Brain Natriuretic Peptide as a Novel Cardiac Hormone in Humans

Evidence for an Exquisite Dual Natriuretic Peptide System, Atrial Natriuretic Peptide and Brain Natriuretic Peptide

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Abstract

Using a specific radioimmunoassay for human brain natriuretic peptide (hBNP) with a monoclonal antibody, we have investigated its synthesis, secretion, and clearance in comparison with those of atrial natriuretic peptide (ANP) in normal subjects and patients with congestive heart failure (CHF). Mean BNP-like immunoreactivity (-LI) levels in normal atrium and ventricle were 250 and 18 pmol/g, respectively. The plasma BNP-LI level in normal subjects was 0.90±0.07 fmol/ml, which was 16% of the ANP-LI level. In contrast, the plasma BNP-LI level markedly increased in patients with CHF in proportion to its severity, and surpassed the ANP-LI level in severe cases. There was a significant step-up of the plasma BNP-LI level in the coronary sinus (CS) compared with that in the aortic root (Ao) and the difference between these BNP-LI levels, $\Delta_{(CS-Ao)}$BNP, also increased with the severity of CHF. In addition, the step-up of the BNP-LI level in the anterior interventricular vein [$\Delta_{(AIV-Ao)}$BNP] was comparable to $\Delta_{(CS-Ao)}$BNP, indicating that BNP is secreted mainly from the ventricle. Predominant BNP synthesis in the ventricle was also confirmed by Northern blot analysis. Catheterization and pharmacokinetic studies revealed that hBNP is cleared from the circulation more slowly than α-hANP; this was in part attributed to lower (about 7%) binding affinity of hBNP to clearance receptors than that of α-hANP. A predominant molecular form of BNP-LI in the heart and plasma was a 3-kD form corresponding to hBNP. These results indicate that BNP is a novel cardiac hormone secreted predominantly from the ventricle, and that the synthesis, secretion and clearance of BNP differ from those of ANP, suggesting discrete physiological and pathophysiological roles of BNP in a dual natriuretic peptide system. (*J. Clin. Invest.* 1991. 87:1402–1412.) Key words: congestive heart failure • gene expression • monoclonal antibody • pharmacokinetics • radioimmunoassay

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Introduction

Since the discovery of atrial natriuretic peptide (ANP)[1] in the heart (1–5) and subsequently in the brain (5–10), ANP has been implicated in body fluid homeostasis and blood pressure control as a hormone and as a neuropeptide (1–11). We and others have previously demonstrated that the synthesis and secretion of ANP in the heart are increased in patients with congestive heart failure (CHF) in relation to its severity (12–18).

More recently, brain natriuretic peptide (BNP) was isolated from the porcine brain (19), which has either 26 or 32 amino acid residues, porcine (p) BNP-26 and pBNP-32, respectively (20), with a remarkable sequence homology to ANP and has peripheral and central actions similar to those of ANP (19, 21–23). BNP is also synthesized in, and secreted into the circulation from, the porcine heart (24, 25). Subsequently, we and others isolated rat BNP (rBNP) with 45 amino acid residues from the rat heart (26–28). To date, however, the information on BNP in humans is scarce, mainly for lack of cross-reactivity of human BNP (hBNP) with antisera against pBNP or rBNP.

Recently, after the elucidation of the cDNA sequence encoding the hBNP precursor, hBNP[1–108] (29), we have isolated hBNP from the human atrium and determined its amino acid sequence (30). hBNP comprises 32 amino acid residues (30), which is identical to the sequence [77–108] of the hBNP precursor.

In the present study, we have developed a monoclonal antibody against hBNP and established a specific radioimmunoassay (RIA) for hBNP. Using the RIA, we have investigated the synthesis, secretion, and clearance of hBNP in normal subjects and patients with CHF, comparing them with those of ANP.

Methods

Peptides

hBNP (hBNP[77–108]), hBNP[83–108], and [Tyr^{82}]-hBNP[83–108] were gifts from Professor H. Matsuo at the National Cardiovascular

1. *Abbreviations used in this paper:* AIV, anterior interventricular vein; ANP, atrial natriuretic peptide; Ao, aorta; BNP, brain natriuretic peptide; similarly, hBNP, pBNP, and rBNP, human, porcine, and rat BNP; C, clearance (receptor); CAD, coronary artery disease; CHF, congestive heart failure; CS, coronary sinus; DCM, dilated cardiomyopathy; HP-GPC, high-performance gel permeation chromatography; -LI, -like immunoreactivity; NYHA, New York Heart Association; VHD, valvular heart disease.

Center, Suita, Japan. A carboxy-terminal fragment of hBNP, or hBNP[103–108], pBNP-32 (pBNP[75–106]), and rBNP (rBNP[51–95]) were synthesized by the solid-phase method. α-Human ANP (α-hANP, or human ANP[99–126]) and α-rat ANP (α-rANP) were purchased from Peptide Institute, Inc., Minoh, Japan. An ANP analogue des[Gly^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}]-α-rANP [4–23]-NH_2 (C-ANF[4–23]) was generously provided by Professor T. Maack, Cornell University Medical College, New York.

Preparation and characterization of monoclonal antibody

hBNP[83–108] (2.3 mg) was conjugated to bovine thyroglobulin (9.1 mg, Sigma Chemical Co., St. Louis, MO) using the carbodiimide coupling procedure (31). 10 BALB/c mice were immunized with subcutaneous injections of the conjugate containing 20 μg of the peptide emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) over a period of 2 mo at 2–3-wk intervals. Sera were periodically screened for the antibody titer by RIA with ^{125}I-[Tyr^{82}]-hBNP[83–108] as described below. Elevation of the antibody titer was observed in two mice, and the one with higher response (final dilution, 1:50,000) was selected for cell fusion. Fusion of spleen cells from the immunized mouse with mouse myeloma cells, X63-Ag8.653, was performed in a ratio of 5:1 using 50% polyethylene glycol 4000 (Merck & Co., Darmstadt, FRG) (32). Hybridomas were screened for their ability to produce antibody by the RIA for hBNP, cloned by the limiting dilution technique, and expanded intraperitoneally in BALB/c mice (32).

Isotyping of the monoclonal antibody was performed by the Ouchterlony technique (Mouse Monoclonal Typing Kit, Miles Laboratories, Inc., Elkhart, IN). Binding affinity was analyzed by a Scatchard plot using the RIA for hBNP.

Tissues and extraction procedure

Cardiac tissues were obtained from patients without cardiac complications (n = 6) at autopsy (normal hearts), and from those with CHF (n = 6) at autopsy or cardiac surgery (failing hearts). Among the patients with CHF, five suffered from coronary artery disease (CAD) and one from dilated cardiomyopathy (DCM). One patient with CAD and one with DCM were autopsy cases. Samples were immediately frozen in liquid nitrogen and stored at −70°C until extraction. Cardiac tissues were boiled for 5 min in 10 vol of 1 M acetic acid containing 20 mM HCl to abolish intrinsic proteolytic activity and extraction of BNP from tissues was carried out as described previously (15, 16, 31).

