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INCREASED SECRETION OF BRAIN NATRIURETIC PEPTIDE AND ATRIAL NATRIURETIC PEPTIDE, BUT NOT SUFFICIENT TO INDUCE NATRIURESIS IN RATS WITH NEPHROTIC SYNDROME

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SUMMARY : The levels of immunoreactive brain natriuretic peptide (ir-BNP) and immunoreactive atrial natriuretic peptide (ir-ANP) were evaluated by radioimmunoassay in both the atrium, ventricle and plasma of adriamycin-induced nephrotic rats and control rats. There was no difference in right and left atrial concentrations of ir-BNP, however, a higher right atrial concentration of ir-ANP was observed in nephrotic rats than in controls ($p < 0.01$). The ventricular ir-BNP and ir-ANP were increased in nephrotic rats compared to controls (BNP : $p < 0.001$, ANP : $p < 0.001$). Cardiac BNPs were composed of pro-BNP (γ-BNP) and its C-terminal 45-amino-acid peptide (BNP-45). The ratio of BNP-45/γ-BNP in nephrotic rats was higher than that of controls in both atria and in the ventricle. Plasma ir-BNP and ir-ANP were significantly higher in nephrotic rats than in controls (BNP : $p < 0.001$, ANP : $p < 0.001$), and each level was negatively correlated with urinary sodium excretion in nephrotic rats (BNP : $r = -0.84$, $p < 0.001$, ANP : $r = -0.88$, $p < 0.001$). These results suggest that production and secretion of both BNP and ANP are concomitantly stimulated by a decreased renal ability to eliminate sodium and water, but this secretion is insufficient to induce effective natriuresis in nephrotic rats.

Brain natriuretic peptide (BNP), distinctly differs from atrial natriuretic peptide (ANP) in its amino acid sequence, is considered to be a hormonal regulator of water and salt excretion because of its potent diuretic and natriuretic activities (1). We have recently isolated two distinct forms of BNP in the rat heart: pro-BNP (γ-BNP) and BNP-45. The γ-form is a 95-amino-acid peptide with a molecular weight of 13,000 daltons, and BNP-45 is a C-terminal 45-amino-acid peptide with a molecular weight of 5,000 daltons (2,3). Chromatographic analysis of plasma extracts indicates that BNP-45 is the predominant circulating molecular form (to be published). Thus, BNP-45 is considered to be formed from its precursor γ-BNP, stored in cardiac

ABBREVIATIONS : BNP, brain natriuretic peptide ; ANP, atrial natriuretic peptide ; TEA, trifluoroacetic acid ; RIA, radioimmunoassay ; ir-, immunoreactive ; RP-HPLC, reverse phase high performance liquid chromatography.

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myocytes, and secreted into the circulation. Cardiac rat ANP is uniformly composed of the γ-form, and has a molecular weight of 13,000 daltons (4). These results suggest that rat BNP is a cardiac hormone whose synthetic pathway differs from that of rat ANP. However, its pathophysiological significance in the regulation of blood volume through its production and release remains obscure. In the present study, BNP secretion behavior in rats with nephrotic syndrome, a sodium retaining disorder, is investigated.

MATERIALS AND METHODS

Experimental protocol : Seven-week-old, male Wistar rats (n=30) weighing 200-230 g at the start of the study were purchased from Charles River Japan (Nagoya). The animals were fed standard rat food (Nihon CLEA, CE-2, Japan ; NaCl, 0.85g/100 g) ad libitum. Fifteen rats in the nephrotic rat group were intravenously injected with 7.5 mg/kg of adriamycin (Kyowa Hakko Co., Tokyo,Japan) dissolved in 0.9% saline, under light ether anaesthesia. The remaining control rats (n=15) were administered 0.9% saline. Twenty days after injection, each rat was kept in an individual metabolic cage for 24h urine collection. Systolic blood pressure was measured in conscious, nonanesthetized rats by the tail-cuff method. The rats were then decapitated, and their hearts were quickly removed. Whole blood was collected in a chilled tube containing aprotinin (500 kIU/ml) and ethylenediamine tetraactetic acid (EDTA)-2Na (1mg/ml). After centrifugation at 3,000 rpm for 10 min at 4℃, plasma was diluted two-fold with 0.9% saline containing 0.15% EDTA (pH 6.0).

