Pro-B-type natriuretic peptide is cleaved intracellularly: impact of distance between O-glycosylation and cleavage sites

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Abstract

We investigated the molecular mechanism underlying the processing of pro-B-type natriuretic peptide (proBNP). Rat neonatal atrial and ventricular myocytes were cultured separately. We examined the molecular forms of secreted and intracellular BNP in atrial and ventricular myocytes; levels of corin and furin mRNA in atrial and ventricular myocytes; the effect their knockdown on proBNP processing; plasma molecular forms of BNP from rats and humans with and without heart failure; and the impact of the distance between the glycosylation and cleavage sites in wild-type and mutant human proBNP expressed in rat myocytes transfected with lentiviral vectors. BNP was the major molecular form secreted by atrial and ventricular myocytes. Transfection of furin siRNA reduced proBNP processing in both atrial and ventricular myocytes; however, transfection of corin siRNA did not reduced it. BNP was the major molecular form in rat plasma, whereas proBNP was the major form in human plasma. The relative fraction of human BNP in rat myocytes expressing human proBNP was about 60%, but increasing the distance between the glycosylation and cleavage sites through mutation, increased the processed fraction correspondingly. These results suggest that proBNP is processed into BNP intracellularly by furin. The level of proBNP processing is lower in humans than rats, most likely due to the smaller distance between the O-glycosylation and cleavage sites in humans.

Key words: B-type natriuretic peptide; atrial natriuretic peptide; furin; processing; corin
INTRODUCTION

B-type (also known as brain) natriuretic peptide (BNP) is a cardiac hormone mainly expressed in the ventricles (14). Ventricular expression of BNP increases in response to pressure overload, volume overload and ischemic injury, and plasma BNP levels increase in proportion to disease severity in patients with heart failure, myocardial infarction, pulmonary hypertension or cardiac hypertrophy (13). Whereas tissue concentrations of BNP are much higher in the atria than the ventricles (1), and BNP secreted from the atria is also important in cases of atrial fibrillation and atrial overload (6). The precursor proBNP is believed to be cleaved to BNP and N-terminal proBNP as it is secreted from myocytes (13), and both molecules have been used for more than a decade as biochemical markers of heart failure. However, several recent studies have shown that in addition to BNP and N-terminal proBNP, levels of uncleaved proBNP are also greatly increased in heart failure (15,23,28).

The precise mechanism by which proBNP is processed is not fully understood at present. Specifically, the molecular form of BNP within cells, the mechanism of proBNP processing, the enzymes catalyzing proBNP processing, whether the patterns of BNP secretion differ between the atria and ventricles, and whether the pattern of BNP secretion differs from that of ANP all remain poorly understood. Interestingly, recombinant proBNP derived from mammalian cells has seven sites of $O$-linked oligosaccharide attachment within the N-terminal portion of the peptide (20). Western blot analysis of a proBNP-expressing cell line and primary human cardiomyocytes showed the predominant intracellular form of BNP is nonglycosylated proBNP, whereas the major extracellular form in culture medium is glycosylated proBNP (27). Moreover, using site-directed mutagenesis, it was shown that $O$-glycosylation of Thr71 suppresses proBNP processing in HEK293 cells (24). By contrast,
Peng et al (18) recently reported that proBNP glycosylation differs in HEK293 cells and murine cardiac myocyte cell line, HL-1 cells. They showed that Thr71 O-glycosylation inhibited the proBNP processing in HEK293 cells, but T71A mutation had little effect on proBNP processing in HL-1 cells because T71 was not glycosylated in them (18). Thus, the results are controversial in part because UDP-N-acetyl-α-d-galactosamine:polypeptide N-acetylgalactosaminyltransferases (GalNAc-Transferases) are differentially expressed in cells and tissues (2). Consequently, the experiment is best performed in cardiac ventricular and/or atrial myocytes. In addition, measurement of BNP and proBNP within cells and in medium requires the use of a highly sensitive, specific and accurate method, as their concentrations are very low. Assessing proBNP processing is important because elevation of inactive proBNP caused by impairment of its processing may be associated with the development of heart failure (5,8,11). Thus, a complete understanding of proBNP processing may yield a better understanding of the molecular basis of heart failure, and lead to the development of new therapeutic approaches to the treatment of heart failure.

In the present study, therefore, we investigated [1] the molecular forms of BNP in culture medium conditioned by rat atrial and ventricular myocytes; [2] the intracellular forms of BNP in rat atrial and ventricular myocytes; [3] the roles of furin and corin in the processing of rat proBNP; [4] the molecular forms of BNP in atrial and ventricular tissue and plasma in rats with and without heart failure; [5] the molecular forms of BNP in human plasma; [6] the impact of the distance between its glycosylation and cleavage sites on proBNP processing; and [7] the proBNP/total BNP ratios in coronary sinus and aorta in mild and severe heart failure patients.

**MATERIAL AND METHODS**
All patients provided written informed consent for all blood sample analyses, and the protocol was approved by the Ethical Committee of Kyoto University Graduate School of Medicine. Our study was performed conforming with the principles outlined in the Declaration of Helsinki.

