

# Co-elevation of brain natriuretic peptide and proprotein-processing endoprotease furin after myocardial infarction in rats

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**Abstract** We investigated the expression of the yeast Kex2 family endoproteases furin and PACE4, and brain natriuretic peptide (BNP) in the atrium and ventricle after infarction as well as the conversion of the BNP precursor  $\gamma$ BNP to BNP-45. In a rat heart failure model, plasma BNP rose in two phases – first at day 3, and again at day 14. BNP mRNA, as measured by Northern blot analysis, increased strongly at day 3, then at days 14 and 28 less strongly in the atrium, and in the ventricle it increased weakly at day 3, then strongly at days 14 and 28. Furin mRNA showed the same pattern of expression as that of BNP message, whereas PACE4 message stayed unchanged after the infarction. Both furin and BNP were immunostained in the myocardium adjacent to the infarcted tissue. We suggest that after myocardial infarction, furin is co-expressed with BNP in both the atrium and ventricle, and that furin may be responsible for the conversion of  $\gamma$ BNP to BNP-45.

**Key words:** Brain natriuretic peptide; Furin; PACE4; Processing; Myocardial infarction

## 1. Introduction

Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) regulate blood pressure, diuresis, natriuresis, and dilation of vascular smooth muscles [1,2]. In congestive heart failure and hypertrophic cardiomyopathy, plasma levels of ANP and BNP rise quickly [3,4]. In those conditions BNP is extensively produced in the atrium and ventricle, whereas ANP is produced mostly in the atrium, and in severe cases in the ventricle – where ANP is not expressed in a normal state [5]. Plasma ANP declines gradually with the recovery of cardiac function while plasma BNP continues elevated for a while [3,6]. To close scrutiny, plasma BNP increases rapidly after the infarction, decreases gradually, then increases after 2 weeks in patients with myocardial infarction [3]. A similar diphasic rise in BNP was also observed in rats with congestive heart failure caused by experimental arteriovenous fistula between the infrarenal aorta and the vena cava, although the first rise peaked at day 2 and the second rise at day 8 after the operation [7]. Since the rise of BNP levels is more marked and longer than those of ANP in congestive heart failure and hypertrophic cardiomyopathy, BNP is thought to be a more valuable marker for those disorders than ANP [3,6].

BNP is synthesized from its precursor,  $\gamma$ BNP, primarily in

the atrium and ventricle, although BNP was initially isolated from the porcine brain [8,9].  $\gamma$ BNP and its processed form, positioned at the carboxy-terminal side of  $\gamma$ BNP, exhibit different sizes in different species [9].  $\gamma$ BNP consists of 108 amino acids in humans, 105 in pigs, and 95 in rats. This precursor is converted to 32 amino acid BNP in humans, 26 and 32 amino acid BNPs in pigs, and 45 amino acid BNP in rats. The cleavage site of BNP in these species consists of a consensus amino acid sequence of -Arg-X-X-Arg↓Ser- [9]. The Arg-X-X-Arg (RXXR) motif is found also in other cardiovascular peptide precursors including the amino-terminus of BNP and C-type natriuretic peptide (CNP) [9,10], and both amino- and carboxyl-termini of adrenomedullin [11] and big endothelin [12]. This motif is cleaved by yeast Kex2 family endoproteases, furin and PACE4 [13,14]. Furin is thought to have much wider substrate specificity than PACE4 [13,14]. These two endoproteases are reported to be localized in cardiocytes [15]. Especially PACE4 is abundant in both the atrial and ventricular cardiocytes. Thus, furin and/or PACE4 are the most plausible endoprotease candidates for the conversion of  $\gamma$ BNP to BNP. If furin and/or PACE4 function as a processing endoprotease for BNP, we thought these enzymes might follow the expression of BNP in the atrium and ventricle after infarction.

In this paper we demonstrate that furin was co-expressed with BNP in both atrium and ventricle after myocardial infarction by the ligation of coronary arteries in rats. In contrast, PACE4 stayed unchanged in infarcted atrium and ventricle. Furin and BNP were immunostained in the same region close to the infarcted tissues. We suggest that furin plays an essential role in the processing of  $\gamma$ BNP to BNP.

## 2. Materials and methods

### 2.1. Surgical procedures

Male Wistar rats weighing approximately 250 g were purchased from the Oriental Yeast Co., Tokyo. The left anterior descending coronary artery was ligated to cause left ventricular infarction as described previously [16]. Briefly, rats were anesthetized with diethyl ether. After left thoracotomy the heart was everted, and the left coronary artery was then ligated. Sham operations were performed using an identical procedure without ligation of the coronary artery. The rats with coronary artery ligation (MI rats) and those with sham operations were killed with a lethal injection of sodium pentobarbital on day 1, 3, 7, 10, 14, or 28 after the operation ( $n=3-7$  on each indicated day). In most MI rats, the infarcted area occupied 30–50% of the ventricular wall as determined by histological examination. After death, the heart was removed and the atrium and left ventricle were dissected out. The weight of each tissue was as follows: sham-operated atrium 0.35–0.41 g, sham-operated left ventricle 0.55–0.67 g,

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MI rat atrium 0.41–0.60 g, MI rat left ventricle 0.61–0.80 g. In MI rats, the weight of each tissue increased over a period of days after the operation. The tissues were stored at  $-80^{\circ}\text{C}$  until use.