Plasma sampling

Plasma samplings were performed in healthy subjects and patients. Informed consent was obtained from them and the study was approved by the ethical committee on human research of Kyoto University (No. 61-9). Plasma BNP levels at various sampling sites were measured and compared with ANP levels simultaneously determined.

Sampling from the antecubital vein. Peripheral plasma samples were obtained from 11 healthy male subjects (aged 25–33 yr, mean 29.6 yr) with normal salt intake (163±15 meq/d) and from 39 patients with heart disease (26 men and 13 women, aged 16–78 yr, mean 54.5 yr). Among them, 12 patients had CAD, 9 had valvular heart disease (VHD), 7 had DCM, 7 had hypertensive heart disease, 2 had congenital heart disease, 1 had hypertrophic obstructive cardiomyopathy, and 1 had myocarditis. According to the classification of the New York Heart Association (NYHA), 8 patients were classified as class I, 10 as class II, 16 as class III, and 5 as class IV. None of the patients had evidence of renal failure. Blood was withdrawn at 9:00 a.m. from the antecubital vein in a recumbent position after an overnight fast, immediately transferred to chilled siliconized glass tubes containing Na_2EDTA (1 mg/ml) and aprotinin (1,000 KIU/ml, Ohkura Pharmaceutical, Kyoto, Japan), and centrifuged at 4°C. Plasma was immediately frozen and stored at −20°C until assay.

Sampling during cardiac catheterization. Blood sampling was performed from various sites in two series of cardiac catheterization. In the first series, plasma samples were taken from the coronary sinus, the aortic root and the femoral vein through a catheter in six patients (four men and two women, aged 29–74 yr, one in NYHA class I, four in NYHA class II, and one in NYHA class III; see Table II).

In the second series of catheterization, plasma samples were also obtained from the anterior interventricular vein (AIV), which drains the left ventricle (17, 33), in addition to the coronary sinus (CS) and the aorta (Ao) in 11 patients (eight men and three women, aged 21–69 yr). According to our previous study (17), the difference in the plasma ANP level between the AIV and the aorta [$\Delta_{(AIV\text{-}Ao)}$ANP], which reflects the amount of ANP released from the left ventricle, was calculated, and the patients were classified into two groups of normal (group I) and increased ventricular secretion of ANP (group II). $\Delta_{(AIV\text{-}Ao)}$ANP in group I was < 50 fmol/ml. Among six patients in group I with no or mild CHF (two in NYHA class I and four in class II), four patients had CAD, one had VHD, and one had chest pain syndrome. Five patients in group II (four in NYHA class II and one in class III) consisted of three patients with VHD, one with CAD, and one with DCM.

Pharmacokinetics of hBNP

Six healthy male volunteers (aged 27–32 yr, body weight of 65.2±2.2 kg) participated in the pharmacokinetic study after giving their informed consent. After an overnight fast, the subject was kept recumbent for 30 min, and then received the constant intravenous infusion of synthetic hBNP at a rate of 0.1 μg (29 pmol)/kg per min for 15 min using an infusion pump (model SP-50, Nipro, Osaka, Japan) with a flow rate of 1.0 ml/min. Blood samples were obtained 0, 1, 3, 5, 10, 15, 30, 45, 60, 75, and 90 min after the cessation of infusion through an indwelling cannula in the antecubital vein to determine plasma hBNP levels. Pharmacokinetic analysis of hBNP was performed using a two-compartment open model as previously reported (34).

RIA for hBNP

[Tyr^{82}]-hBNP[83–108] (1 μg) was radioiodinated by the chloramine-T method as previously described (31). The specific activity of ^{125}I-[Tyr^{82}]-hBNP[83–108] ranged from 500 to 900 μCi/μg. The monoclonal antibody named KY-hBNP-I (final dilution of ascites, 1:5 × 10^6) was incubated with either standard hBNP or samples in 0.2 ml of assay buffer (50 mM phosphate buffer, pH 7.4, containing 0.1% gelatin (Merck), 0.1% Triton X-100, 1 mM Na_2EDTA, 0.2 mM L-cystine, and 0.1% NaN_3) for 24 h at 4°C. Then, 0.05 ml ^{125}I-[Tyr^{82}]-hBNP[83–108] (10,000 cpm) was added and the mixture was further incubated for 24 h at 4°C. Bound and free ligands were separated by adding 1.0 ml of a suspension of dextran-coated charcoal consisting of 250 mg of Norit SX Plus (Norit Vereenging N.V., The Netherlands) and 25 mg of Dextran T-70 (Pharmacia, Uppsala, Sweden) in 100 ml of 50 mM phosphate buffer, pH 7.4, containing 0.01% merthiolate, as previously described (31).

Measurement of plasma BNP-like immunoreactivity (BNP-LI) concentrations was performed with or without extraction. In the assay without extraction, 25 μl of plasma was added to the incubation mixture. Hormone-free plasma, prepared by passing normal plasma through a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) (12, 35), was used for constructing the standard curve and diluting plasma samples. For the RIA with extraction, peptides were extracted from 5–10 ml of plasma using a Sep-Pak C_{18} cartridge, as described previously (12, 24, 35). The mean recovery of 3–15 fmol/ml hBNP added to plasma was 70%. The minimal detectable concentrations of BNP-LI in plasma with and without extraction were 0.4 and 10 fmol/ml, respectively.

RIA for ANP

The RIA for ANP was performed as already reported (12, 31). This RIA recognizes the carboxy-terminal portion of α-hANP, α-ANP[17–28]. The cross-reactivity with hBNP was < 0.01% on a molar basis.

High-performance gel permeation chromatography (HP-GPC)

HP-GPC was performed on a TSK-GEL G2000 SW column (7.5 × 600 mm, Toyo Soda, Tokyo, Japan), eluted with 10 mM trifluoroacetic

acid containing 0.3 M sodium chloride and 30% acetonitrile as a solvent as previously reported (12, 15, 24, 26). The flow rate was 0.3 ml/min and the fraction volume was 0.36 ml.

Reverse-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC was carried out on a Nucleosil $5C_{18}$ column (4.6 × 150 mm, Macherey-Nagel, Duren, FRG) as we described elsewhere (27, 30). Elution was performed with a linear gradient of acetonitrile from 15% to 30% in 0.1% trifluoroacetic acid. The flow rate was 1.0 ml/min and the fraction volume was 1.0 ml.