Rat ventricular and atrial myocyte cultures

Primary cultures of rat neonatal ventricular and atrial myocytes were prepared as described previously (4). Briefly, to obtain the ventricular myocytes, apical halves of cardiac ventricles from 1- to 2-day-old Wistar rats were separated, minced and dispersed with 0.1% collagenase type II (Worthington Biochemical Corp). To obtain atrial myocytes, right and left atrial appendages were resected, minced, and dispersed in the same manner as the ventricular cells. Myocytes were then segregated from nonmyocytes on a discontinuous Percoll (Sigma Chemical Co) gradient prepared as described previously (4). We used Dulbecco’s minimum essential medium (DMEM) as a basal medium.

Heart failure and control rat

Male inbred Dahl salt-sensitive (DS) rats were obtained from SLC Corp (Shizuoka, Japan). After weaning, DS rats were fed a 0.3% NaCl (low-salt) diet until they were 6 weeks old, after which they were fed a diet containing 8% NaCl (high-salt). Age-matched male Dahl salt-resistant (DR) rats fed the same diet served as a control group. At 18 weeks of age, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg) after measuring their body weight. A polyethylene catheter (PE-50) was then inserted into the thoracic aorta via the right carotid artery to measure the heart rate, mean arterial pressure and
left ventricular end-diastolic pressure as previously reported (17). In addition, blood samples were collected. Immediately thereafter the heart was arrested by injection of 2 mmol/L KCl, and the right and left atria and left ventricle were excised, weighed, frozen in liquid nitrogen and stored at -80°C.

**Measurement of the molecular forms of rat BNP and ANP in culture medium, cell lysates and atrial and ventricular tissue samples**

After incubating neonatal ventricular and atrial myocytes in serum-free DMEM for 48 h, the culture medium was collected and stored at -80°C. The cells were then rapidly collected into glass tubes, where they were exposed to hot water (95°C) for 10 min to inhibit protease activity. After cooling, the cells were homogenized at 4°C using a polytron mixer. The resultant homogenate was centrifuged for 15 min at 15,000 × g, and the supernatant containing dissolved peptides including the molecular forms of natriuretic peptides was collected and stored at -80°C. In addition, atrial and ventricular tissues were boiled in 10 volumes of 1 mol/L acetic acid and then homogenized and centrifuged as described above, after which the supernatant was stored at -80°C.

Samples of plasma, medium and supernatant from extracted tissues and cell lysates were loaded onto Sep-Pak C18 cartridges (Waters, Milford, MA, USA) as described previously (12). The eluate was then lyophilized, dissolved in 30% acetonitrile containing 0.1% TFA and separated by gel filtration high performance liquid chromatography (HPLC) on a TSK gel G2000SWXL column (7.8 × 300 mm, Tosoh) at a flow rate of 0.2 mL/min or on tandemly connected 2 Superdex 75 10/300columns (10 × 300 mm, GE Healthcare) at a flow rate of 0.4 mL/min, as described previously (15). The column effluent was fractionated every minute and
lyophilized with BSA. Each fraction was then dissolved in radioimmunoassay buffer, and BNP and ANP were measured using a radioimmunoassay as previously reported (1,10). In brief, the rat ANP radioimmunoassay system is highly sensitive and half-maximum inhibition by ANP is 60 pg/tube and can measure ANP as low as 10 pg/tube. This ANP radioimmunoassay system uses rabbit antiserum recognizing the sequence flanked by two cysteine residues (positions 7 and 23) in ANP molecule. Because both rat ANP and human ANP are composed of 28-amino acid and only one residue is replaced, this ANP radioimmunoassay system can measure both rat ANP and human ANP (10). The rat BNP radioimmunoassay system is also highly sensitive and half-maximum inhibition of is 30 pg/tube and can measure rat BNP as low as 5 pg/tube. This rat BNP radioimmunoassay system uses rabbit antiserum recognizing the middle to C-terminal portion of the rat BNP. Because BNP sequence is largely different among the species, this rat BNP radioimmunoassay system does not cross-react with porcine BNP, human BNP, rat ANP, human ANP or chicken ANP (1).

The BNP-45/total BNP and proBNP/total BNP ratios-% were calculated based on the summation of high molecular weight (MW)-immunoreactive (IR-) proBNP and low MW IR BNP-45 (IR-BNP-45) using the formulas: proBNP/total BNP ratio-% = IR-proBNP/(IR-proBNP+IR-BNP-45) × 100% and BNP-45/total BNP ratio-% = IR-BNP-45/(IR-proBNP+IR-BNP-45) × 100%. The ANP-28/total ANP ratio-% and proANP/total ANP ratio-% were calculated in analogous fashion.

**RNA preparation and real-time PCR analysis**

Total RNA was extracted from atrial and ventricular myocytes and tissue samples using the acid guanidinium thiocyanate-phenol-chloroform method, and first strand complementary
DNA was then synthesized as previously described (17). The gene expression levels of BNP, ANP, furin, corin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by real-time quantitative reverse-transcription polymerase chain reaction using an ABI 7700 system and specific primers, as reported previously (17). The relative level of each mRNA was assessed as the amount of original template of each molecule normalized to the amount of original template of GAPDH.

