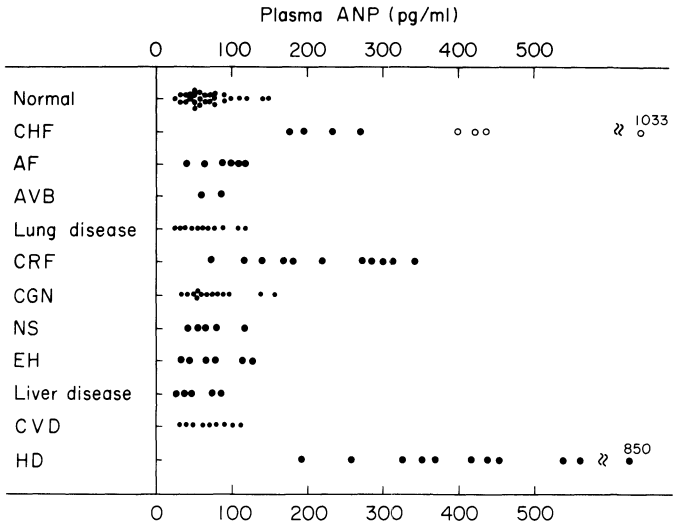

Fig. 2. Plasma ANP levels in various diseases. CHF, congestive heart failure (●, NYHA class III; ○, NYHA class IV); AF, atrial fibrillation; AVB, complete A-V block with pace maker implantation; CRF, chronic renal failure; CGN, chronic glomerulonephritis; NS, nephrotic syndrome; EH, essential hypertension; CVD, cerebrovascular disease; HD, maintenance hemodialysis (values before hemodialysis).

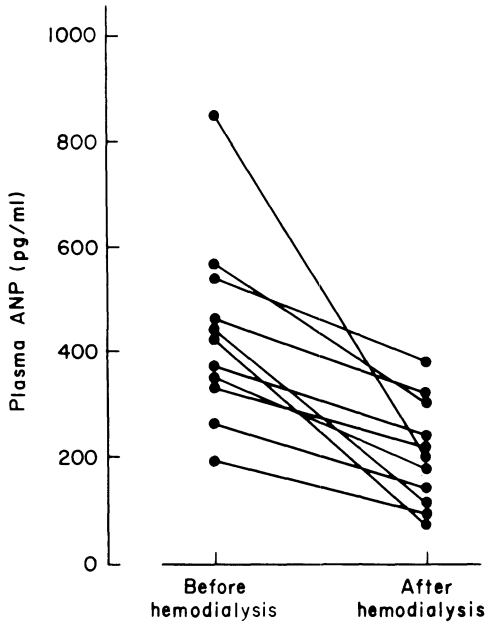

Fig. 3. Effects of hemodialysis on plasma ANP levels. Plasma ANP levels are high before homodialysis and decrease after hemodialysis in all patients.

TABLE 1. *ANP concentrations in the inlet blood (CBi), in the outlet blood (CBo) and in the outlet dialysate (CDo) of dialyzer*

	CBi (pg/ml)	CBo (pg/ml)	CDo (pg/ml)	CBo/CBi
Case 1	275	212	17	0.77
Case 2	490	350	35	0.71
Case 3	437	342	43	0.78
Case 4	456	383	5	0.84
Case 5	551	435	15	0.79
Case 6	277	230	12	0.83
mean±S.D.	414±104	325±80	21±13	0.79±0.04

ANP concentration in the inlet dialysate (CDi)=0 pg/ml
Dialysate flow=500 ml/min
Blood flow=120−200 ml/min

plasma, were ranged from 63% to 84% (72±7%, n=6).

Plasma ANP levels in various diseases are shown in Fig. 2. Plasma ANP levels in normal subjects were ranged from 27 to 152 pg/ml (70±32 pg/ml). There was no significant difference in plasma ANP levels between men (70±23 pg/ml) and wemen (71±43 pg/ml). Plasma ANP levels were significantly elevated in patients with congestive heart failure (394±260 pg/ml, $p<0.001$ compared with normal). In these patients, plasma ANP levels were significantly higher in NYHA class IV (571±267 pg/ml) than those in NYHA class III (217±36 pg/ml) ($p<0.05$). Plasma ANP levels in patients with atrial fibrillation (84±25 pg/

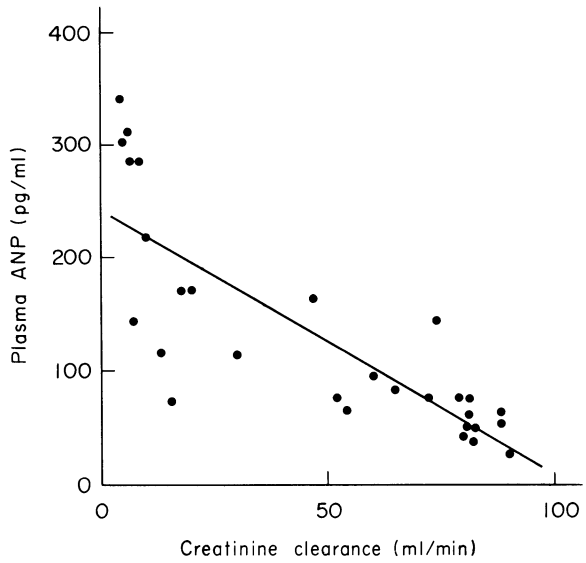

Fig. 4. Correlation between plasma ANP level and creatinine clearance. Plasma ANP level is conversely correlated with creatinine clearance ($r=-0.812$, $p<0.001$).