## 2.2. Radioimmunoassay for BNP

RIA was carried out using a radioimmunoassay (RIA) kit for rat (r) BNP-45 (RIK 9085, Peninsula Laboratories, Belmont, CA, USA) [17]. The antibody was added to test samples and incubated for 16–24 h at  $4^{\circ}\text{C}$ .  $^{125}\text{I}$ -labeled BNP (10 000–15 000 cpm) was then added, mixed, and incubated again at  $4^{\circ}\text{C}$  for 16–24 h. The second antibody (goat anti-rabbit immunoglobulin G mixed with normal goat serum) was added to the above mixture, then incubated at room temperature for 90 min. RIA buffer supplied with the kit was added, and the samples were vortexed and centrifuged for 20 min at 3000 rpm. The supernatant was removed and the radioactivity in the pellets was counted in a gamma counter.

## 2.3. Northern blot analysis

Total RNA isolation from the atrium and left ventricle and Northern blotting were performed as described previously [18]. After transferring RNA onto a nylon membrane (Hybond-N, Amersham Japan, Tokyo), hybridization was carried out in a solution consisting of  $5\times\text{SSPE}$  ( $20\times\text{SSPE}=3.6\text{ M NaCl}$ ,  $200\text{ mM NaH}_2\text{PO}_4$ ,  $20\text{ mM EDTA}$ , pH 7.4),  $10\times\text{Denhardt's reagent}$  (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA), 50% formamide, 1.4% sodium dodecyl sulfate (SDS), and 0.1 mg/ml salmon sperm DNA with either a  $^{32}\text{P}$ -labeled rat BNP cDNA fragment (628 bp) [19], mouse furin cDNA fragment (924 bp) [20], or rat PACE4 cDNA fragment (797 bp) [14]. After hybridization, the membrane was washed with  $0.1\times\text{SSC}$  and 0.1% SDS at room temperature for 5 min two to three times, then at  $55^{\circ}\text{C}$  for 30 min. The membrane was exposed to an X-ray film (Kodak, XAR 5) with an intensifying screen at  $-80^{\circ}\text{C}$ .

## 2.4. Enzyme assay

Processing enzyme activities were measured for the homogenate of atrium at day 3 and ventricle at day 14 after the operation using the three substrates pyroglutamyl-arginyl-threonyl-lysyl-arginyl methylcoumarylamide (pyr-Arg-Thr-Lys-Arg-MCA), *t*-butyloxycarbonyl-alanyl-glycyl-prolyl-argininyl methylcoumarylamide (boc-Ala-Gly-Pro-Arg-MCA), and *t*-butyloxycarbonyl-glycyl-lysyl-argininyl methylcoumarylamide (boc-Gly-Lys-Arg-MCA) (Peptide Institute, Inc., Osaka, Japan). The substrates pyr-Arg-Thr-Lys-Arg-MCA, boc-Ala-Gly-Pro-Arg-MCA, and boc-Gly-Lys-Arg-MCA are designed to assess furin, rat ANP precursor processing enzyme, and paired basic residue cleavage enzyme, respectively. The enzyme activity was measured by the production of liberated fluorescent aminomethylcoumarin (AMC) from the carboxyl-terminus of a synthetic peptide substrate [21]. Enzyme source was prepared by sonicating and homogenizing the myocardial tissues in PBS containing 1 mM  $\text{CaCl}_2$  and 2% *n*-octylglucoside, a detergent. To the homogenate of 0.20 ml, 20 nmol of a synthetic substrate was added to make a total volume of 0.25 ml, containing 1 mM  $\text{CaCl}_2$ , 50 mM HEPES (pH 7.3), and 1% *n*-octylglucoside. The enzyme reaction was performed at  $37^{\circ}\text{C}$  for 2 h, then 2.0 ml of 5 mM EDTA was added to stop the reaction. The liberated AMC was fluorometrically measured with excitation at 380 nm and emission at 460 nm. Protein concentration was measured using the Bradford method (Bio-Rad, Richmond, CA, USA) using bovine serum albumin as a standard.