Northern blot analysis

Cardiac tissues obtained from patients at autopsy were also subjected to Northern blot analysis of tissue BNP mRNA. Total RNA was extracted from atrial and ventricular tissues and levels of BNP mRNA and ANP mRNA were measured, as described elsewhere (16, 36, 37). The hBNP cDNA probe (462 bp) including the entire coding region for hBNP was prepared by the cDNA synthesis and the polymerase chain reaction method (37, 38). The hANP cDNA probe was 581-bp SacI-PstI fragment (16). The human β-actin probe was purchased from Wako Pure Chemical Industries, Osaka, Japan. These probes were labeled by the random priming method (38) with [α-^{32}P]dCTP (Amersham International, Buckinghamshire, UK). Specific activities of the probes were ~ 1×10^9 cpm/μg. Actin mRNA levels were almost equivalent among different RNA samples.

Binding study with human clearance (C) receptor

Crude human C receptors for natriuretic peptides were prepared from the fresh human lung tissue obtained at surgery (39), according to the procedure reported by Shimonaka et al. (40). α-rANP was radioiodinated by the chloramine-T method (31). Solubilized C receptors were incubated with ^{125}I-α-rANP (10,000 cpm) and various concentrations of α-hANP, hBNP, or C-ANF[4–23] in 0.5 ml of assay buffer (40) for 48 h at 4°C, and bound and free ligands were separated by the dextran-coated charcoal method (31, 39). Nonspecific binding was defined as binding in the presence of 10^{-7} M unlabeled α-hANP.

Statistical analysis

Data were expressed as means±SE. Statistical analysis was performed using Student's *t* test or Duncan's multiple range test when appropriate. Linear regression analysis was used to determine correlations between results.

Results

Preparation and characterization of monoclonal antibody. After the fusion, three clones among 288 wells of hybridoma gave a positive antibody response. After further culture and cloning, one clone that produced antibody with the strongest response was selected for expansion and characterization. The established monoclonal antibody, named KY-hBNP-I, belonged to the IgG_1 subclass. Analysis by a Scatchard plot revealed a high affinity for hBNP, with an association constant of 1.0×10^{11} M^{-1}. Specificity of the monoclonal antibody, KY-hBNP-I, in the RIA for hBNP is shown in Fig. 1 *A*. hBNP[83–108], which is a 26-amino acid fragment of hBNP lacking amino-terminal six amino acids, showed an equimolar cross-reactivity with standard hBNP. There was no cross-reactivity (< 0.001%) with the extreme carboxy-terminal fragment of hBNP, hBNP[103–108]. These results indicate that the antibody recognizes the ring structure of hBNP.

RIA for hBNP. In the standard curve of the RIA for hBNP (Fig. 1 *A*), the minimal detectable quantity was 0.3 fmol per tube, and the 50% binding intercept was 3 fmol per tube. The

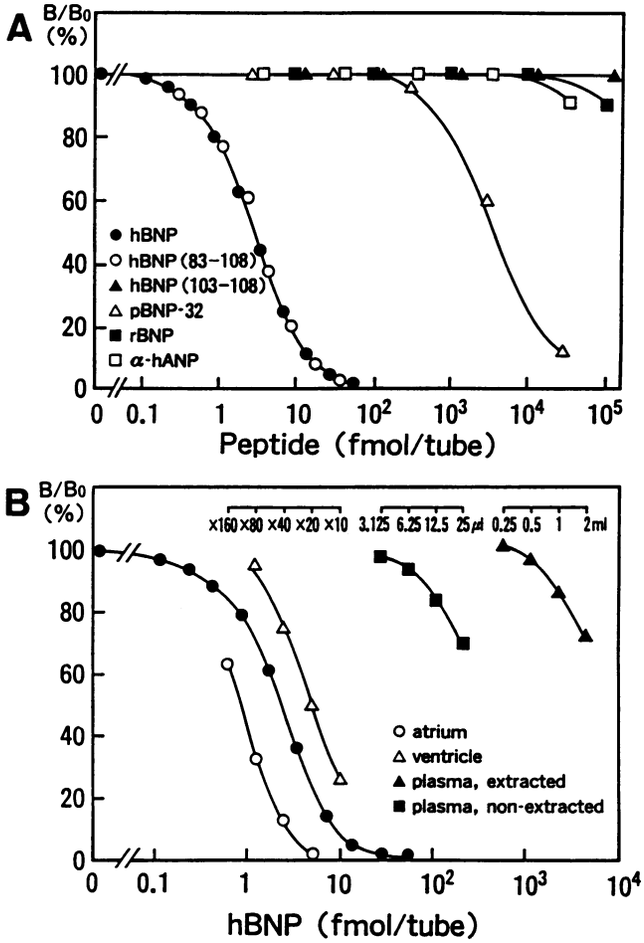

Figure 1. Typical standard curves of hBNP and cross-reactivity profiles of its related peptides (*A*) and dilution curves of various samples (*B*) in the RIA for hBNP with a monoclonal antibody, KY-hBNP-I.

cross-reactivity with α-hANP or rBNP was < 0.005%. pBNP-32 showed a cross-reactivity of 0.1% on a molar basis. The intra- and interassay coefficients of variation were 8.4% (n = 8) and 6.4% (n = 8), respectively.

BNP-LI in the normal heart. Serial dilution curves of human atrial and ventricular extracts were parallel to the standard curve as depicted in Fig. 1 *B*. The tissue levels and contents of BNP-LI in the normal atrium and ventricle are shown in Table I. There was no significant difference in the BNP-LI level between right and left sides of the heart. The mean concentration of BNP-LI in the atrium was 250 pmol/g, and a considerable amount of BNP-LI (18±3 pmol/g) was also detected in the ventricle. The ventricular BNP-LI level was 7.2% of the atrial level. Taking tissue weight into account, the total amount of BNP-LI in the ventricle (4.5 nmol) was ~ 30% of that in the whole heart (15.3 nmol). The ratio of BNP-LI to ANP-LI was much higher in the ventricle (49%) than in the atrium (2.6%).

Fig. 2 *A* shows a representative HP-GPC profile of the extract from a normal human atrium. BNP-LI was composed of two components with approximate molecular masses of 12 and 3 kD in the atrium, in which the 3-kD form was predominant. The elution position of 3-kD BNP-LI was identical to that of synthetic hBNP. The 12-kD component corresponded to the

Table I. Tissue Levels and Total Amounts of BNP and ANP in Normal and Failing Human Hearts

	Normal heart			Failing heart		
	BNP	ANP	BNP/ANP	BNP	ANP	BNP/ANP
	pmol/g		%	*pmol/g*		%
Tissue level						
Atrium	250±60	9,600±3,100	2.6	180±60	12,300±3,700	1.5
Ventricle	18±3	37±14	49	40±11	330±94*	12
V/A (%)	7.2	0.4	—	22	2.7	—
	nmol		%	*nmol*		%
Total amount						
Atrium	10.8±2.7	420±140	2.6	14.7±4.7	1,000±310	1.5
Ventricle	4.5±0.8	9.1±3.5	49	15.9±4.2*	135±31‡	12
V/A (%)	42	2.2	—	108	14	—
A/A + V (%)	70	98	—	48	88	—
V/A + V (%)	30	2	—	52	12	—