RNA interference

Rat neonatal ventricular and atrial myocytes in 6-cm dishes were incubated in serum-free medium for 24 h before being transfected with 50 nmol/L ON-TARGET plus® small interfering (si)RNA targeting rat furin (Dharmacon), Silencer® Select siRNA targeting rat corin (Ambion) or control scrambled siRNA (Thermo Scientific) using Dharmafect 3 (9). After 24 h, the culture medium was aspirated and stored at -80°C, and the cells were collected, homogenized and stored as described above.

Plasmid

cDNA encoding full-length human pre-proBNP was amplified by PCR using a human cDNA library as a template, and cloned into pBluescript II SK(+) (Agilent Technologies, Santa Clara, CA, USA). After confirming the sequence, cDNA encoding human pre-proBNP was inserted into pLVSIN-CVM Neo vector, which is a SIN type lentiviral vector plasmid. ProBNP is reportedly O-glycosylated at Thr36, Ser37, Ser44, Thr48, Ser53, Thr58 and Thr71 in its N-terminal region. To investigate the effect of Thr71 glycosylation and the effect of the distance between the cleavage and glycosylation sites during proBNP processing, we generated
four human proBNP mutants (T71A, L69T/T71A, M67T/T71A, T36A/S37A/S44A/T48A/S53A/T58A/T71A) using QuikChange Lightning Site-Directed Mutagenesis Kits (Agilent Technologies, Santa Clara, CA, USA) with human pre-proBNP incorporated into pBluescript II as a template. After confirming the sequences, the mutant genes also inserted into pLVSIN-CVM Neo vector.

**Lentiviral vector production and transfection**

HIV-based lentiviral vectors were generated using a Lenti-XTM HTX Ecotropic packaging system (Clontech Laboratories, Inc., CA, U.S.A.) according to the manufacturer’s guidelines with pLVSIN-CMV Neo vectors harboring wild and mutant human pre-proBNP (lenti-proBNP). Rat neonatal ventricular myocytes were transfected with each lentivirus 24 h after plating, and the medium was exchanged after an additional 24 h. Culture medium and cell lysates were then harvested 96 h after transfection. In a similar manner, rat cardiac fibroblasts and NIH3T3 cells were also transfected with lenti-proBNP, and the conditioned culture medium was collected.

**Deglycosylation enzyme treatment**

Samples of medium and supernatant from extracted tissues were lyophilized and were dissolved in with 250 µl of 50 mM phosphate buffer (pH 6.0), after which portions of the dissolved phosphate buffer (228 µl) were added to 12 µl of phosphate buffer, with or without a cocktail of deglycosylating enzymes, and incubated for 24 h at 37°C. The enzyme cocktail included O-glycosidase (Roche Diagnostics) and neuraminidase (Roche Diagnostics) at final concentrations of 4.25 and 42.5 mU/ml, respectively, as described previously (12).
Plasma levels of BNP and proBNP in heart failure patients

We collected venous blood samples from heart failure patients (n=38) and healthy subjects (n=101). Patients clinical characteristic are presented in Table 1.

Measurement of human proBNP and total BNP in culture medium and plasma

Blood sample was immediately transferred to chilled disposable tubes containing aprotinin (500 kallikrein inactivator units/ml) and EDTA (1.5 mg/ml) and centrifuged at 4°C, and the plasma was frozen and stored at -80°C until required for assay. ProBNP and total BNP (BNP + proBNP) in plasma from control and heart failure patients and in culture medium conditioned by proBNP-transfected myocytes were measured using our recently developed direct immunochemiluminescent assay for proBNP and total BNP (16). This is a two-step immunochemiluminescent assay for total BNP and proBNP using monoclonal antibodies and glycosylated proBNP as a standard. In both assays, the antibody recognizing a common epitope of C-terminal region of proBNP and BNP was used as a capture antibody. The antibody recognizing an epitope of N-terminal portion of proBNP was used as a signal antibody in proBNP assay, whereas antibody recognizing an epitope of ring structure of BNP was used as a signal antibody in BNP assay. The assay enables measurement of plasma total BNP and proBNP within 7 h, without prior extraction of the plasma. The detection limit was 0.4 pmol/L for a 50 µl plasma sample. The dilution curves for plasma samples showed good linearity, and analytical recovery was 90-101%. Importantly, since the affinity of two signal antibodies for each epitope was the same, we could measure accurately the proBNP/total BNP ratio in plasma or conditioned medium as an index assessing the processing of proBNP. We
also calculated the processing fraction as follows: processed fraction (%) = (total BNP – proBNP) / total BNP × 100. These assays are specific for human BNP and proBNP, and do not cross-react to rat BNP-45 or rat proBNP-95. We previously reported about it in detail (16).

Statistical Analysis

All values are expressed as means ± SD. The statistical significance of differences between two groups was evaluated using Fisher’s exact test or paired Student’s t-test, as appropriate. Variables were compared among three groups using one-way analysis of variance followed by Bonferroni’s multiple comparison test. Correlation coefficients were calculated using linear regression analysis. Values of P < 0.05 were considered significant.