Preparation of plasma samples : Plasma was loaded into a Sep-pak C18 cartridge (Waters Associates, Millipore Co., Milford, Massachusetts, U.S.A.), and washed with 0.1% trifluoroacetic acid (TFA). Absorbed materials were eluted with 60% CH_3CN in 0.1% TFA, and further purified by rat BNP immunoaffinity chromatography as described previously (5).

Tissue extraction : The apical half of the cardiac ventricle was dissected avoiding contamination with atrial tissue, and the atrial auricle was then dissected. After weighing, cardiac tissues were boiled for 10 min in 3 ml water to inactivate intrinsic proteases, and glacial acetic acid was added (1M final concentration). The resulting mixture was homogenized with a Polytron mixer for 2 min. The supernatant, obtained by centrifugation at 15,000 × g for 10 min, was stored at 4 ℃ until assay.

Radioimmunoassays (RIA) for rat BNP and ANP : RIA for rat BNP was performed by the method reported previously (3). The antiserum used for this RIA system exhibited no crossreactivity with α-rat ANP. The intra- and inter-assay coefficients of variance were 5.3% and 7.8%, respectively. RIA for rat ANP was performed as described previously (4). This system showed 0.003% crossreactivity with rat BNP-32.

Characterization of rat BNP and ANP in the heart : Acid extracts of rat cardiac tissue were directly applied to Sephadex G-50 gel filtration (1.8 × 134 cm, Pharmacia) using 1M acetic acid as a solvent. An aliquot of each fraction was used for RIAs of rat BNP and ANP. Fractions exhibiting BNP and ANP immunoactivity were further analyzed by reverse phase high performance liquid chromatography (RP-HPLC) on a Hi-Pore RP-318 column (4.6 × 250 mm, Bio-Rad) with a linear gradient elution of CH_3CN from 10%-60% in 0.1% TFA. Aliquots of all fractions were submitted to RIAs for rat BNP and ANP.

Other assays : Plasma and urine samples were analyzed for total protein, albumin, urea nitrogen, creatinine, and electrolytes with an automatic analyzer (Model 736, Hitachi, Tokyo, Japan).

Analytical methods : Student's t-test was used for comparison between control and nephrotic groups. Linear regression was evaluated by the least-squares method. Data are represented as mean ± standard error of the mean (S.E.M.). Statistical significance was defined as $p < 0.05$.

RESULTS

Physiological and biochemical data : All rats receiving adriamycin developed nephrotic syndrome characterized by proteinuria, hypoalbuminemia, and ascites 3 weeks

Table 1. Physiological, biochemical characteristics, and ir-BNP and ir-ANP in plasma and cardiac tissue of control and adriamycin-induced nephrotic rats

	Control rats (n=15)	Nephrotic rats (n=15)
Body weight (g)	357 ± 3.51	303 ± 4.61***
Systolic blood pressure (mmHg)	126 ± 6.47	134 ± 5.50
heart rate (min^{-1})	362 ± 10.9	380 ± 26.7
Plasma		
Total protein (g/dl)	6.04 ± 0.23	5.02 ± 0.03***
Albumin (g/dl)	4.12 ± 0.12	3.03 ± 0.09***
Urea nitrogen (mg/dl)	15.9 ± 2.01	19.4 ± 2.19
Creatinine (mg/dl)	0.48 ± 0.07	0.52 ± 0.04*
Urine		
UProE (mg/day/100g BW)	2.80 ± 0.24	123 ± 6.64***
UNaE (μEq/day/100g BW)	563 ± 17.0	372 ± 23.5**
Plasma		
Ir-BNP (fmol/ml)	0.73 ± 0.16	1.40 ± 0.23***
Ir-ANP (fmol/ml)	29.8 ± 3.00	66.4 ± 8.94***
Right atrium		
Ir-BNP (pmol/g wet wt.)	316 ± 22.7	331 ± 22.0
Ir-ANP (nmol/g wet wt.)	60.0 ± 3.16	74.2 ± 3.72**
Left atrium		
Ir-BNP (pmol/g wet wt.)	283 ± 18.0	299 ± 23.3
Ir-ANP (nmol/g wet wt.)	42.0 ± 3.30	44.9 ± 3.61
Ventricle		
Ir-BNP (pmol/g wet wt.)	3.61 ± 0.21	5.49 ± 0.45***
Ir-ANP (pmol/g wet wt.)	48.9 ± 4.45	137 ± 15.0***