Values are expressed as the mean±SE from six normal and six diseased cardiac tissues. Abbreviations: V, ventricle; A, atrium. * $P < 0.05$, ‡ $P < 0.01$ compared with values in normal hearts.

precursor form of hBNP. Similar HP-GPC profiles were obtained in other atrial extracts. The extract from the ventricle showed essentially the same HP-GPC profile as in Fig. 2 *A*. Such an HP-GPC profile of BNP-LI contrasted well with that of ANP-LI in the normal human heart, which consisted of γ-hANP, the precursor of α-hANP, as shown in Fig. 2 *A*. To further characterize 3-kD BNP-LI, we analyzed RP-HPLC profile of the atrial extract. As shown in Fig. 2 *B*, the retention time of 3-kD BNP-LI was identical to that of synthetic hBNP. Furthermore, even under an isocratical elution condition in RP-HPLC this component co-migrated with synthetic hBNP.

Plasma BNP-LI in normal subjects. The serial dilution curve of plasma extract was parallel to the standard curve of hBNP as shown in Fig. 1 *B*. The plasma BNP-LI level in 11 normal subjects in a recumbent position after an overnight fast was 0.90±0.07 fmol/ml, whereas the ANP-LI level in the same plasma samples was 6.4±0.9 fmol/ml. The molar ratio of plasma BNP-LI to ANP-LI was 16±2%.

BNP-LI in the failing heart. The tissue BNP-LI levels in human failing hearts are also shown in Table I. When compared with those in normal hearts, the BNP-LI level in the atrium did not change significantly, whereas that in the ventricle increased more than double. Thus, the ventricular BNP-LI level increased to 22% of the atrial level in the failing heart. Taking tissue weight into consideration, the total amount of BNP-LI in the ventricle surpassed that in the atrium, reaching 52% of the total BNP-LI content in the whole heart. Tissue levels of ANP-LI increased both in the atrium and ventricle of the failing heart, where the ventricular level was 2.7% of the atrial level. The total content of ANP-LI in the ventricle was

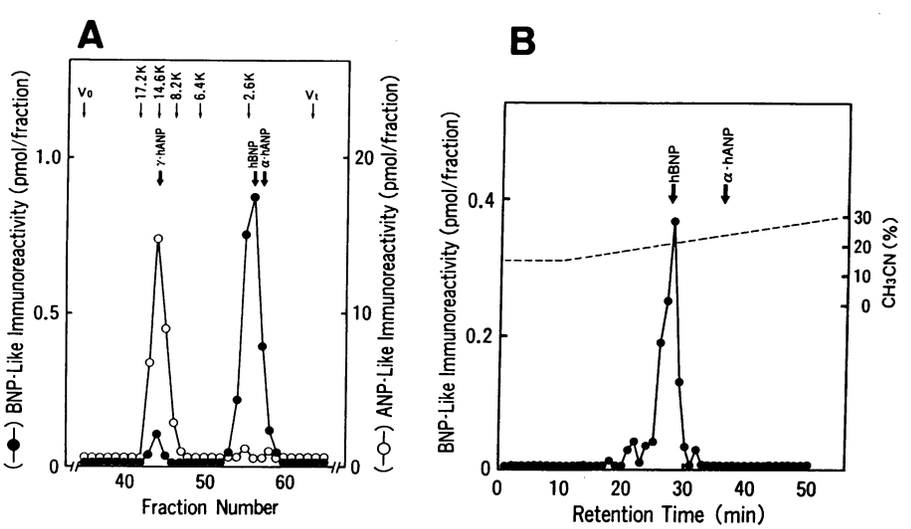

Figure 2. (*A*) Typical HP-GPC and (*B*) RP-HPLC profiles of the extract from a normal atrium. (*A*) Arrows denote elution positions of a series of myoglobins of a polypeptide molecular weight calibration kit (Pharmacia, Uppsala, Sweden), void volume (*Vo*) and total volume (*Vt*). Elution positions of γ-hANP, synthetic hBNP, and α-hANP are also indicated. (*B*) Arrows denote retention times of synthetic hBNP and α-hANP.

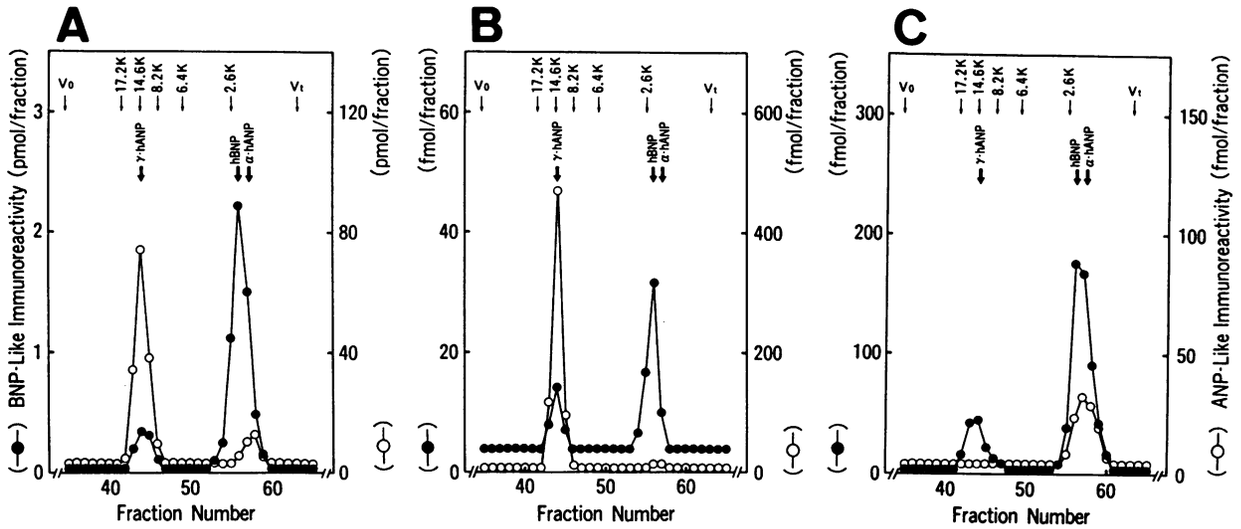

Figure 3. Typical HP-GPC profiles of extracts from the atrium (*A*) and ventricle (*B*) of the failing heart, and of plasma (*C*) taken from a patient with CHF. Symbols and arrows are the same as in Fig. 2A.

12% of that in the whole heart. The ratio of BNP-LI to ANP-LI was reduced in the failing heart compared with that in the normal heart.