## 2.5. Tissue extraction

For extracting BNP from the tissues, the dissected samples were boiled for 10 min in 10 volumes of water to inactivate intrinsic proteases [17]. After cooling, glacial acetic acid was added to a final concentration of 1 M, and the resultant mixture was homogenized for 4 min with a Polytron homogenizer. The supernatants, obtained after centrifugation at 8000 rpm for 20 min, were each diluted two-fold with water and loaded onto a Sep-pak C18 cartridge (Millipore, Milford, MA, USA). After washing the cartridge with 0.5 M acetic acid, adsorbed peptides were eluted with 60% acetonitrile containing 0.1% trifluoroacetic acid (TFA). Each eluate was evaporated under vacuum prior to lyophilization. Lyophilized samples were dissolved in 0.1% TFA and applied to a reverse-phase high performance liquid chromatography (HPLC). HPLC analysis was carried out using a Nucleosil 10C18 column ( $4.6\times 150\text{ mm}$ , Chemco Pak, Osaka, Japan). The column was equilibrated with 0.1% TFA, and eluted with a linear

gradient of 0–60% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. Aliquots of all fractions were subjected to RIA for BNP.

For extracting BNP from blood, a heparinized syringe was used to collect blood samples. The sample was transferred to a chilled test tube containing EDTA (7.5 mg/5 ml), centrifuged (3000 rpm for 10 min at  $4^{\circ}\text{C}$ ), and the plasma was stored at  $-80^{\circ}\text{C}$ . BNP was extracted according to the method previously reported [22]. Briefly, 1 ml of plasma samples were acidified with 1 ml of 0.1% TFA. After centrifugation, the solution was passed through a Sep-pak C18 cartridge. After the cartridge was washed with 0.1% TFA, the absorbed peptides were eluted with 60% acetonitrile in 0.1% TFA. After evaporating each eluate via a centrifugal evaporator, the dried residue was dissolved in an assay buffer for RIA.

## 2.6. Immunocytochemistry

For furin immunostaining, the ventricular myocardium, including infarcted tissue, was fixed with 4.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at  $4^{\circ}\text{C}$ , then transferred sequentially to 10–20% sucrose in the phosphate buffer overnight at  $4^{\circ}\text{C}$ . The myocardium was cryoprotected with Tissue-Tek OCT. Radial sections of the myocardium were cut 3  $\mu\text{m}$  thick at  $-20^{\circ}\text{C}$  using a Jung Cryo-cut 3000 (Leica Instruments GmbH, Germany). Sections were mounted on gelatin-coated slides and dried at  $37^{\circ}\text{C}$ . The fixed samples were immunostained with first antibody anti-furin serum raised in a rabbit, and second antibody indocarbocyanide (Cy3)-conjugated affinity-purified donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA), as described before [18].

For BNP immunostaining, the ventricular myocardium was fixed with formalin, then embedded in paraffin and sectioned 4  $\mu\text{m}$  thick. The sections were deparaffinized and incubated for 30 min in a solution of methanol containing 0.5% hydrogen peroxide to denature endogenous peroxidase. The sections were incubated with first antibody anti-BNP serum raised in a rat, and second antibody horseradish peroxidase-conjugated affinity-purified donkey anti-rat IgG (Jackson ImmunoResearch). The peroxidase reaction was carried out using 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Ltd., Kumamoto, Japan) and 0.005% hydrogen peroxide. After the reaction samples were stained with hematoxylin to visualize nuclei.

## 3. Results

### 3.1. Plasma BNP after ligation of the left coronary artery

To examine the possibility that the pro-protein processing endoproteases furin and/or PACE4 are responsible for the cleavage of  $\gamma\text{BNP}$ , we generated experimental myocardial infarction by ligating the left anterior descending coronary artery in rats. The rate of successful operation was approximately 70–80% in the ligation of the coronary artery, and 100% in the sham operation. Rats in which 30–50% of the left ventricular myocardium was infarcted were used in this

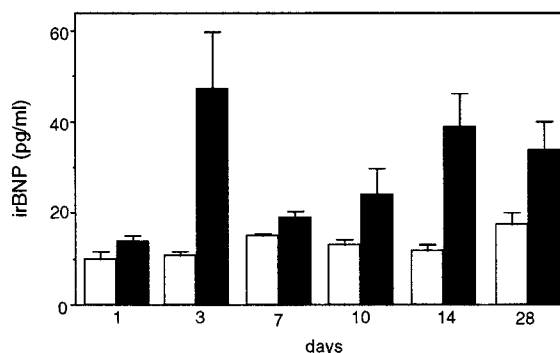


Fig. 1. Serial changes in plasma immunoreactive BNP over 4 weeks in MI and sham-operated rats. MI rats were produced by ligating the descending left coronary artery. Filled bars indicate plasma BNP in MI rats. Open bars indicate plasma BNP in the sham-operated rats. Bars represent the mean  $\pm$  S.E.M.