UProE : urinary protein excretion UNaE : urinary sodium excretion
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. control. mean ± SEM

after administration (Table 1). There was no difference in plasma creatinine, urea nitrogen, and blood pressure between the two gropus. Daily urinary sodium excretion in nephrotic rats was significantly reduced ($p < 0.01$) compared to controls.

Atrial and ventricular concentration of ir-BNP and ir-ANP: The ir-BNP and ir-ANP concentrations in both atria and the ventricle are shown in Table 1. The ir-BNP concentrations of the right and left atria were higher in nephrotic rats than in controls, but the difference was not statisticaly significant. A significantly higher ir-BNP concentration was found in the ventricle of nephrotic rats compared with controls ($p < 0.001$). The ir-ANP concentration in the left atrium of nephrotic rats did not differ from that of controls, however, in the right atrium, it was significantly higher compared with controls ($p < 0.01$), and also in the ventricle ($p < 0.001$). Ir-BNP levels were correlated with ir-ANP levels in the right atrium ($r = 0.37$, $p < 0.05$), in the left atrium ($r = 0.56$, $p < 0.001$), and in particular in the ventricle ($r = 0.83$, $p < 0.001$), as shown in Fig. 1.

Characterization of atrial and ventricular ir-BNP and ir-ANP: In each group, two peaks of ir-BNP were observed in fractions #26-30 and #36-40, corresponding to molecular weights (MW) 13k daltons and 5k daltons, respectively, in the Sephadex G-50 profile (Fig. 2). Each peak was analyzed by RP-HPLC on a C-18 column. The

