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Changes in production and metabolism of brain natriuretic peptide in rats with myocardial necrosis

Wei Jiang[a,b,c], Da-Yong Cai[a], Chun-Shui Pan[b], Yong-Fen Qi[b], Hong-Feng Jiang[b], Bing Geng[b], Chao-Shu Tang[a,b,c,*]

[a]*Institute of Cardiovascular Diseases, Peking University First Hospital, Beijing, No. 8, Xi Shiku street, Beijing 100034, PR China*
[b]*Department of Physiology, Peking University Health Science Center, Beijing 100083, PR China*
[c]*The Reference Laboratory on Cardiovascular Molecular Biology and Regulatory Peptides, Ministry of Health, Beijing 100083, PR China*

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Abstract

In this study, we employed rat model of acute myocardial necrosis induced by isoproterenol (ISO) to study the possible roles of corin, the protease uniquely distributing in myocardium to convert pro-brain natriuretic peptide (proBNP) to BNP, and neutral endopeptidase (NEP), the major enzyme to degrade BNP, in changing the levels of BNP. In rats with isoproterenol alone, the myocardium necrosis occurred and the cardiac function was inhibited; the BNP contents in plasma and myocardium were upregulated, so did the myocardial corin mRNA level; the NEP activity in plasma and myocardium were downregulated. Omapatrilat (OMA) treatment relieved myocardial lesions and improved cardiac function. In the plasma and myocardium, omapatrilat treatment increased BNP contents, reduced NEP activity; in myocardium, mRNA level of proBNP and corin decreased, but NEP mRNA expression increased. Our study confirmed that omapatrilat treated myocardial necrosis effectively and suggested that increased BNP in rats with myocardial necrosis could depend on increased production and conversion as well as decreased degradation.

Keywords: Brain natriuretic peptide; Corin; Neutral endopeptidase; Myocardial necrosis

1. Introduction

Brain natriuretic peptide (BNP), originally isolated from porcine brains by Sudoh et al. in 1988, is constitutively expressed in the adult heart and primarily a ventricular hormone (Stein and Levin, 1998). BNP is composed of 32 amino acids in human and dog, and 45 amino acids in rat, which has diuretic and vasodilator properties (Maisel, 2001). The BNP gene is overepressed in the myocardium after myocardial infarction, and its production and secretion are increased in various cardiovascular diseases such as cardiac hypertrophy, congestive heart failure, and hypertension (Magga et al., 1998). BNP levels, recognized currently as important markers for ventricular dysfunction, increase and are associated with the severity and prognosis in these cardiovascular diseases (Maisel, 2001). Administration of BNP peptide (Doggrell, 2001) or BNP gene delivery (He et al., 2001) results in a marked improvement in cardiovascular hemodynamics, reduction in blood pressure, elevation of plasma level of cyclic guanosine monophosphate (cGMP), and inhibition of the secretion of aldosterone, which is an effective therapy against cardiac hypertrophy and heart failure.

In human cardiac ventricles, BNP is synthesized as a 134-amino acid polypeptide containing a signal peptide, i.e., prepro-BNP, which is processed to 108-residue gamma-BNP [pro-brain natriuretic peptide (proBNP; 1–108)] in the heart by removal of the signal peptide (Shimizu et al., 2003), prepro-BNP and proBNP (1–108) show a lower potency than the mature form of BNP (BNP-32; Hunt et al., 1997). The mature BNP consisting of 32 amino acid

* Corresponding author. Tel.: +8610 82802183; fax: +8610 66551036.
E-mail address: tangchaoshu@263.net.cn (C.-S. Tang).

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residues [i.e., proBNP (77–108)] is hydrolyzed from proBNP in its carboxyl terminus (Shimizu et al., 2003).