Results

Molecular forms of rat ANP and BNP in the medium and within the cells in atrial and ventricular myocytes:

As shown in Figure 1-A and Table 2, BNP-45 is the major molecular form of BNP in medium conditioned by rat atrial myocytes. BNP-45 is also the major molecular form in medium conditioned by rat ventricular myocytes (Figure 1-B). Intracellular forms of BNP in the atrial and ventricular myocytes are shown in Figure 1-C and D. Both BNP-45 and proBNP are present in atrial and ventricular myocytes. The ANP-28 is also the major molecular form of ANP in medium conditioned by rat atrial myocytes. (Figure 1-E and Table 3). Whereas the ANP-28/total ANP ratio-% was lower in medium from ventricular myocytes (Figure 1-F), as compared with atrial myocytes. Regarding the intracellular molecular forms of ANP, proANP is the only major molecular form in atrial and ventricular myocytes (Figure 1-G, H).
The mRNA levels of furin and corin in rat atrial and ventricular myocytes and the effects of siRNA targeting furin and corin on the processing of proBNP and proANP:

The corin mRNA was expressed primarily in atrial myocytes (Figure 2-B), whereas levels of the furin transcript were similar in atrial and ventricular myocytes (Figure 2-A). The levels of BNP mRNA were stronger in ventricular than atrial myocytes (Figure 2-C), while levels of ANP mRNA were higher in atrial myocytes (Figure 2-D).

To further investigate their respective roles in processing BNP and ANP, we next used specific siRNAs to knock down expression of furin and corin. The siRNA targeting furin significantly reduced the BNP-45/total BNP ratio-% in medium conditioned by either atrial or ventricular myocytes (Figure 2-E, F). On the other hand, siRNA targeting corin had no effect on BNP-45/total BNP ratios-% (data not shown). Conversely, the corin-specific siRNA significantly reduced the ANP-28/total ANP ratio-% in medium conditioned by atrial or ventricular myocytes (Figure 2-G, H), while furin-specific siRNA had no effect on ANP-28/total ANP ratios-% (data not shown). Intriguingly, the furin siRNA also significantly reduced the intracellular BNP-45/total BNP ratio-% in both ventricular and atrial myocytes (Figure 2-I, J), suggesting proBNP is cleaved intracellularly to BNP-45 by furin. By contrast, corin-specific siRNA had no effect on the intracellular BNP-45/total BNP ratio-% in either ventricular or atrial myocytes (data not shown). Corin siRNA also had no effect on the intracellular molecular forms of ANP in atrial and ventricular myocytes (Figure 2-K, L), and furin siRNA showed a similar lack of effect (data not shown).

The relationships between the levels of furin and corin mRNA expression and BNP-45/total BNP ratios-% in medium conditioned by rat atrial or ventricular myocytes are
shown in Figure 2-M. Expression of furin mRNA correlated significantly with the BNP-45/total BNP ratio-% in medium from both atrial and ventricular myocytes, but no similar correlation between corin mRNA and BNP-45/total BNP ratio-% was detected. Conversely, expression of corin mRNA correlated significantly with the ANP-28/total ANP ratio-% in medium from atrial or ventricular myocytes, but expression of furin mRNA did not (Figure 2-N).

**Molecular forms of BNP and ANP in atrial and ventricular tissue in the control and heart failure rat:**

Levels of the molecular forms of BNP in atrial and ventricular tissues from rats with and without heart failure are shown in Figure 3-A and in Table 3. There were no differences in the atrial concentrations of BNP between the two groups. The BNP-45/total BNP ratio-% also did not differ between groups. On the other hand, ventricular BNP concentrations were higher in rats with heart failure than in control rats, though the BNP-45/total BNP ratio-% did not differ between the groups. The molecular forms of ANP in atrial and ventricular tissues from rats with and without heart failure are shown in Figure 3-B in Table 3. There were no differences in the atrial concentrations of ANP between the two groups, but the ventricular concentrations were markedly higher in rats with heart failure than in control rats. In addition, the ANP-28/total ANP ratio-% did not differ between rats with and without heart failure, or between atrial and ventricular tissues.

**Plasma concentrations and molecular form of BNP in rat and human:**
Plasma concentrations of the different molecular forms of BNP in rats and humans are shown in Figure 4-A, B and Table 3. BNP-45 was the major molecular form in the plasma of rats with and without heart failure, whereas the major molecular form was proBNP in both healthy humans and heart failure patients. Thus, the major circulating forms of BNP in plasma differ between humans and rats.

Comparison of the amino acid sequences between rat proBNP and human proBNP:

Comparison of the amino acid sequences showed that the N-terminal regions and C-terminal ring structures of proBNP are well conserved between humans and rat, but the sequences in the middle regions largely differ between the human and rat molecules (Figure 4-C). In humans, proBNP is cleaved to N-terminal proBNP[1-76] and BNP[77-108]. Within the N-terminal region of the human proBNP sequence, there are seven O-glycosylation sites (T36, S37, S44, T48, S53, T58 and T71), and there is a five-residue sequence between T71 and cleavage site at R76-S77. In rat proBNP, there are two possible O-glycosylation sites, at S35 and T41, and there are nine residues between T41 and the cleavage site. We hypothesized that the distance between the O-glycosylation site and the cleavage site may determine the processing rate. To test that idea, we first determined whether rat proBNP is actually O-glycosylated by using gel-filtration chromatography in combination with a specific radioimmunoassay to examine proBNP molecular size before and after treatment with deglycosylating enzymes. The proBNP peak obtained with samples of rat left ventricular extract shifted slightly to the right after deglycosylation (Figure 4-D), suggesting that rat proBNP is actually O-glycosylated.