Fig. 3, *A* and *B* shows representative HP-GPC profiles of extracts from the atrium and ventricle, respectively, of the failing human heart. BNP-LI in the atrial extract of the failing heart comprised two components. The major component was hBNP and the minor component was the precursor form, which showed the same profile as that in the normal atrium shown in Fig. 2 *A*. BNP-LI in the ventricular extract of the failing heart exhibited essentially the same HP-GPC profile (Fig. 3 *B*). As for ANP-LI, α-hANP, which is hardly detectable in the normal heart (15), was increased in the atrium of the failing heart (Fig. 3 *A*), while γ-hANP was almost exclusive in the ventricle (Fig. 3 *B*).

Plasma BNP-LI in CHF. The serial dilution curve of plasma was parallel to the standard curve of hBNP as shown in Fig. 1 *B*. Fig. 4 shows plasma BNP-LI levels in 39 patients with cardiac disease as well as in normal subjects. Of eight patients without CHF (NYHA class I), plasma BNP-LI levels were below 10 fmol/ml in four patients and were slightly increased in other four (14–22 fmol/ml, mean±SE; 14.3±1.8 fmol/ml). Out of 10 patients in NYHA class II, BNP-LI levels were increased in six patients (12–399 fmol/ml), although they were below 10 fmol/ml in four patients (mean±SE; 68.9±37.9 fmol/ml). In 21 patients with severe CHF (NYHA class III or IV), plasma BNP-LI levels were markedly elevated (NYHA class III, 15–539 fmol/ml, mean±SE; 155.4±39.1 fmol/ml, NYHA class IV, 119–563 fmol/ml, mean±SE; 267.3±79.9 fmol/ml). Thus, a progressive rise in the plasma BNP-LI level was observed in accordance with the severity of CHF.

The plasma ANP-LI level in these four classes significantly increased according to the severity of the disease (NYHA class I, 24.9±7.2 fmol/ml; class II, 52.4±19.4 fmol/ml; class III, 109.3±21.8 fmol/ml; class IV, 164.4±20.3 fmol/ml), and correlated well with the plasma BNP-LI level ($r = 0.747$, $P < 0.01$). However, the ratio of BNP-LI to ANP-LI in plasma varied markedly depending on the severity of CHF. In normal subjects, the plasma BNP-LI level was six times lower than the ANP-LI level, whereas the mean plasma BNP-LI level in patients with severe CHF (NYHA class III or IV) was much higher than the ANP-LI level determined simultaneously, with mean plasma BNP/ANP ratios of 1.44 and 1.72 in NYHA classes III and IV, respectively. Therefore, when compared with normal levels, the augmentation of plasma BNP-LI in severe CHF was much more prominent (200–300 times) than that of ANP-LI (20–30 times).

A typical HP-GPC profile of plasma extract from a patient with CHF is depicted in Fig. 3 *C*. Plasma BNP-LI consisted of two components. The main molecular form was hBNP, although the precursor was also present as the minor component. ANP-LI in plasma was composed of α-hANP as shown in Fig. 3 *C*.

BNP-LI in plasma obtained during cardiac catheterization. Table II shows BNP-LI and ANP-LI levels in plasma samples taken from various sites in six patients in the first series of cardiac catheterization. When we compared plasma BNP-LI levels in the coronary sinus with those in the ascending aorta near the coronary ostium, there was a two- to threefold step-up

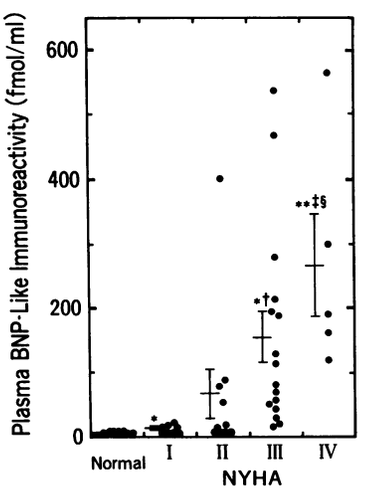

Figure 4. Plasma BNP-LI levels of 11 normal subjects and 39 patients with heart disease. Patients are classified according to the functional classification of NYHA and the mean±SE of the BNP-LI level in each group is indicated. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with values in normal group. $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ compared with NYHA class I group. $^{\S}P < 0.05$ compared with NYHA class II group.

Table II. BNP and ANP Levels in Plasma Taken from Various Sites during Cardiac Catheterization

Case	Age/sex	Disease	NYHA class	Coronary sinus			Aorta			Femoral vein			$\Delta_{(CS-Ao)}$		
				BNP	ANP	BNP/ANP	BNP	ANP	BNP/ANP	BNP	ANP	BNP/ANP	BNP	ANP	BNP/ANP
				fmol/ml			*fmol/ml*			*fmol/ml*			*fmol/ml*		
1	52/M	OMI	I	22	266	0.08	<5	27	<0.18	<5	19	<0.26	<22	189	<0.12
2	29/M	MS + TR	II	38	255	0.15	17	96	0.18	11	41	0.27	21	159	0.13
3	74/M	OMI	II	88	358	0.25	34	92	0.37	12	28	0.43	54	266	0.20
4	45/M	DCM	II	202	629	0.32	81	105	0.77	78	76	1.03	121	524	0.23
5	32/F	ASR	II	813	637	1.28	419	287	1.46	399	219	1.82	394	350	1.13
6	51/F	HOCM + MR	III	303	328	0.92	175	154	1.14	113	65	1.73	128	174	0.74

Abbreviations: $\Delta_{(CS-Ao)}$, difference of the levels between the coronary sinus and the aorta; OMI, old myocardial infarction; MS, mitral stenosis; TR, tricuspid regurgitation; DCM, dilated cardiomyopathy; ASR, aortic stenosis and regurgitation; HOCM, hypertrophic obstructive cardiomyopathy; MR, mitral regurgitation.

of the plasma BNP-LI level in the coronary sinus in all cases, demonstrating that BNP-LI is secreted into the circulation through the coronary sinus from the heart.

The BNP-LI concentration in the coronary sinus showed a rise proportional to the severity of CHF. Moreover, a similar increase was observed in the difference of the BNP-LI level between the coronary sinus and the aorta, $\Delta_{(CS-Ao)}$BNP, which is an indicator of its secretory rate (17, 41). These findings indicate that the secretion of BNP-LI is augmented in severe CHF. The ANP-LI level in the coronary sinus and $\Delta_{(CS-Ao)}$ANP tended to increase in severer cases, but the increment was rather smaller than that of the BNP-LI level. Accordingly, the ratio of $\Delta_{(CS-Ao)}$BNP to $\Delta_{(CS-Ao)}$ANP, which reflects their ratio of secretion, became higher in severe cases than in less severe ones, indicating that the secretion of BNP is more markedly augmented than that of ANP in accordance with the severity of CHF.