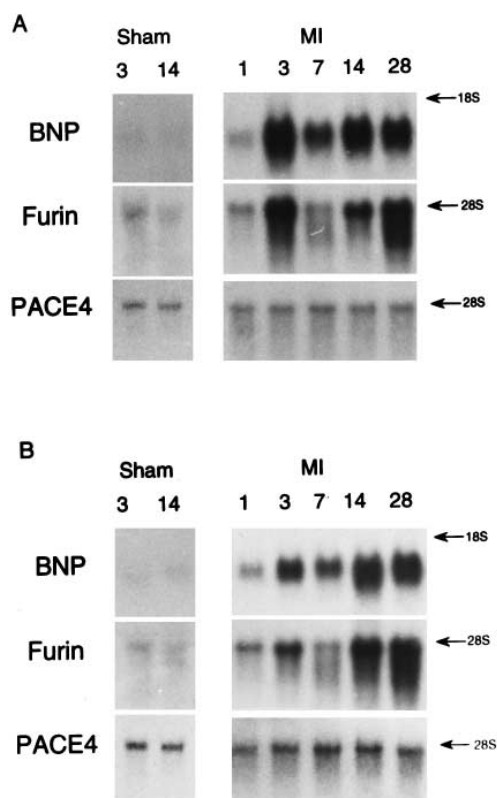


Fig. 2. Northern blot analysis of BNP, furin, and PACE4 mRNAs in the atrium (A) and left ventricle (B). In sham-operated rats total RNA was isolated from the atrium and left ventricle at days 3 and 14 after the operation. In MI rats total RNA was isolated at days 1, 3, 7, 14, and 28. Upper panel: BNP mRNA in the atrium (A) and left ventricle (B) of sham-operated and MI rats. Middle panel: furin mRNA in the atrium (A) and left ventricle (B) of sham-operated and MI rats. Lower panel: PACE4 mRNA in the atrium (A) and left ventricle (B) of sham-operated and MI rats. Ribosomal RNAs are indicated as 28S and 18S.

experiment. Serial changes in plasma immunoreactive BNP (irBNP) over 4 weeks after the operation are shown in Fig. 1. Plasma BNP increased at day 3 and fell by day 7, then began to increase again until day 14. Increased levels of plasma BNP continued as late as day 28. In sham-operated rats, plasma BNP stayed at low levels. BNP levels were 4.5-fold higher at day 3 and 2.0-fold higher at day 14 in the MI rats than in sham-operated rats.

### 3.2. Northern blot analysis of BNP, furin, and PACE4

Since plasma BNP was elevated at days 3 and 14 in MI rats, we examined BNP expression in the atrium and left ventricle by Northern blotting (Fig. 2A,B). In sham-operated rats, BNP mRNA stayed consistently faint at days 3 and 14 in the atrium as well as in the left ventricle. Likewise, in MI rats BNP mRNA was faint at day 1 in both atrium and left ventricle. In contrast, in the atrium of MI rats BNP expression was markedly elevated at days 3, 14, and 28. However, it was distinctly decreased at day 7. In the left ventricle of MI rats the expression of BNP message was similarly elevated at days 3, 14, and 28, and it was higher at days 14 and 28 than at day 3 (Fig. 2A,B). At day 7 BNP mRNA decreased like in the MI atrium. Thus, in both MI atrium and ventricle BNP mRNA rises at day 3, decreases at day 7, then increases again at days 14 and 28. The BNP expression in the MI atrium was

a little higher at day 3 than at days 14 and 28, whereas the expression in the MI ventricle was more striking at days 14 and 28 than at day 3. Thus, elevated levels of plasma BNP in MI rats on days 3, 14, and 28 reflected the result of the rise of BNP expression in both atrium and left ventricle.

We next investigated furin and PACE4 expression in correlation with BNP expression. In sham-operated rats, furin was expressed at low levels in both the atrium and left ventricle. In MI rats furin was highly expressed in both the atrium and ventricle. Atrial furin expression was higher at days 3, 14, and 28, but decreased to the control level at day 7 (Fig. 2A). The furin expression in the MI ventricle was also strikingly high at days 3, 14, and 28, but the level was much higher at days 14 and 28 than at day 3 (Fig. 2B). The furin mRNA level at day 7 was diminished to a level similar to day 1. The profile of furin expression correlated strongly with that of BNP both in the atrium and in the ventricle.

On the other hand, expression of PACE4 was low in both the atrium and left ventricle of sham-operated rats. In the MI atrium PACE4 mRNA stayed constant at the level of sham-operated atrium even at days 3, 14 and 28, when both BNP and furin expression were markedly elevated. In the MI ventricle the mRNA was a little elevated at days 7 and 14. However, compared with the striking up-and-down movement of BNP and furin, PACE4 expression was rather motionless in both the atrium and ventricle of MI rats.