The effects of the distance between O-glycosylation sites and cleavage site on the processing of proBNP:
We then examined the processing of human proBNP expressed in rat cardiac myocytes. The processed fraction of wild-type human proBNP was 62 ± 6% (Figure 5-A, F). After T→A^{71} and L→T^{69} double substitution, which increased the number of residues between the O-glycosylation and cleavage sites to seven, the processed fraction was increased slightly (Figure 5-B, F). Moreover, after T→A^{71} and M→T^{67} double substitution, which further increased the intervening sequence to nine residues, the processed fraction was increased (Figure 5-C, F). Then after single T→A^{71} substitution, which increased the intervening sequence to 18 residues, the processed fraction was even greater (Figure 5-D, F). Finally, when all of the glycosylation sites were replaced with Ala, the processing was nearly complete (Figure 5-E, F).

We also analyzed the processing of human proBNP expressed in rat cardiac fibroblasts and NIH3T3 cells. The processed fraction of human proBNP was 36 ± 6% in the cardiac fibroblasts and 74 ± 7% in the NIH3T3 cells.

To confirm that the molecular weight is reduced when T^{71} or all the glycosylation sites were replaced with Ala, reflecting the loss of the sugar moiety, we used gel filtration chromatography in combination with a BNP immunochemiluminescent assay to examine the human proBNP peak. Following T→A^{71} substitution, the human proBNP peak was shifted slightly to the right, as compared to human proBNP without substitution of T^{71}. When all the glycosylation sites were replaced with Ala, the peak was shifted o the right further (Figure 5-G).

Discussion
In the present study, we investigated which molecular forms of BNP are secreted by cultured rat atrial and ventricular myocytes, and compared them with the forms of ANP. Cultured rat atrial and ventricular myocytes mainly secrete BNP-45, and that cultured rat atrial myocytes secrete mainly ANP-28, while the ventricular myocytes secrete similar amounts of ANP-28 and proANP. ProANP is the only form present intracellularly in both atrial and ventricular myocytes. By contrast, both BNP-45 and proBNP were observed in both atrial and ventricular myocytes, which suggests that a certain percentage of proBNP is cleaved to BNP-45 and N-terminal proBNP intracellularly, and subsequently they are co-secreted with proBNP. Thus, the intracellular molecular profile and secretion pattern of BNP differs substantially from those of ANP.

Recent studies have shown that proANP is cleaved into ANP-28 and N-terminal proANP by corin, a transmembrane serine protease (29). Our current finding that proANP is nearly the only molecular form present within either atrial and ventricular myocytes is consistent with those earlier findings (26). By contrast, both proBNP and BNP-45 are present in atrial and ventricular myocytes, and BNP-45 is the dominant form in culture medium. This suggests proBNP is cleaved intracellularly, though the mechanism involved in proBNP processing is still not well understood. Yan et al (29) showed that cotransfection of proBNP and corin expression vectors into HEK293 cells resulted in slight cleavage of proBNP, whereas no proteolytic cleavage of proBNP was detected after cotransfection of proBNP and control vectors. A recent study showed that reducing furin activity using a specific inhibitor or siRNA significantly impairs proBNP processing in HEK293 cells expressing proBNP (25); however, intracellular BNP is not detected in HEK293 cells overexpressing proBNP, which suggests intracellular proBNP is resistant to furin-catalyzed cleavage (27). In the current study we
transfected cardiac fibroblasts and NIH3T3 cells with lenti-proBNP and then measured proBNP and total BNP in medium conditioned by the two cell types. Our finding that both proBNP and BNP were present in culture medium from both cardiac fibroblasts and NIH3T3 cells supports the idea that proBNP is cleaved by the ubiquitously expressed enzyme furin, rather than by corin, because corin is a cardiomyocyte specific enzyme (29).

In addition, we found that a furin-specific siRNA significantly reduced proBNP processing in both atrial and ventricular myocytes, and the level of furin mRNA closely correlated with the BNP-45/proBNP ratio in both atrial and ventricular myocytes. Intriguingly, the intracellular BNP-45/proBNP ratio-% was significantly reduced after treatment with a siRNA targeting furin. Since furin is localized mainly in the trans-Golgi network (19), furin is considered to be the enzyme that cleaves proBNP. This idea is consistent with a recent study, which showed the presence of BNP within the Golgi apparatus (27). Moreover, a corin-specific siRNA reduced proANP processing in both atrial and ventricular myocytes, but had no effect on proBNP processing. Furin mRNA is expressed at similar levels in atrial and ventricular myocytes, whereas corin mRNA is dominantly expressed in atrial myocytes. The weak expression of corin mRNA in ventricular myocytes may explain the lower level of proANP processing in ventricular myocytes.