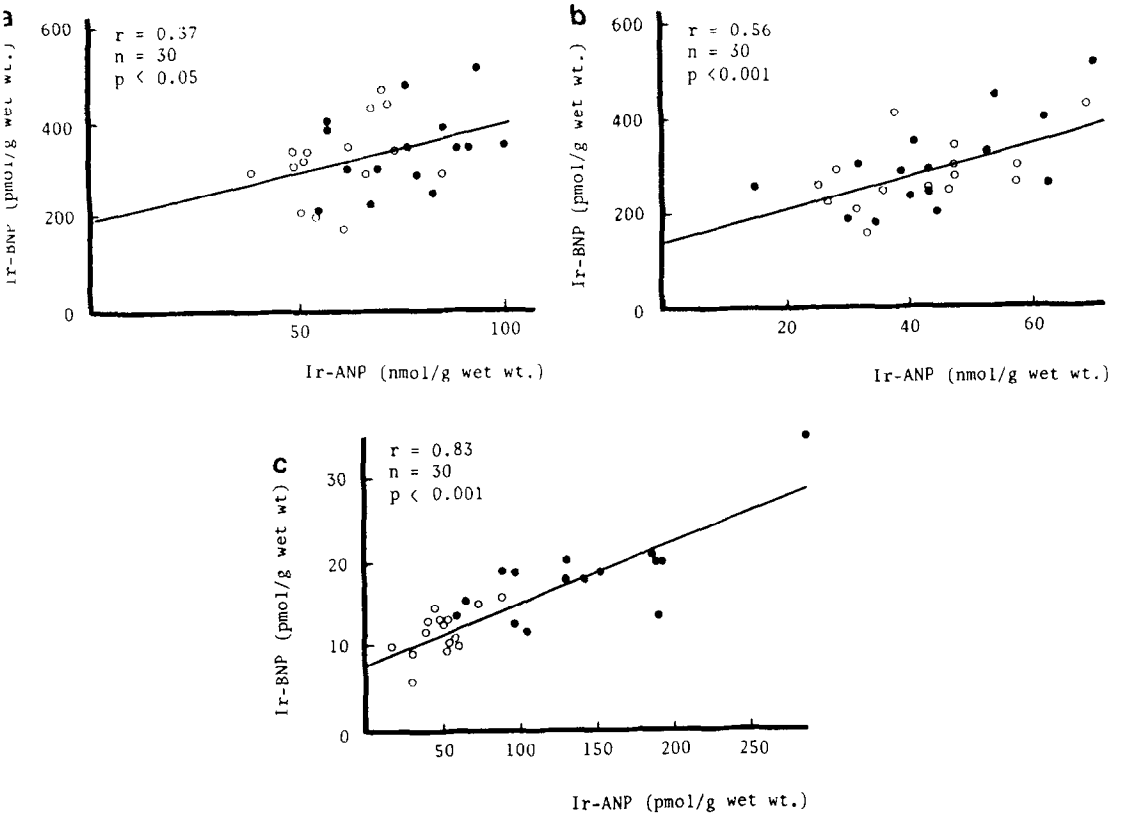

Fig. 1. (a) Relationship between ir-BNP and ir-ANP concentration in the right atrium, (b) left atrium, and (c) ventricle. ○ = control rats, ● = nephrotic rats

low MW ir-BNP was eluted as a single peak at an elution time identical to that of synthetic BNP-45. High MW ir-BNP fractions also emerged as a single peak, which was identical to that of γ-BNP (data not shown). However, ir-ANP emerged as one peak of 13k daltons in both atria and the ventricle, which was identified as γ-ANP by the RP-HPLC profile. The relative ratio of low MW form BNP (BNP-45) to high MW form BNP (γ-BNP) were : right atrium, 1.70 in controls and 3.85 in nephrotic rats ; left atrium, 1.61 in controls and 2.51 in nephrotic rats ; ventricle, 0.49 in controls and 1.04 in nephrotic rats.

Plasma concentration of ir-BNP and ir-ANP, and relation to urinary sodium excretion : The plasma concentration of ir-BNP and ir-ANP are shown in Table 1. It was impossible to obtain enough blood from controls to determine ir-BNP levels in individual plasma samples because of its extremely low concentration. Thus, plasma ir-BNP levels in control rats were measured after they were divided into five groups. A significant higher plasma ir-BNP concentration was observed in nephrotic rats than in controls ($p < 0.001$). A similar result was obtained for ir-ANP ($p < 0.001$). There was a significant negative correlation between plasma ir-BNP level and daily urinary sodium excretion ($r = -0.84$, $p < 0.001$), and between plasma ir-ANP level and daily urinary sodium excretion ($r = -0.88$, $p < 0.001$) in nephrotic rats (Fig. 3).