### 3.3. Furin-like protease activities in the atrium and ventricle

Since expression of furin and BNP was highly correlated in the atrium and ventricle of MI rats, we assayed the proteolytic activities contained in the atrial and ventricular lysate. We used three enzyme substrates: boc-Ala-Gly-Pro-Arg-MCA, a substrate for rat ANP precursor processing enzyme; boc-Gly-Lys-Arg-MCA, a substrate for dibasic site cleavage enzyme; and pyr-Arg-Thr-Lys-Arg-MCA, a substrate for furin. In the sham-operated atrium and ventricle, proteolytic activity to the

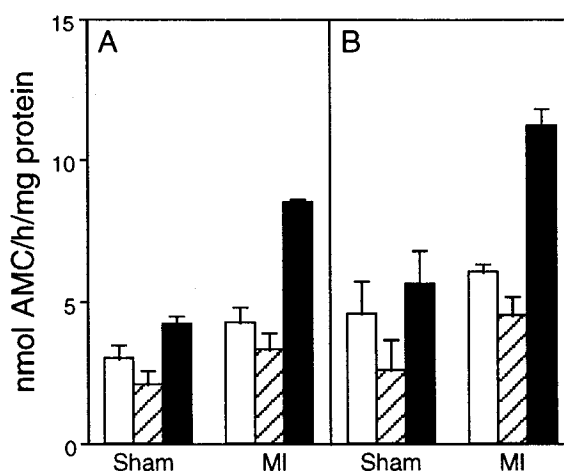


Fig. 3. Proteolytic activities in the extracts from the atrium and ventricle of sham-operated and MI rats. Proteolytic activity of atrial and ventricular lysates were measured to the three synthetic substrates: boc-Ala-Gly-Pro-Arg-MCA, boc-Gly-Lys-Arg-MCA, and pyr-Arg-Thr-Lys-Arg-MCA. The activity was assayed in the reaction containing 1 mM CaCl<sub>2</sub> at pH 7. Open bar, proteolytic activity for the ANP processing enzyme substrate boc-Ala-Gly-Pro-Arg-MCA; hatched bar, proteolytic activity for the dibasic site cleavage enzyme substrate boc-Gly-Lys-Arg-MCA; filled bar, proteolytic activity for the furin substrate pyr-Arg-Thr-Lys-Arg-MCA.

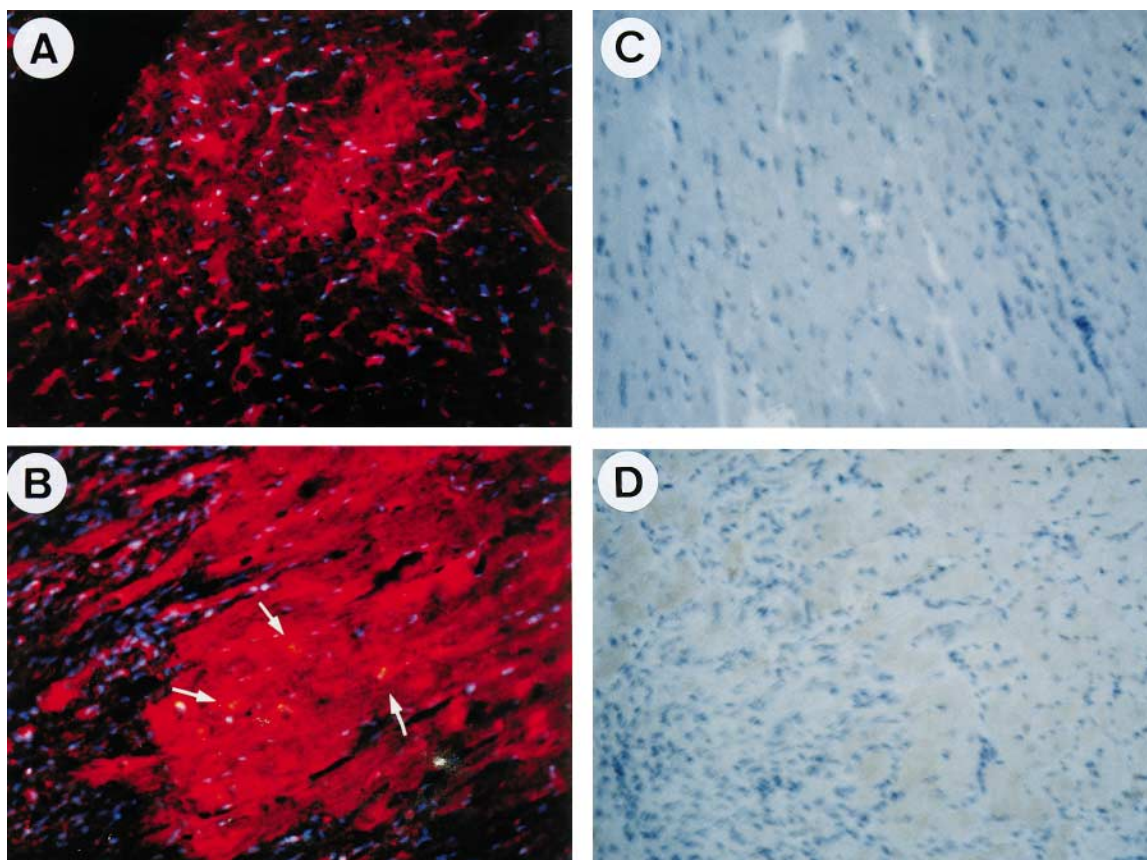


Fig. 4. Immunocytochemical staining of furin and BNP in the ventricle of sham-operated and MI rats at day 14. A and B: Furin staining. Ventricles from a sham-operated rat (A) and a MI rat (B) were stained with rabbit antibody to furin and Cy3-labeled donkey anti-rabbit IgG (red). Healthy myocardium appears red, nuclei are stained blue with 4,6-diamidino-2-phenylindole, and furin is stained orange (indicated by arrows). C and D: BNP staining. Ventricles from a sham-operated rat (C) and a MI rat (D) were incubated with rat antibody to BNP and horseradish peroxidase-labeled donkey anti-rat IgG. Peroxidase reaction was performed with 3,3'-diaminobenzidine and hydrogen peroxide to produce light-brown staining.