We showed that the molecular forms of BNP in atrial and ventricular tissues from rats with and without heart failure were similar to the intracellular forms seen in cultured atrial and ventricular myocytes, and that there were no differences in the atrial and ventricular molecular forms of BNP between rats with and without heart failure. In plasma, BNP-45 is the major molecular form in control rats and rats with heart failure, whereas proBNP is the major molecular form in both healthy subjects and heart failure patients (16). To determine why the
BNP forms in rat plasma differ from those in human plasma, we compared the amino acid sequences of human and rat proBNP. In the N-terminal region of human proBNP, there is a consensus sequence $R^{73}X^{74}X^{75}R^{76}$, which is cleaved by furin (21). Interestingly, in rat proBNP one amino acid is deleted between $R^{61}$ and $R^{63}$, as compared to the corresponding human sequence ($R^{61}X^{62}R^{63}$ vs. $R^{73}X^{74}X^{75}R^{76}$), so that the rat sequence cannot serve as cleavage site. In addition, human E$^{59}$ corresponds to rat R$^{47}$, which contributes to the $R^{47}X^{48}X^{49}R^{50}$ consensus sequence (2,21,22). Consequently, rat proBNP is cleaved at $R^{50}S^{51}$ to form BNP-45, while human proBNP is cleaved at $R^{76}S^{77}$ to form BNP-32. Thus, the R-X-X-R consensus sequence appears to determine the proBNP cleavage site in both rat and human.

One recent study has shown that little human proBNP is cleaved into BNP-32 and N-terminal proBNP in HEK293 cells transfected with wild-type proBNP (21). However the T71A variant exhibited a marked increase in processing, as compared to wild-type proBNP (56.7% vs. 7.6%) (21). This suggests that a glycosylation site nearer placed to the cleavage site plays an important role in the processing of proBNP. Whereas human proBNP has seven O-glycosylation sites, rat proBNP has only two, located in the mid-region of the molecule ($S^{35}$ and $T^{41}$). When we evaluated the molecular size of rat proBNP using gel-filtration chromatography combined with a specific BNP radioimmunoassay to determine whether rat proBNP is actually O-glycosylated, our results were compatible with the idea that rat proBNP may have two O-glycosylated sites. In addition, it is noteworthy that there are nine amino acids between the closer glycosylation site ($T^{41}$) and the cleaving site ($R^{50}S^{51}$) in rat proBNP. By contrast, human proBNP has only five amino acids between the closest glycosylation site ($T^{71}$) and the cleavage site ($R^{76}S^{77}$).
To investigate the processing mechanism of human proBNP, we transfected rat myocytes with lentiviral vectors encoding human proBNP. We then used our recently developed immunochemiluminescent assay to measure proBNP and total BNP secreted into the medium (16). Our findings show that the processed fraction of wild-type human proBNP was about 60%. Given the aforementioned difference between rat and human proBNP, we assessed the impact of the distance between the cleavage and O-glycosylation sites on the processing of human proBNP using four proBNP mutants (L69T/T71A, M67T/T71A, T71A and T36A/S37A/S44A/T48A/S53A/T58A/T71A). Our results show that O-glycosylation plays a key role in the processing of proBNP, and that increasing the length of the intervening amino acid sequence between the O-glycosylation and cleavage sites increased the processed fraction.

Thus, the distance between the O-glycosylation site and the cleavage site appears to be a key determinant governing the processing of proBNP, and differences in proBNP processing among the species may be explained by differences in the distance between the O-glycosylation and cleavage sites.

We have limitation of the study. We measured proBNP and total BNP levels in the plasma and medium and calculated the processing fraction as an index of processing of proBNP. However, proBNP and total BNP levels in the plasma and medium are results of production and degradation. For example, previous study has shown that neprilysin-dependent degradation of BNP is species-specific (3). In addition, neprilysin was shown to degrade truncated rat BNP activity in rat kidney membranes, whereas neprilysin was ineffective against human BNP (3). Thus, since the rat BNP degradation by neprilysin is greater than human BNP, this study might underestimate the processing fraction of rat proBNP. However, since the effects of neprilysin are not so large (7), it does not affect our conclusion.
In conclusion, we showed that proBNP is processed into BNP and N-terminal proBNP intracellularly, most likely by furin. The level of proBNP processing is lower in humans than rats, most likely due to the smaller distance between the O-glycosylation and cleavage sites in humans.

**Perspective and Significance**

In heart failure, BNP immunoreactivity is greatly increased; however, it cannot compensate heart failure. Recent studies revealed the proBNP level is higher than of BNP in heart failure (23,28), and that proBNP/BNP ratios are widely distributed depending on heart failure status (15). The proBNP/BNP is reported to be higher in heart failure with ventricular overload or with renal failure (15). This study demonstrated that the distance between O-glycosylation site and the cleavage site plays an important role in the processing of proBNP. Thus, elucidation of the molecular mechanism of proBNP processing may help to clarify the pathogenesis of heart failure and/or pave the way towards novel therapies.

**GRANTS**

This study was supported in part by Scientific Research Grants-in-Aid 20590837, 23126511, 23591041, 25126712, 15K09138 (to T. Nishikimi), 26293187, 26670400 (to K. Kuwahara), 25461107 (to Y. Nakagawa), and 15K19377 (to T. Minami) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, is declared by the authors.