The conversion process from proBNP is very important for the physiological action of human BNP-32 (BNP-45 in rats). Furin, a calcium-dependent Yeast Kex2 family serine endoprotease with ubiquitous expression (Steinhelper, 1993; Sawada et al., 1997), and a factor in serum (Hunt et al., 1997), are capable of converting proBNP into BNP. Recently, Yan et al. (1999) cloned a novel cDNA from the human heart that encodes a putative transmembrane serine protease, later designated as corin. Subsequently, the authors proved that corin uniquely distributing in myocardium and can convert proBNP to BNP too, with the cleavage being highly sequence specific (Yan et al., 2000). BNP levels in plasma and tissues are regulated by the balance between its production and degradation (Smith et al., 2000). Neutral endopeptidase (E.C.3.4.24.11; NEP) is the major enzyme for the degradation of BNP (Smith et al., 2000). Seymour et al. (1992) treated spontaneously hypertensive rats (SHRs) by combining human BNP-32 with the NEP inhibitor SQ 28,603 (*N*-[2-(mercaptomethyl)-1-oxo-3-phenylpropyl]-beta-alanine) and observed that the NEP inhibitor potentiated the renal and depressor activities of BNP by protecting BNP from degradation. NEP inhibition represents a novel approach for the treatment of cardiovascular diseases (Nawarskas et al., 2001).

Corin is also the long-sought pro-atrial natriuretic polypeptide (ANP)-converting enzyme and mediates pro-ANP activation (Yan et al., 2000). Tran et al. (2004) have observed that corin gene was upregulated in hypertrophic cardiomyocytes and failing left ventricle (LV) myocardium induced by ligation of the left coronary artery, which suggested that increased corin expression might contribute to the elevation of ANP in the pathophysiological processes of cardiac hypertrophy and heart failure. Langenickel et al. (2004) studied the stretch-induced ANP release from the atria in rat heart failure model established by an infrarenal aortocaval shunt; they found that corin mRNA expression was decreased in both atria in shunt animals and that downregulation of atrial corin mRNA expression may be a novel mechanism for the blunted ANP release in heart failure to deteriorate the pathologic status. In addition, our previous studies in SHRs (Jiang et al., 2004a) and rats with septic shock (Jiang et al., 2004b) indicated that the decreased activity and expression of NEP in the myocardium and vessels might contribute to the elevation of adrenomedullin in local cardiovascular tissues. Currently, it is not clear whether the distributions of corin and NEP in myocardial necrosis also change and whether such changes affect the biological effects of endogenous BNP and reveal some beneficial therapeutic implication. In the current study, we used rats with acute myocardial necrosis induced by isoproterenol (ISO) to study the mRNA expression of corin, NEP, and BNP; the activity of NEP and the level of BNP in the plasma and ventricular myocardium, to study the possible roles of corin and NEP in altering the levels of endogenous BNP. In addition, we used omapatrilat (OMA), a dual NEP and angiotensin-converting enzyme inhibitor, to verify the pharmacological effect of NEP inhibition and further elucidate the potential pharmacological implication of corin and NEP in the therapy of cardiovascular diseases.

2. Materials and methods

2.1. Materials

The radioimmunoassay (RIA) kit for rat BNP was kindly provided by Phoenix Pharmaceutical (Belmont, CA, USA). Isoproterenol, aprotinin, *N*-dansyl-Ala-Gly-D-nitro-Phe-Gly (DAGNPG), dansyl-D-Ala-Gly (DAG), and thiorphan were purchased from Sigma (St. Louis, MO, USA); trizol was from GIBCO BRL (Gaithersburg, MD, USA); dNTP was from Clontech Laboratories (Palo Alto, CA, USA); and moloney murine leukemia virustranscriptiase (MMLV), Taq, RNAsin, and oligo (dT) 15 primer were from Promega (Madison, WI, USA). Oligonucleotides were synthesized by Sai Baisheng Biotechnology (Beijing, China). The sequences of oligonucleotide primers were proBNP-S, 5′ –TCTGCTCCTGCTTTTCCTTA–3′ , and proBNP-A, 5′ –GAACTATGTGCCATCTTGGA–3′ , for the amplification of proBNP cDNA; corin-S, 5′ –CATCGCGGCTGAGTGGGTGTG–3′ , and corin-A, 5′ –GGGACGCGCTGGCCTGTATTC–3′ , for the amplification of corin cDNA; NEP-S, 5′ –CATTGAACTATGGGGGCATC–3′ , and NEP-A, 5′ –CCTGAAATTGCCAGGACTGT–3′ , for the amplification of NEP cDNA; and β-actin-S, 5′ –CATCCGTAAAGACCTCTATGCCAAC–3′ , and β-actin-A, 5′ –CAAAGAAAGGGTGTAAAACGCAGC–3′ , for the amplification of β-actin for calibrating sample loading. The specific goat polyclonal antibody against rat NEP was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); omapatrilat was kindly provided by Bristol-Myers Squibb (New York, USA). Other chemicals and reagents were of analytical grade.