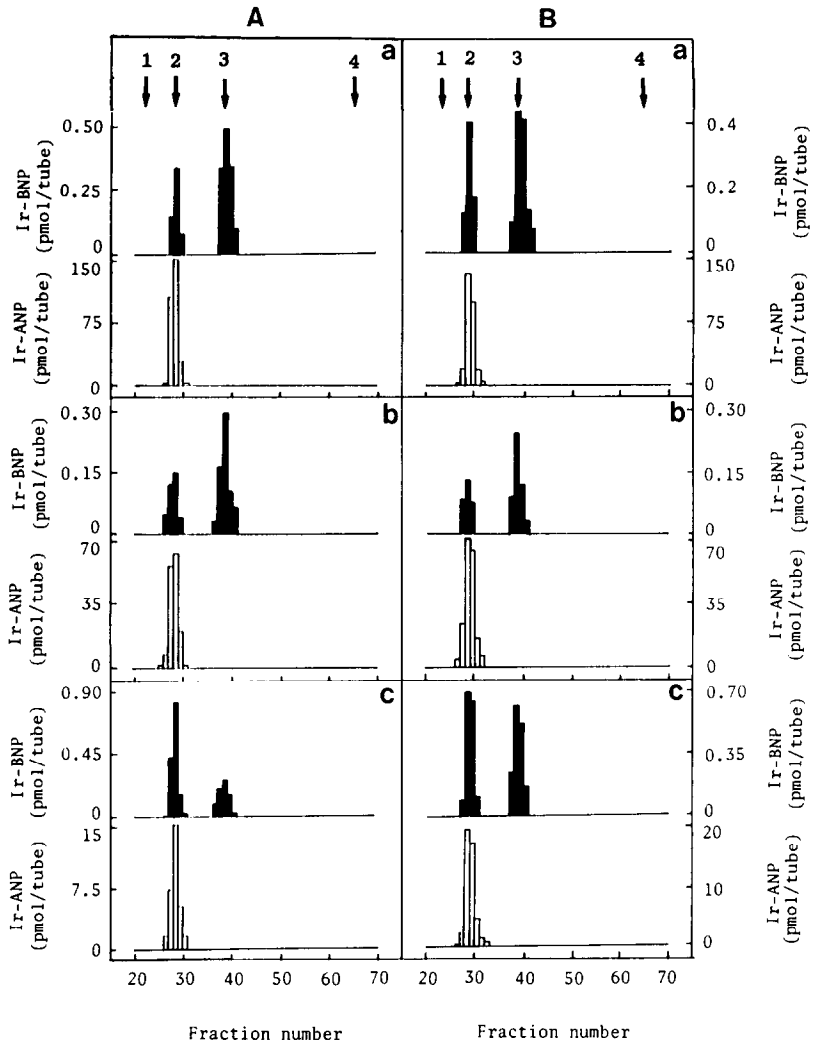

Fig. 2. (a) Sephadex G-50 gel filtration of acid extracts of right atrium, (b) left atrium, and (c) ventricle in control (A) and nephrotic rats (B).

Sample, A : control rats, B : nephrotic rats
(a) right atrium, A : 7.67 mg wet wt. B : 5.82 mg wet wt.
(b) left atrium, A : 6.56 mg wet wt. B : 6.07 mg wet wt.
(c) ventricle, A : 452 mg wet wt. B : 419 mg wet wt.
Column : Sephadex G-50 (1.8 × 134 cm, Pharmacia). Flow rate : 8 ml/h. Fraction size : 5 ml/tube. Solvent : 1M acetic acid. Arrows indicate elution positions of 1) Vo, 2) γ-ANP and γ-BNP, 3) BNP-45, and 4) Vt.

DISCUSSION

The results of this study showed that nephrotic syndrome was fully developed 3 weeks after a single intravenous injection of adriamycin, which causes extensive fusion of foot process of glomerular epithelial cells and loss of the glomerular basement membrance charge barrier (6). Glomerular changes were not observed by light microscopy, and no immunoglobulin deposits were observed by immunofluorescence (data not shown). There was no difference in plasma creatinine concentration and blood pressure between control and adriamycin-injected rats. These biochemical,