**AUTHOR CONTRIBUTIONS**
Author contributions: TN: design the study and wrote the manuscript; YN, AF, KK: analyzed the human proBNP and mutant experiment; KA, TI: analyzed the rat culture experiment; KT, MI: analyzed the RIA for rat ANP and BNP; NM, KT: interpreted results of gel-filtration analysis; CY, Kazuhiro N, TM, YK, HK, TT: analyzed the human study; KK, KN.: supervise the study.

ACKNOWLEDGMENTS

We thank Prof. Kazuhisa Nakayama and Prof. Shogo Oka for helpful advice. We thank Ms. Machiko Sakata, Ms. Masako Minato and Ms. Masako Matsubara for their excellent technical assistance, and Ms. Yukari Kubo for her excellent secretarial work.
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**Figure legends**

Figure 1-A, B, C, D, E, F, G, H

Secreted molecular forms of BNP and ANP in medium conditioned for 48 h by rat atrial or ventricular myocytes and intracellular molecular forms of BNP and ANP within atrial and ventricular myocytes. Data are means ± S.D. from 4~6 independent experiments. Representative gel-filtration analyses are also shown. Arrows indicate the elution position of authentic rat proBNP (a), rat BNP-45 (b), rat proANP (c), and rat ANP-28(d).

Figure 2-A, B, C, D, E, F, G, H, I, J, K, L, M, N

A – D. Expression of furin, corin, BNP and ANP mRNAs in atrial and ventricular myocytes. White bar graph shows ventricular myocytes. Black bar graph shows atrial myocytes. Data are means ± S.D. from 2 independent experiments (n = 8).

E – L. Effects of siRNA targeting furin and corin on BNP and ANP processing in atrial and ventricular myocytes. White bar graph shows control group. Black bar graph shows siRNA-treated group. Data are means ± S.D. from 3 independent experiments (n = 9~12). M, N.

Relationships between corin or furin mRNA expression and BNP-45/total BNP and ANP-28/total ANP ratios-% in medium conditioned by atrial and ventricular myocytes.

Figure 3-A, B, C, D, E, F, G, H

Molecular forms of BNP and ANP in atrial or ventricular tissue in control and heart failure rat. Black bar graph shows BNP-45. White bar graph shows proBNP. Data are means ± S.D. (control rat, n = 10, heart failure rat, n = 8 ).
Figure 4- A, B, C, D

A. Plasma proBNP and BNP-45 levels in control and heart failure rats. Data are means ± S.D.

B. Plasma proBNP and BNP-32 levels in control and heart failure subjects. Data are means ± S.D.

C. Comparison of the amino acid sequences of rat and human proBNP.

D. Gel-filtration analysis of rat ventricular proBNP before and after treatment with a cocktail of deglycosylating enzymes.

Figure 5-A, B, C, D, E, F, G, H

A, B, C, D, E. Schematic representation of human proBNP and its mutants.

F. Processed fractions (%) of human proBNP and its mutants in the medium in myocytes. Data are means ± S.D. from 5 ~ 7 independent experiments.

G. Gel-filtration analysis of human proBNP, a human proBNP mutant in which T^{71} was replaced with Ala, a human proBNP mutant in which all T^{36}, T^{48}, T^{58}, T^{71}, S^{37}, S^{44}, S^{53} were replaced with Ala, recombinant proBNP with and without glycosylation and BNP-32.
Table 1. Clinical characteristics in patients with heart failure and control subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>Heart failure (n=38)</th>
<th>Normal subjects (n=111)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62±13</td>
<td>52±11</td>
<td>0.023</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>20/18</td>
<td>53/48</td>
<td>0.785</td>
</tr>
<tr>
<td>NYHA I-II</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-IV</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etiology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valvular heart disease</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD, NYHA: New York Heart Association
Table 2. BNP and ANP concentrations in medium and lysates from rat cultured atrial and ventricular myocytes

<table>
<thead>
<tr>
<th>Variables</th>
<th>BNP-45</th>
<th>proBNP</th>
<th>ANP-28</th>
<th>proANP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrial cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium (pg/10⁴ cells/day)</td>
<td>114 ± 30</td>
<td>24 ± 6</td>
<td>1865 ± 365</td>
<td>312 ± 15</td>
</tr>
<tr>
<td>Intracellular (pg/10⁶ cells)</td>
<td>5.5 ± 2.2</td>
<td>3.6 ± 0.8</td>
<td>5 ± 1</td>
<td>441 ± 32</td>
</tr>
<tr>
<td>Ventricular cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium (pg/10⁴ cells/day)</td>
<td>563 ± 129</td>
<td>42 ± 7</td>
<td>3376 ± 573</td>
<td>3195 ± 557</td>
</tr>
<tr>
<td>Intracellular (pg/10⁶ cells)</td>
<td>3.3 ± 0.8</td>
<td>5.0 ± 1.0</td>
<td>4 ± 1</td>
<td>424 ± 56</td>
</tr>
</tbody>
</table>

Values are means ± SD
**Table 3.** Concentrations of BNP and ANP in atrial and ventricular tissue from control rats and rats with heart failure and in plasma from rats and humans with heart failure