dibasic substrate was low compared with that to the other two substrates. Between the two, furin-type enzyme activity was a little higher than ANP-processing-type enzyme activity. In the MI atrial and ventricular lysates proteolytic activity to any of the three substrates was increased extensively. Especially furin-like activity was most enhanced compared with monobasic and dibasic site cleavage enzymes (Fig. 3). Thus, enhanced furin mRNA expression in the MI atrium and ventricle reflected the increase of furin-like proteolytic activity.

### 3.4. Immunocytochemical staining of furin and BNP

A high level of furin and BNP expression was noted in the left ventricle at day 14 in MI rats. Histological observation showed that the myocardium of MI rats exhibited characteristics typical of infarction: on a macroscopic scale, there was fibrosis and thinning of the ventricular wall, and on a microscopic scale, there was an absence of the nucleus, infiltration of inflammatory cells, and hyperplasia of fibroblasts. In the ventricle of MI rats stained with Cy3-conjugated antibody, the myocardium was observed to be red, and the infarcted tissue appeared as a dark shadow with light blue-stained nuclei (Fig. 4B). Furin was stained scatteringly as orange-colored spots (indicated by arrows) in red-colored myocardium. These orange-colored spots were not observed in the control ventric-

ular tissue (Fig. 4A). The furin-positive myocardium was located close to the infarcted area.

BNP was also localized in the same region adjacent to the infarcted tissue (Fig. 4D). Since ventricular myocytes do not possess secretory granules, BNP was stained light-brown in the whole cytoplasm. This light-brown staining was not observed in the control ventricular tissues (Fig. 4C). This finding is similar to the previous report that BNP immunostaining was localized in the cardiocytes surrounding the infarcts and in the surviving cardiocytes in the infarcts [4]. Thus, co-elevated furin and BNP were shown to be localized in the same region.

### 3.5. Molecular forms of BNP in the atrium and left ventricle

If furin converts  $\gamma$ BNP to BNP-45, a higher level of furin expression may result in the increased production of BNP-45. To investigate this possibility we analyzed irBNP from the atrium and ventricle, differentiating the two species by the fact that BNP-45 elutes first,  $\gamma$ BNP last, from an HPLC column. The ratio of  $\gamma$ BNP to BNP-45 in the normal atrium was approximately 1:2, whereas that in the atrium of MI rats was 1:3.4 at day 3 and 1:3.5 at day 14 (Fig. 5A). Likewise, the ratio of  $\gamma$ BNP to BNP-45 in the normal ventricle was approximately 1:1, whereas this ratio became 1:3 at day 3 and 1:3.5

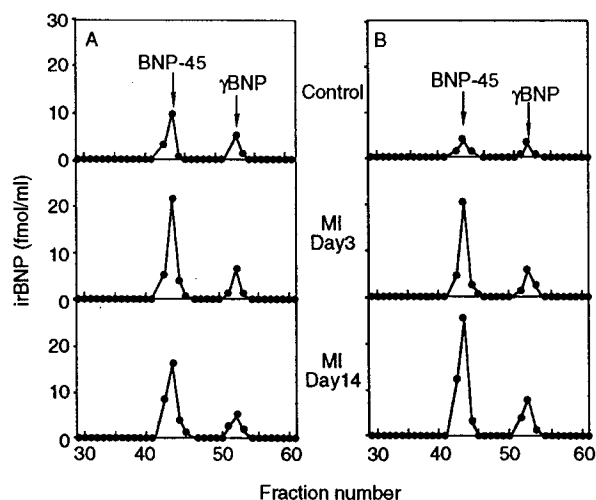


Fig. 5. Molecular forms of BNP in the atrium and left ventricle of control and MI rats. Immunoreactive BNP was separated into  $\gamma$ BNP and BNP-45 via reverse phase HPLC. A: BNP in the atrium; top, control (without MI operation); middle, at day 3; bottom, at day 14. B: BNP in the ventricle; top, control (without MI operation); middle, at day 3; bottom, at day 14. In each assay three to five samples were combined for BNP extraction. Similar elution profiles were obtained in at least three other experiments.

at day 14 in the MI ventricles (Fig. 5B). Thus, the ratio of  $\gamma$ BNP to BNP-45 fell in both the atrium and ventricle of MI rats, indicating that the increase in plasma BNP-45 after myocardial infarction likely derives from increased processing of  $\gamma$ BNP in the myocardium.