2.2. Animal model of myocardial necrosis

Male Sprague–Dawley rats (animal center, Health Sciences Center, Peking University) weighing 200–250 g were housed under standard conditions (room temperature 20±1 °C, humidity 60±10%, lights from 6 a.m. to 6 p.m.) and given standard rodent chow and water freely. All experiment procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China.

We established the myocardial necrosis model of rats by subcutaneous injection of isoproterenol as described (Rona, 1985). Animals were divided randomly into three groups (10 animals per group) and were treated as following:

(a) isoproterenol group (ISO group): rats were subcutaneously injected with 20 mg/kg isoproterenol in 10 mg/

ml normal saline (Kung and Blau, 1978), once a day for 2 days;

(b) omapatrilat group (ISO+OMA group): rats were pre-injected with 20 mg/kg omapatrilat in 10 mg/ml normal saline through the caudal vein once a day for 7 days (Lapointe et al., 2003), then injected with isoproterenol as in group (a) and omapatrilat continuously for 2 days;

(c) control group: rats were subcutaneously injected with normal saline alone for 2 days. Then, all the rats were intraperitoneally anesthetized with urethane (1 g/kg).

Two polythene catheters, which connected to a four-channel physiological recorder (PowerLab, Australia), were placed into the left femoral artery and left ventricle from the left common carotid artery to measure mean arterial blood pressure and ventricular pressure, respectively. Left ventricular end systolic pressure and diastolic pressure and LV dP/dt_{max}($\pm$LV dP/dt_{max}) were derived from the ventricular pressure. At the end of hemodynamic measurement, the blood was collected from the artery catheter, and plasma was prepared for assay of malondialdehyde (MDA) content and lactic dehydrogenase (LDH) activity. All animals were killed by an overdose of anesthetics; the hearts were removed and placed in cold normal saline (4 °C).

The histological sections were prepared from the cardiac apex of all three groups (n=3/group) and stained with hematoxylin and eosin. Pathological changes in the myocardium were viewed under a light microscope.

2.3. Determination of malondialdehyde content in plasma and myocardium and lactic dehydrogenase activity in plasma

The myocardium sample was homogenized by use of a Polytron set and centrifuged. The protein content of myocardial homogenate was determined by Coomassie blue staining. Malondialdehyde levels in the plasma and myocardial homogenate were measured as described (Pompella et al., 1987) by use of 2-thiobarbituric acid with isobutyl alcohol extraction. The contents of malondialdehyde were expressed as μM for plasma and pmol/mg protein for tissues. The activity of lactic dehydrogenase in plasma, expressed as U/l, was detected at 30 °C as the change in absorbance at 340 nm on chromatometer, using 0.18 mM NADH and 0.72 mM pyruvate as substrates in 50 mM K-phosphate buffer, pH 7.4 (Webster et al., 1995).