In order to further investigate the secretion of BNP from the heart, we measured BNP-LI levels in plasma taken from the AIV as well as the levels in the aorta and the coronary sinus in 11 patients of the second series of catheterization. The plasma BNP-LI level in the AIV was 138±56 fmol/ml, and was significantly higher than that in the aorta (66±33 fmol/ml) ($P < 0.05$). There was no significant difference in the plasma BNP-LI level between the AIV and the coronary sinus (132±53 fmol/ml). To further analyze the BNP secretion in relation to the severity of CHF, patients were classified into groups I and II according to the degree of ventricular secretion of ANP, as described in Methods. Fig. 5 illustrates the step-up of the BNP-LI level in the AIV [$\Delta_{(AIV-Ao)}$BNP] and that in the coronary sinus [$\Delta_{(CS-Ao)}$BNP] as well as those of the ANP-LI level in these two groups and total patients. In group I patients with normal $\Delta_{(AIV-Ao)}$ANP, there was a small but significant step-up of BNP-LI level in the AIV (21±8 fmol/ml). $\Delta_{(CS-Ao)}$BNP in these patients (18±7 fmol/ml) was almost equal to $\Delta_{(AIV-Ao)}$BNP. Thus, a predominant step-up of the BNP-LI level in the coronary circulation occurred between the aorta and the AIV and not between the AIV and the coronary sinus in these patients. In contrast to BNP secretion, a main step-up of the ANP-LI level was observed between the AIV and the coronary sinus, and $\Delta_{(AIV-Ao)}$ANP was < 10% of $\Delta_{(CS-Ao)}$ANP. $\Delta_{(AIV-Ao)}$BNP was almost equal to or even higher than $\Delta_{(AIV-Ao)}$ANP in group I patients. In group II, $\Delta_{(AIV-Ao)}$BNP as well as $\Delta_{(CS-Ao)}$BNP increased significantly compared with those of group I, and there was also no difference between $\Delta_{(AIV-Ao)}$BNP and $\Delta_{(CS-Ao)}$BNP. Both $\Delta_{(AIV-Ao)}$ANP and $\Delta_{(CS-Ao)}$ANP increased in these patients, and the increase of $\Delta_{(AIV-Ao)}$ANP was evident. In total patients of groups I and II, there was no difference between $\Delta_{(AIV-Ao)}$BNP and $\Delta_{(CS-Ao)}$BNP, while $\Delta_{(CS-Ao)}$ANP was about double of $\Delta_{(AIV-Ao)}$ANP. Thus, the pattern of secretion of BNP is clearly different from that of ANP.

In Table II, the plasma BNP/ANP ratio was larger in the aorta than in the coronary sinus for each patient. A further

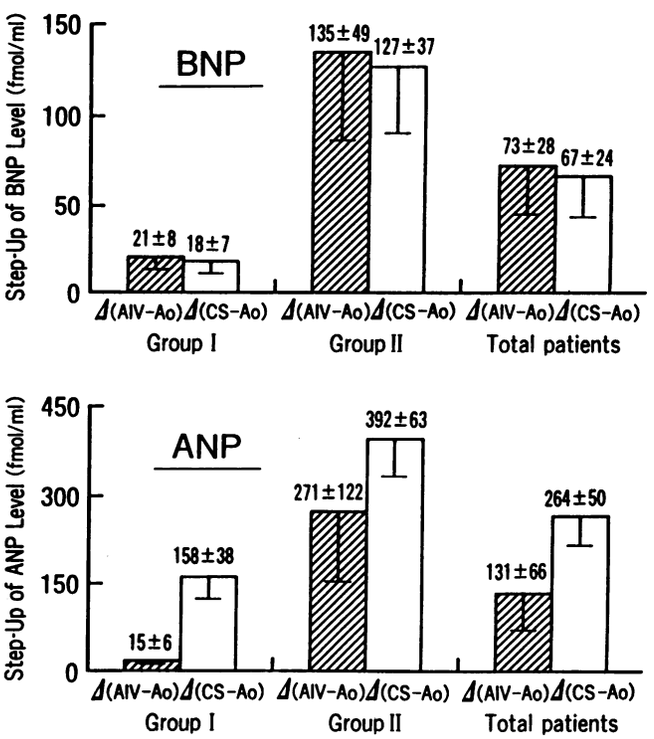

Figure 5. Step-ups of BNP (*top*) and ANP (*bottom*) levels in the AIV and in the coronary sinus. Patients are classified into groups I and II according to the level of $\Delta_{(AIV-Ao)}$ANP, as described in Methods. $\Delta_{(AIV-Ao)}$, the difference in the plasma level between the AIV and the aorta; $\Delta_{(CS-Ao)}$, the difference in the plasma level between the coronary sinus and the aorta.

increase of the ratio was invariably observed in the femoral vein. Such a rise in the BNP/ANP ratio was also noted when compared with that in $\Delta_{(CS-Ao)}$. In case nos. 4 and 6, BNP-LI levels surpassed ANP-LI levels in the femoral vein although their relation was reverse upon secretion ($\Delta_{(CS-Ao)}$). These results suggest that BNP is retained longer in the circulation after secretion than ANP.

Pharmacokinetics of hBNP. In order to further verify the hypothesis on a slow clearance of hBNP in human circulation, we investigated pharmacokinetic analysis of hBNP infused into six healthy volunteers. The plasma BNP-LI levels after the cessation of hBNP infusion are shown in Table III. The plasma half-times of hBNP for the fast and slow components were 3.9±0.23 and 20.7±1.87 min, respectively. These values were significantly longer than those of α-hANP (fast, 1.7±0.07 min and slow, 13.3±1.69 min) in our previous report (34).

Northern blot analysis. To further assess the site of BNP synthesis in the heart, we measured BNP mRNA levels in atria and ventricles of normal and failing hearts. Fig. 6 shows Northern blot analysis of BNP mRNA and ANP mRNA from normal and failing hearts of representative cases.

RNA extracted from normal atrial and ventricular tissues contained a hybridizing BNP mRNA band of ~ 0.9 kb. The BNP mRNA level in the ventricle corresponded to ~ 40% of that in the atrium. Taking tissue weight into account, the total amount of BNP mRNA in the ventricle represented ~ 70% of that in the whole heart. By contrast, the ANP mRNA level in the ventricle was only 0.6% of that in the atrium, and the total amount of ANP mRNA in the ventricle was about 3.5% of that in the whole heart.

Northern blot analysis of RNA from a DCM heart is also shown in Fig. 6. The patient suffered from severe CHF of NYHA class IV and the plasma BNP and ANP levels were

Table III. Clearance Profile and Plasma Half-Times of hBNP

Plasma hBNP levels	
min	*fmol/ml*
0	923.1±85.3
1	983.6±106.5
3	741.1±49.8
5	532.0±44.6
10	299.8±16.5
15	178.7±13.4
30	69.1±5.9
45	48.1±3.5
60	34.1±4.3
75	23.5±3.1
90	18.8±2.7
	min
Half-times	
Fast	3.9±0.23
Slow	20.7±1.87

Plasma hBNP levels were measured over a period of 90 min after the cessation of hBNP infusion at a rate of 0.1 μg (29 pmol)/kg per min for 15 min in six healthy subjects. Values are expressed as the mean±SE.