#### 4. Discussion

The present results indicate that plasma BNP in MI rats rose to a high level at day 3 and again at days 14–28 after ligating the descending left coronary artery (Fig. 1), and that both BNP and furin mRNAs were highly expressed in the atrial and ventricular myocardia at day 3 and again at days 14–28 (Fig. 2). The expression level of BNP or furin in the atrium was similar at days 3, 14, and 28, whereas the level in the ventricle was higher at days 14 and 28 than at day 3. Thus, BNP and furin mRNAs were highly expressed earlier in the atrium than in the ventricle. The time shift of each expression may be explained by the difference between the pressure loads in the atrium and the ventricle [23]. Since the atrial wall is thinner than that of the ventricle, increasing pressure load may initially stretch the atrial wall more strongly than the ventricular wall, so that the first rise of plasma BNP at day 3 contributes considerably to high BNP expression in the atrium. On the other hand, since the mass of ventricular tissue is larger than that of the atrium, and the expression of BNP mRNA is similar in the two tissues in a normal state [6,10], BNP produced from the ventricle will contribute much more to the rise in plasma BNP than BNP from the atrium at days 14–28.

Furin mRNA rose simultaneously with that of BNP in the atrium and ventricle of MI rats. This, together with the fact that rat, pig, and human gBNPs contain furin cleavage sites RXXR at the amino-termini of their respective BNP molecules, suggests that furin may be the physiologic generator of BNP from  $\gamma$ BNP [9,24]. If  $\gamma$ BNP is a substrate for furin, BNP and furin should be expressed in the same cells.

Although we attempted to show this co-localization in the same cells, we could not demonstrate it because of a technical immunostaining problem. However, immunostaining study demonstrated that BNP and furin were distributed in the same myocardial region adjacent to the infarcted tissue (Fig. 4). The expression of PACE4 stayed rather constant compared with a dramatic elevation of furin with BNP both in the atrium and in the ventricle after experimental myocardial infarction. Thus, we think furin but not PACE4 is a primary endoprotease for the conversion of  $\gamma$ BNP to BNP-45 at the RXXR motif, although PACE4 is produced at a high level in the atrial and ventricular cardiocytes in normal condition [15].

Mononuclear inflammatory cells accumulated in the infarcted tissue. Cardiocytes surviving the infarct are likely exposed to a variety of cytokines and growth factors, some of which are probably produced from these mononuclear cells [25]. Along with these humoral stimulants, cardiocytes are also affected by the stretch force and pressure load resulting in the rapid secretion of BNP [4,26]. Thus, the expression of both BNP and furin is regulated by these physical and humoral stimulants. Recently we found that mechanical stretch of cultured cardiocytes induced extensive expression of both BNP and furin (Y. Sawada et al., unpublished data).

In atrial cardiocytes BNP is stored in the secretory granules and secreted through a pathway regulated by extracellular signals [1,2]. In general, pro-peptide hormones are processed by prohormone convertases such as PC2 or PC3 in the freshly formed immature secretory granules [27]. However, atrial granules do not contain such prohormone convertases [15]. ANP is contained as a precursor in the atrial granules and processed when it is secreted [28]. A processing enzyme for ANP appears to be granule membrane-associated. BNP precursor may be converted to BNP-45 by furin at the TGN, then stored in the secretory granules in the atrium. In contrast, ventricular cardiocytes do not possess secretory granules, and secrete BNP into the blood stream through a constitutive pathway without retention in the cytoplasm. The conversion to BNP-45 appears to be more efficient in the atrium than in the ventricle because irBNP extracted from the atrium showed approximately a 2:1 proportion of BNP-45 to  $\gamma$ BNP whereas irBNP from the ventricle had almost a 1:1 proportion (Fig. 5). In the MI atrium and ventricle the proportion of BNP-45 to  $\gamma$ BNP was increased over 3:1 presumably due to the increase of furin expression.

In the infarcted myocardium a number of genes are expressed in a cascade fashion, and cardiocytes surviving the infarct undergo hypertrophic growth in an autocrine, juxtacrine, and/or paracrine fashion [29,30]. We suggest that the genes for both furin and BNP are among the genes induced early after the myocardial infarction, and BNP converted by furin compensates for cardiac overload by inducing dilation of vascular smooth muscles, natriuresis, and decrease of blood pressure.

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#### References

- [1] Rosenzweig, A. (1991) *Annu. Rev. Biochem.* 60, 229–255.
- [2] Espiner, E.A. (1994) *J. Intern. Med.* 235, 527–541.