<table>
<thead>
<tr>
<th>Variables</th>
<th>BNP (pg/mg)</th>
<th>proBNP (pg/mg)</th>
<th>ANP (pg/mg)</th>
<th>proANP (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atrial Tissue (rat)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>698 ± 474</td>
<td>672 ± 437</td>
<td>77131 ± 27079</td>
<td>1389 ± 1210</td>
</tr>
<tr>
<td>heart failure</td>
<td>719 ± 553</td>
<td>644 ± 357</td>
<td>59236 ± 30485</td>
<td>1767 ± 837</td>
</tr>
<tr>
<td><strong>Ventricular tissue (rat)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>13 ±6</td>
<td>7 ± 3</td>
<td>83 ± 31</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>heart failure</td>
<td>38 ± 12*</td>
<td>20 ± 10*</td>
<td>871 ± 416*</td>
<td>34 ± 18*</td>
</tr>
<tr>
<td><strong>Rat plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>3.5 ± 0.5</td>
<td>0.3 ± 0.2</td>
<td>96 ± 20</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>heart failure</td>
<td>48 ± 18*</td>
<td>3.8 ± 0.5*</td>
<td>730 ± 230*</td>
<td>74 ± 25*</td>
</tr>
<tr>
<td><strong>Human plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>3.6 ± 1.7</td>
<td>8.5 ± 2.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>heart failure</td>
<td>102 ± 85*</td>
<td>156 ± 78*</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Note that rat BNP is BNP-45, whereas human BNP is BNP-32. Rat proBNP is proBNP-95, whereas human proBNP is proBNP-108. ND: not determined. *P < 0.05 vs. control. Values are means ± SD
<table>
<thead>
<tr>
<th></th>
<th>atrial cells</th>
<th>medium</th>
<th>intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP</td>
<td><a href="#">Diagram</a></td>
<td><a href="#">Diagram</a></td>
<td></td>
</tr>
<tr>
<td>ventricular cells</td>
<td><a href="#">Diagram</a></td>
<td><a href="#">Diagram</a></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>atrial cells</th>
<th>medium</th>
<th>intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP</td>
<td><a href="#">Diagram</a></td>
<td><a href="#">Diagram</a></td>
<td></td>
</tr>
<tr>
<td>ventricular cells</td>
<td><a href="#">Diagram</a></td>
<td><a href="#">Diagram</a></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2

(A) Furin/GAPDH

(B) Corin/GAPDH

(C) BNP/GAPDH

(D) ANP/GAPDH

(E) BNP-45/total BNP ratio
- Atrial cells
  - Control
  - Furin siRNA (+)
  - Statistical significance indicated

(F) BNP-45/total BNP ratio
- Ventricular cells
  - Control
  - Furin siRNA (+)
  - Statistical significance indicated

(G) ANP-28/total ANP ratio
- Atrial cells
  - Control
  - Corin siRNA (+)
  - Statistical significance indicated

(H) ANP-28/total ANP ratio
- Ventricular cells
  - Control
  - Corin siRNA (+)
  - Statistical significance indicated

(M) BNP
- Atrial cells
- Ventricular cells
- Corin mRNA (%) vs. BNP-45 total BNP ratio
- Linear regression equations shown

(N) ANP
- Atrial cells
- Ventricular cells
- Corin mRNA (%) vs. ANP-28 total ANP ratio
- Linear regression equations shown
<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>heart failure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BNP</strong></td>
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<td>atrial tissue</td>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
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<tr>
<td>ventricular tissue</td>
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<tr>
<td><strong>ANP</strong></td>
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<tr>
<td>ventricular tissue</td>
<td><img src="image7.png" alt="Graph" /></td>
<td><img src="image8.png" alt="Graph" /></td>
</tr>
</tbody>
</table>
Figure 4

(A) Rat

Plasma BNP levels (pg/mL)

Control | Heart Failure

- BNP-45
- proBNP

(B) Human

Plasma BNP levels (pg/mL)

Control | Heart Failure

- BNP-32
- proBNP

(C) Rat/Human BNP Comparison

HUMAN

RAT

(D) Rat proBNP-95

Fraction Number

- BNP immunoreactivity (pg/fraction)
- Rat proBNP before deglycosylation
- Rat proBNP after deglycosylation

-Cleaving site
- Oligosaccharide
- Ring structure
Figure 5

: oligosaccharide

: processing

* : p < 0.01 vs proBNP

† : p < 0.05 vs proBNP 69Th+71Ala

# : p < 0.05 vs proBNP 67Th+71Ala

$ : p < 0.01 vs proBNP 71Ala

(F)

Processed fraction (%)

<table>
<thead>
<tr>
<th></th>
<th>proBNP</th>
<th>proBNP 69Th+71Ala</th>
<th>proBNP 67Th+71Ala</th>
<th>proBNP 71Ala</th>
<th>All Ala</th>
</tr>
</thead>
<tbody>
<tr>
<td>Processed fraction (%)</td>
<td>60 ± 5</td>
<td>70 ± 5</td>
<td>80 ± 5</td>
<td>90 ± 5</td>
<td>100 ± 5</td>
</tr>
</tbody>
</table>

ventricular myocytes

(G)

BNP immunoreactivity (pg/fraction)

- human wild proBNP
- human mutant proBNP (T71→A)
- human mutant proBNP (T36, T42, T58, T71, S37, S44, S53→all A)

glycosylated proBNP-108

nonglycosylated proBNP-108

Fraction Number