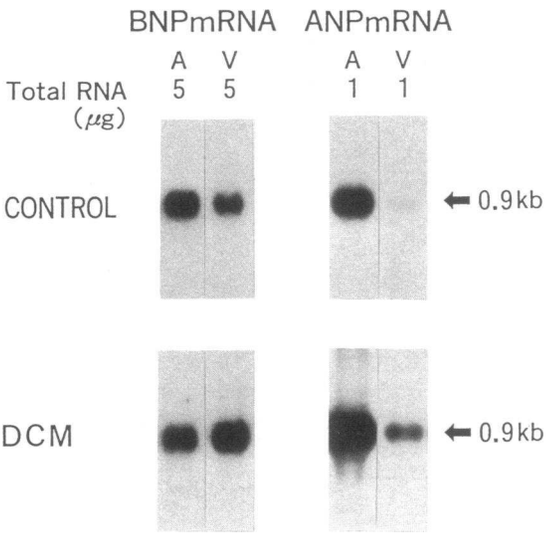

Figure 6. Northern blot analyses of BNPmRNA and ANPmRNA from normal and failing hearts. 5 μg of total RNA from atria or ventricles was fractionated on agarose gel for BNPmRNA, and 1 μg of total RNA from atria or ventricles was fractionated for ANPmRNA. Results of representative cases are shown. *A*, atrium; *V*, ventricle.

increased to 522 and 235 fmol/ml, respectively. BNP mRNA from the atrium or ventricle of the DCM heart was the same size (0.9 kb) as that from the normal heart. The ventricular BNP mRNA level was about double the atrial BNP mRNA level and, therefore, the total amount of BNP mRNA in the ventricle reached up to 88% of that in the whole heart. As compared with the normal heart, the ventricular BNP mRNA level in the DCM heart showed a fivefold increase, while the atrial level was almost equivalent. Similar results were obtained in other cases with CHF. ANP mRNA levels in the DCM heart increased significantly in the atrium and more prominently in the ventricle relative to those in the normal heart, and the total amount of ANP mRNA in the ventricle was 20% of that in the whole heart. ANP mRNA levels in normal and DCM hearts were consistent with the results of our previous study (16).

Binding study with human C receptor. To explore the mechanism of a slow clearance of hBNP in human circulation, we performed the binding study with human C receptors. Fig. 7 shows displacement curves of ^{125}I-α-rANP by α-hANP, hBNP, and C-ANF[4–23] in the binding assay with C receptor preparation from the human lung. C-ANF[4–23], a ligand specific for C receptor (42), effectively competed for the majority (> 98%) of specific binding sites of ^{125}I-α-rANP, confirming that this represents a crude preparation of human C receptors. hBNP almost completely inhibited the specific binding of ^{125}I-α-rANP to this receptor preparation, but at a higher dose than did α-hANP or C-ANF[4–23]. The apparent K_i value of α-hANP was 10 pM, whereas that of hBNP was 140 pM. Thus, the apparent binding affinity of hBNP to human C receptors was estimated to be ~ 7% of that of α-hANP.

Discussion

Previous studies including ours have shown that BNP, originally isolated from the brain (19, 20), is also synthesized in and secreted from the heart in the pig (24, 25) and rat (26–28).

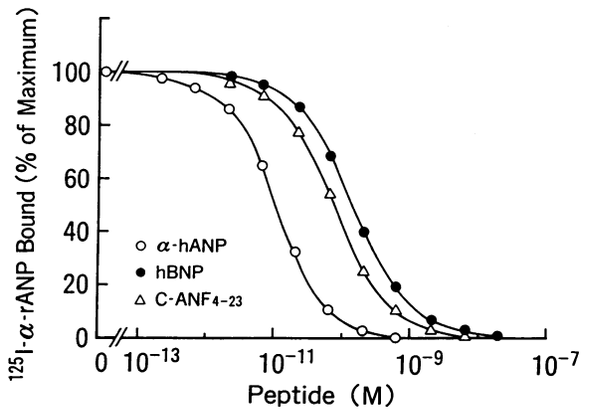

Figure 7. Displacement curves of ^{125}I-α-rANP by α-hANP (○), hBNP (●) and C-ANF[4-23] (△) in the binding assay with human C receptor preparation.

However, little is known on BNP in humans, because antisera against pBNP or rBNP failed to detect BNP-LI in human tissues and the structure of hBNP remained undetermined. Recently, we have succeeded in the isolation and sequence determination of hBNP (30). In the present study, we have developed a monoclonal antibody against hBNP, KY-hBNP-I, and established a specific and sensitive RIA for hBNP. Since KY-hBNP-I recognizes the ring structure of hBNP, which is essential to the biological action of natriuretic peptides, our RIA can detect not only hBNP but amino-terminally or carboxy-terminally modified (elongated or deleted) forms of hBNP including the precursor, suggesting the usefulness of this RIA for studying the biosynthesis, processing and metabolism of hBNP.

The present study demonstrates that BNP is a novel cardiac hormone in humans. The step-up of the BNP concentration in plasma taken at the coronary sinus (Table II) clearly indicates that the major source of circulating BNP is the heart and that most of BNP secreted into the circulation emerges through the coronary sinus from the heart in the same way as ANP (12, 35). To further elucidate the site of BNP production in the heart, we investigated the atrial and ventricular levels of BNP. In general, the peptide hormone concentration of a given endocrine tissue is determined by the difference between its production and secretion, in other words, the capacity for hormone storage. Therefore, the BNP concentration in the atrium or ventricle does not necessarily tell of the source of circulating BNP. The present study has revealed that in the normal heart the tissue BNP level in the ventricle is much lower (7.2%) than that in the atrium (Table I). However, the BNP/ANP ratio in the ventricle was much higher than that in the atrium (Table I) and the share of ventricular BNP in its total amount in the heart was one order of magnitude larger than that of ventricular ANP (Table I). These findings raise the possibility that the ventricle produces and secretes a considerable amount of BNP and that the contribution of the ventricle as the source of circulating BNP is much larger than that of circulating ANP. This idea prompted us to investigate BNP secretion from the ventricle. In the present study, we have demonstrated a significant step-up in the plasma BNP level between the aorta and the AIV, which is located in the anterior interventricular groove and drains the left ventricle but not the atrium (17, 33), showing that BNP is secreted from the ventricle. Moreover, there was no significant difference in the plasma BNP level between the AIV and the coronary sinus, suggesting that BNP secreted from the atrium constitutes a minor portion of BNP in the coronary sinus in these patients. This pattern of secretion of BNP shows a striking contrast with that of ANP, which is secreted predominantly from the atrium, as clearly demonstrated in group I (Fig. 5). To support the idea that the ventricle is the major site of production and secretion of BNP, we further investigated the expression of the BNP gene in the heart. As shown in Fig. 6, the total amount of BNP mRNA in the normal ventricle was larger than that in the atrium. These findings in the human heart obtained in the present study are consistent with our recent observations on the secretion of BNP from the rat heart, which show that ~ 60% of rBNP secreted is derived from the ventricle, although the rBNP level in the ventricle is only 1% of that in the atrium (43). These observations on a large amount of secretion with a small storage of BNP in the ventricle are also supported by the previous finding that ventricular cardiocytes secrete ANP more rapidly after its synthesis via the constitutive pathway than atrial cardiocytes (44).