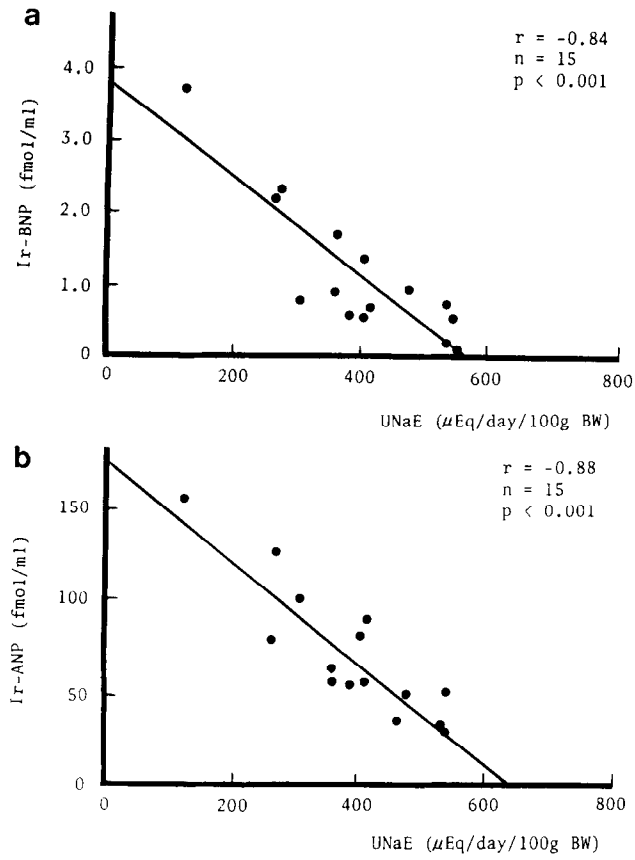

Fig. 3. (a) Relationship between plasma ir-BNP and urinary sodium excretion (UNaE), and (b) plasma ir-ANP and UNaE in nephrotic rats. ○= control rats, ●= nephrotic rats

physiological, and histological findings indicated that the adriamycin-injected rats of this study are a model of minimal-change nephrotic syndrome without renal failure.

In the present study, there was no difference in the ir-BNP concentration of both atria of control and nephrotic rats, whereas the ratio of BNP-45/γ-BNP in nephrotic rats was higher than that of the controls in both atria, in particular in the right atrium, indicating an increase in the secretory form of BNP-45. The ir-ANP concentration in the right atrium of nephrotic rats was significantly higher than that of controls. These results suggest that both BNP-45 and ANP production in the right atria were increased compared to controls.

The ir-BNP concentration in the ventricle was significantly increased, and the ratio of BNP-45/γ-BNP was also increased in nephrotic rats compared to controls. Thus, production of BNP and conversion of γ-BNP to BNP-45 are accelerated, resulting in a large store of secretory-form BNP-45 in the ventricle of nephrotic rats. These changes seem to correspond to probable accelerated BNP secretion from the ventricle. Similary, ventricular ir-ANP concentration was increased in nephrotic rats compared to controls. Common stimulating factors which increase ventricular production of BNP as well as ANP may be involved in nephrotic rats.

A positive correlation between plasma ANP levels and blood volume in humans (7) and rats (8) with nephrotic syndrome has been previously reported. These results suggest that plasma ANP concentration may be a good marker of intravascular volume status in nephrotic syndrome. Although we did not measure intravascular volume in the present study, increased plasma level of ANP associated with decreased urinary sodium excretion may represent blood volume expansion in these nephrotic rats. However, ir-BNP levels were correlated with ir-ANP levels in both atria and the ventricle in this experiment. Although the conversion pathway differs from that of ANP, cardiac production of BNP may also be closely associated with intravascular volume status, and participate in the regulation of body fluid homeostasis similarly to ANP.

Plasma ir-BNP and plasma ir-ANP in nephrotic rats were 1.9 times, and 2.2 times higher than that of controls. These changes were associated with increase BNP and ANP production and secretion in the heart. However, daily urinary sodium excretion was decreased in nephrotic rats compared to controls and the levels of plasma BNP and ANP were negatively correlated with urinary sodium excretion. These results suggest that sodium and water retention in nephrotic syndrome cannot be attributed to a lack of ANP and BNP production and/or secretion, and the elevation of plasma levels of ANP and BNP is considerd to be a compensatory phenomenon induced by a decreased renal ability to eliminate sodium and water in nephrotic rats.

BNP acts on the kidney in a manner identical to that of ANP by binding to ANP receptors in both glomerular and inner medullary collecting duct cells (IMCD), stimulating particulate guanylate cylase, and inhibiting Na^+ uptake in IMCD (9). Previous studies demonstrated attenuated renal responsiveness to diuretics and natriuretics with synthetic ANP in nephrotic rats (8,10-13) and humans (7), despite a comparable increase in glomerular filtration rate compared to controls (8,11). In addition, Perico et al.(8) showed that ANP receptor density and affinity in the inner stripe of the outer medulla and the inner medulla are comparable in normal and adriamycin-induced nephrotic rats. Thus, the existence of intrarenal factors disturbing the ANP-BNP system are suggested in the nephrotic syndrome. To clarify the mechanisms causing retention of sodium and water in the nephrotic syndrome, intrarenal factors decreasing renal response to endogenous ANP and BNP need to be investigated.

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