- [3] Horio, T., Shimada, K., Kohno, M., Yoshimura, T., Kawarabayashi, T., Yasunari, K., Murakawa, K., Yokokawa, K., Ikeda, M., Fukui, T. and Takeda, T. (1993) *Am. Heart J.* 126, 293–299.
- [4] Hama, N., Itoh, H., Shirakami, G., Nakagawa, O., Suga, S.-i., Ogawa, Y., Masuda, I., Nakanishi, K., Yoshimasa, T., Hashimoto, Y., Yamaguchi, M., Hori, R., Yasue, H. and Nakao, K. (1995) *Circulation* 92, 1558–1564.
- [5] Ogawa, Y., Nakao, K., Mukoyama, M., Hosoda, K., Shirakami, G., Arai, H., Saito, Y., Suga, S.-i., Jougasaki, M. and Imura, H. (1991) *Circ. Res.* 69, 491–500.
- [6] Mukoyama, M., Nakao, K., Hosoda, K., Suga, S., Saito, Y., Ogawa, Y., Shirakami, G., Jougasaki, M., Obata, K., Yasue, H., Kambayashi, Y., Inouye, K. and Imura, H. (1991) *J. Clin. Invest.* 87, 1402–1412.
- [7] Hoffman, A., Grossman, E. and Keiser, H.R. (1991) *Am. J. Hypertens.* 4, 597–601.
- [8] Sudoh, T., Kangawa, K., Minamino, N. and Matsuo, H. (1988) *Nature* 332, 78–81.
- [9] Koller, K.J. and Goeddel, D.V. (1992) *Circulation* 86, 1081–1088.
- [10] Kojima, M., Minamino, N., Kangawa, K. and Matsuo, H. (1990) *FEBS Lett.* 276, 209–213.
- [11] Kitamura, K., Sakata, J., Kangawa, K., Kojima, M., Matsuo, H. and Eto, T. (1993) *Biochem. Biophys. Res. Commun.* 194, 720–725.
- [12] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) *Nature* 332, 411–415.
- [13] Rehemtulla, A., Barr, P.J., Rhodes, C.J. and Kaufman, R.J. (1993) *Biochemistry* 32, 11586–11590.
- [14] Hosaka, M., Murakami, K. and Nakayama, K. (1994) *Biomed. Res.* 15, 383–390.
- [15] Beaubien, G., Schäfer, M.K.-H., Weihe, E., Dong, W., Chrétien, M., Seidah, N.G. and Day, R. (1995) *Cell Tissue Res.* 279, 539–549.
- [16] Kanda, T., Nakano, M., Arai, M., Yamamoto, A., Suzuki, T. and Murata, K. (1993) *Exp. Clin. Endocrinol.* 101, 173–177.
- [17] Aburaya, M., Minamino, N., Hino, J., Kangawa, K. and Matsuo, H. (1989) *Biochem. Biophys. Res. Commun.* 165, 880–887.
- [18] Kayo, T., Sawada, Y., Suzuki, Y., Suda, M., Tanaka, S., Konda, Y., Miyazaki, J.-i. and Takeuchi, T. (1996) *J. Biol. Chem.* 271, 10731–10737.
- [19] Kojima, M., Minamino, N., Kangawa, K. and Matsuo, H. (1989) *Biochem. Biophys. Res. Commun.* 159, 1420–1426.
- [20] Hatsuzawa, K., Hosaka, M., Nakagawa, T., Nagase, M., Shoda, A., Murakami, K. and Nakayama, K. (1990) *J. Biol. Chem.* 265, 22075–22078.
- [21] Mizuno, K., Nakamura, T., Takada, K., Sakakibara, S. and Matsuo, H. (1987) *Biochem. Biophys. Res. Commun.* 144, 807–814.
- [22] Suzuki, E., Hirata, Y., Kohmoto, O., Sugimoto, T., Hayakawa, H., Matsuoka, H., Sugimoto, T., Kojima, M., Kangawa, K., Minamino, N. and Matsuo, H. (1992) *Circ. Res.* 71, 1039–1048.
- [23] Yoshimura, M., Yasue, H., Okumura, K., Ogawa, H., Jougasaki, M., Mukoyama, M., Nakao, K. and Imura, H. (1993) *Circulation* 87, 464–469.
- [24] Steinhilper, M.E. (1993) *Circ. Res.* 72, 984–992.
- [25] Palmer, J.N., Hartogensis, W.E., Patten, M., Fortuin, F.D. and Long, C.S. (1995) *J. Clin. Invest.* 95, 2555–2564.
- [26] Nakagawa, O., Ogawa, Y., Itoh, H., Suga, S.-i., Komatsu, Y., Kishimoto, I., Nishino, K., Yoshimasa, T. and Nakao, K. (1995) *J. Clin. Invest.* 96, 1280–1287.
- [27] Halban, P.A. and Irminger, J.-C. (1994) *Biochem. J.* 299, 1–18.
- [28] Sei, C.A., Hand, G.L., Murray, S.F. and Glembotski, C.C. (1992) *Mol. Endocrinol.* 6, 309–319.
- [29] Chien, K.R., Knowlton, K.U., Zhu, H. and Chien, S. (1991) *FASEB J.* 5, 3037–3046.
- [30] Komuro, I. and Yazaki, Y. (1993) *Annu. Rev. Physiol.* 55, 55–75.