In normal subjects, the plasma BNP concentration is much lower than the plasma ANP concentration. This result is consistent with previous observations on plasma BNP in pigs (24, 25) and rats (26, 43). Therefore, the functional significance of circulating BNP under physiological conditions is not clear at present. It is noteworthy, however, that the biologically active receptor (B receptor) relatively specific for BNP has been cloned and sequenced (45, 46). The physiological role of the dual natriuretic peptide system must await further investigation.

The present study demonstrates that the plasma BNP level is markedly increased in patients with CHF in proportion to its severity. The plasma BNP level reached as high as 500 fmol/ml in the severest cases, although its normal level was lower than 1 fmol/ml (Fig. 4). Thus, the percent increment in the plasma BNP concentration is much larger (one order of magnitude or more) than that in the plasma ANP concentration in patients with severe CHF. This elevation in the plasma BNP concentration can be explained mainly by the augmented secretion of BNP from the failing heart, as demonstrated by measuring $\Delta_{(CS-Ao)}$BNP (Table II, Fig. 5). Accumulating evidence indicates that in the failing heart the ANP synthesis and secretion are augmented in the atrium (12–18) and more prominently in the ventricle (16, 17, 36, 47). Therefore, the elevation of the plasma BNP concentration observed in CHF, which is much more prominent than that of the plasma ANP level, suggests the contribution of ventricular BNP. In the present study, we have demonstrated that the BNP mRNA level is increased in the ventricle of the failing heart (Fig. 6), and that the total amount of BNP mRNA in the ventricle is much larger than that in the atrium. This finding agrees well with the idea that the increase of the plasma BNP level in patients with CHF results, at least in part, from the augmented production and secretion of BNP in the ventricle. This notion was further verified by the finding that the augmented secretion of BNP in patients with severe CHF (group II) was mostly attributed to the augmented secretion from the ventricle (Fig. 5). Although an exact share of the ventricle in BNP secretion from the whole heart is not clear, it

is highly likely that BNP is secreted predominantly from the ventricle and that the atrial contribution to BNP secretion is much smaller than that to ANP secretion even in patients with CHF.

Another important finding in the present study is that BNP is retained in the circulation longer than ANP, as suggested by the increase of the BNP/ANP ratio with a rank order as follows: the coronary sinus < the aorta < the femoral vein (Table II). This hypothesis was confirmed by the pharmacokinetic analysis of hBNP (Table III), in which the half-times of plasma disappearance of hBNP were longer than those of α-hANP reported previously by us (34) and by others (48). It is conceivable, therefore, that in patients with CHF a slow clearance of BNP from the circulation further exaggerates the elevation of the plasma BNP level in addition to the augmented secretion of BNP from the heart, and leads to a marked rise in the plasma BNP/ANP ratio. As for the differential clearance of ANP and BNP from the circulation, a possible interpretation that BNP could be synthesized and secreted from extracardiac tissues cannot be excluded completely. However, we have demonstrated that in rats the heart is the only major site of BNP production among peripheral organs (43). It is unlikely, therefore, that BNP secreted from other organs could contribute to the increase of the BNP/ANP ratio in addition to the slower clearance of BNP.

There is a working hypothesis that natriuretic peptide receptors are classified into two categories, C receptors (42, 49) and B receptors coupled with particulate guanylate cyclases (45, 46, 50). Therefore, in order to explore the mechanism of the slow clearance of hBNP, we examined the binding ability of hBNP to C receptors prepared from the human lung and compared with that of α-hANP. The binding ability of hBNP to C receptors was 14 times lower than that of α-hANP (Fig. 7). Since it is postulated that C receptors constitute the vast majority of receptors in the vasculature and kidney (42, 49), this finding can explain the slow clearance of hBNP in human circulation. We further examined cGMP production by hBNP in cultured human mesangial and bovine endothelial cells, and found that the potency for cGMP production of hBNP is equal to that of α-hANP (51). Taken together with a recent discovery of B receptor relatively specific for BNP (45, 46), these biological and clinical characteristics of BNP different from those of ANP provide a clue to the physiological and pathophysiological implication of BNP, especially in pathological states such as CHF.

The posttranslational processing of the hBNP precursor is different from that of the human ANP precursor. ANP is stored in the cardiocyte as the precursor, γ-hANP, and the proteolytic cleavage of γ-hANP occurs at Pro^{97}-Arg^{98} during the secretion to yield α-hANP (12, 35, 52). Although the sequence of hBNP is preceded by the same processing signal, Pro^{75}-Arg^{76}, in the precursor (29, 30), hBNP is the major storage form both in the atrium and in the ventricle, even in the failing heart, and circulates in the body as a mature hormone. This processing pattern of hBNP is the same as that of rBNP as we reported (26, 43) and differs from that of pBNP (24, 25), which shows the ANP-like pattern (12, 35, 52). Further studies are necessary to clarify the mechanism and implication of such a diverse processing pattern of natriuretic peptides, ANP and BNP.

In the present study, we have developed a monoclonal antibody, KY-hBNP-I, with high affinity and specificity for hBNP. We previously reported monoclonal antibodies to ANP (32, 53) and their useful applications, including immunohistochemistry (32, 54–56) and a highly sensitive sandwich enzyme immunoassay for plasma α-hANP (57, 58). In addition, they were used for a neutralization experiment to block endogenous ANP in hypertensive rats, resulting in the aggravation of hypertension (59). Thus, such approaches become possible using the monoclonal antibody, KY-hBNP-I, to further explore the significance of hBNP.

In conclusion, the present study demonstrates that BNP is a novel cardiac hormone secreted predominantly from the ventricle in humans and that the synthesis, secretion and clearance of BNP differ from those of ANP. Combined with recent observations on receptor multiplicity (40, 42, 45, 46, 49, 50), these findings suggest discrete physiological and pathophysiological roles of BNP in cardiovascular control, and the presence of an exquisite dual natriuretic peptide system, consisting of at least two ligands, ANP from the atrium and BNP from the ventricle, and three types of receptors expressed in target tissues with tissue specificity.

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