ml) and with pace maker implantation (78±17 pg/ml) were not significantly different from those in normal subjects. Plasma ANP levels were also significantly elevated in patients with chronic renal failure (219±86 pg/ml, $p<0.001$ compared with normal). Plasma ANP levels in patients with lung disease (63±30 pg/ml), chronic glomerulonephritis (77±38 pg/ml), nephrotic syndrome (72±24 pg/ml), essential hypertension (75±33 pg/ml), liver disease (52±24 pg/ml) and cerebrovascular disease (71±27 pg/ml) were not significantly different from those in normal subjects.

In patients undergoing hemodialysis, plasma ANP levels were markedly high before hemodialysis (433±166 pg/ml, $p<0.001$ compared with normal) and decreased after hemodialysis (204±92 pg/ml, $p<0.01$ compared with values before hemodialysis). Plasma ANP levels were still higher in 8 of 11 patients after hemodialysis than those in normal subjects (Fig. 3). The ratios of plasma ANP levels in the outlet and inlet sides of dialyzer (CBo/CBi) were 0.79±0.04. ANP levels were ranged from 5 to 43 pg/ml (21±13 pg/ml) in the outlet dialysate, but no ANP was detected in the inlet dialysate (Table 1).

The correlation between plasma ANP level and creatinine clearance is shown in Fig. 4. Plasma ANP level was conversely correlated with creatinine clearance ($r=-0.812$, $p<0.001$).

Discussion

Using synthetic human ANP (1-28) and its specific antibodies, we have established a radioimmunoassay system for ANP. The sensitivity was enough, the reproducibility was reliable and the recovery was satisfactory in this system. Dilution curve of a plasma extract paralleled the standard curve, and immunoreactive peaks of plasma extracts and synthetic human ANP (1-28) were eluted in a close vicinity by Sephadex G-50, superfine gelfiltration (data not shown). Thus, this system permitted a specific quantitative analysis of immunoreactive ANP substances in plasma extracts.

In the present study, it is shown that plasma ANP levels are elevated in patients with congestive heart failure and that the levels are higher in a severe group than those in a moderate group of congestive heart failure. Recently similar results have been reported (Tikkanen et al. 1985). These findings suggest that ANP may play an important pathophysiological role in heart failure. It has been reported that plasma ANP levels are elevated in patients with supraventricular tachycardia (Schiffrin et al. 1985 ; Tikkanen et al. 1985 ; Yamaji et al. 1985). In fact our patients with severe congestive heart failure also had supraventricular tachycardia. This may explain partly the reason of high plasma ANP levels in congestive heart failure. The mechanism for secretion of ANP has not been elucidated. In normal rats, ANP-like immunoreactive material is released into the circulation in response to acute volume overload and the increase of right atrial pressure (Lang et al. 1985). The elevated plasma ANP levels in

congestive heart failure may be mediated by these stimulations.

It is shown that plasma ANP levels are elevated in patients with chronic renal failure and maintenance hemodialysis and that the levels elevate in relation to the disturbance of renal function. The reason for increased plasma ANP levels in these disorders is unclear. Although the metabolism of ANP has not been clarified, Tang et al. (1984) have reported that a powerful activity on the degradation of I^{125} labelled atriopeptin III exists in the kidney, suggesting that ANP is destroyed in the kidney. Thus elevated plasma ANP leveles may be mediated by reduced renal degradation of ANP in patients with chronic renal failure and maintenance hemodialysis. What is more, these diseases are often accompanied with volume expansion resulting from decreased excretion of sodium and water, which may also stimulate the secretion of ANP and elevate the levels in plasma.

Plasma ANP levels decrease after hemodialysis in all hemodialysis patients. It is shown that plasma ANP levels are lower in the outlet sides than those in the inlet sides of dialyzer and that ANP is detected in the outlet dialysate. These results indicate that ANP is removed from plasma to dialysate through the membrane of dialyzer. In addition, the reduction of plasma volume in consequence of the removal of fluid may also relate to the decrease of plasma ANP levels after hemodialysis (Rascher et al. 1985).

In nephrotic syndrome with edema and in liver cirrhosis with ascites, plasma ANP levels are not significantly different from those in normal subjects. It is unlikely that circulating volume is increased in these diseases if no congestive heart failure coexists, because the oncotic pressure is usually low in these disorders. Therefore the results mentioned above may be reasonable.

Many reports have shown that ANP has vasodilating and blood pressure lowering activity (Currie et al. 1983; Garcia et al. 1984; Hirata et al. 1984; Kleinert et al. 1984; Oshima et al. 1984; Ishihara et al. 1985). These reports make it interesting to investigate the interaction of ANP in the regulation of blood pressure. However, no abnormality in plasma ANP levels is found in patients with essential hypertension in the present study. In regard to this item, further detailed studies are required.

Acknowledgments

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