2.4. Determination of levels of immunoreactive BNP (ir-BNP) in plasma and ventricular myocardium

Blood samples of each rat were taken with the use of EDTA·Na_2 (2.7 mM), aprotinin (500 KIU) and heparin. The plasma was separated by centrifugation (1600×g for 15 min) and stored at −70 °C for assaying. Chopped myocardial tissues were boiled for 10 min in 1 M acetic acid and homogenized with a Polytron set at 4 °C. The extract solution was centrifuged at 24,000×g for 30 min. The plasma and tissue extract solution were loaded onto a Sep-Pak C_{18} cartridge (Waters, MA, USA) and pre-equilibrated with 0.5 mM acetic acid. The adsorbed material was eluted with 4 ml of 50% CH_3CN containing 0.1% trifluoroacetic acid (TFA). After the samples were lyophilized, the residue was dissolved in radioimmunoassay buffer and assayed according to the manufacturer's instructions. The IC_{50} of the BNP assay was 3.7 pg/tube and the reactivity with rat BNP 100%. No cross-reactivity was found with rat endothelin-1, ANP-32, ANP-45, and C-type natriuretic peptide-22. The within- and between-assay coefficients of variation for the ir-BNP assay were both less than 10%. The content of ir-BNP was calculated and expressed as pM for plasma and pmol/g wet weight for tissues, respectively.

2.5. Assay of NEP activity in plasma and myocardium

NEP activity in the plasma and the particulate fractions of the myocardial tissue homogenates was measured by spectrofluorometric assay with the synthetic peptide DAGPNG, as previously described (Fielitz et al., 2002). The reaction was carried out in the presence or absence of thiorphan (20 nM), a specific inhibitor of NEP, and only the activity inhibited by thiorphan equaled the NEP activity. Blood samples of each rat were mixed with heparin, then centrifuged (1600×g for 15 min, 4 °C), and the plasma was separated for NEP assay. The particulate fractions of the tissues were prepared as described by Wolff and colleagues (Wolff et al., 1989), with a few modifications. Tissues were washed extensively with cold saline solution to remove all of the blood and homogenized three times (5 s each) in a 10-v solution of iced cold Tris-HCl buffer (50 mM, pH 7.4) by a Polytron set at maximum speed. Then, the samples were homogenized in a motor-driven glass–Teflon homogenizer (medium speed, 10 strokes). The homogenate was then filtrated through a four-layered cheesecloth, and the filtrate was centrifuged at 1000×g at 4 °C for 20 min. The pellet was resuspended in fresh iced Tris–HCl buffer and centrifuged at 1000×g (4 °C, 20 min) at least twice. The final pellet was resuspended in fresh iced Tris–HCl buffer with a protein concentration of about 4 mg/ml (the protein content was determined by Coomassie blue staining).

2.6. Reverse-transcriptase polymerase chain reaction (RT-PCR) study

The expression of proBNP, corin, and NEP mRNA was assessed by RT-PCR as described (Suzuki et al., 1998). Total ventricular myocardial tissue RNA of the control, isoproterenol, and omapatrilat groups (n=3/group) were extracted with use of standard techniques. Isolated total tissue RNA was then quantified by use of an ultraviolet spectrophotometer (DU-640, Beckman, USA). Reverse

transcription to cDNA was accomplished by priming 2 μg of total RNA samples with MMLV and oligo (dT) 15 primer. The products were then used for the following PCR amplification: the PCR reaction mixture was in a 25 μl volume containing 2.5 mM dNTP 1 μl, 10×PCR buffer (20 mM $MgCl_2$, 500 mM KCl, 1.5 M Tris–HCl, pH 8.7), 2.5 μl cDNA, 200 nM of the appropriate proBNP-, corin-, or NEP-paired primers and 1.25 unit of Taq DNA polymerase. The following PCR cycles were used: 94 °C for 30 s, 57 °C for 30, s and 72 °C for 40 s, for 30 cycles, then 74 °C for 5 min. As an internal control for each PCR reaction, β-actin mRNA was also amplified with each sample. A total of 200 nM of actin primers and cDNA 2 μl were amplified under the same reaction conditions. All PCR products were loaded onto a 1.5% agarose-Tris-acetate–EDTA gel before electrophoresis, and then the products were visualized on ethidium bromide staining. The ultraviolet illumination photos then underwent computerized densitometric analysis. The final results are expressed as the ratios of proBNP (256 bp), corin (561 bp), or NEP (440 bp) PCR product to the β-actin PCR product (291 bp) for each sample. The amplified proBNP, corin and NEP cDNA and β-actin were confirmed by digestion of the PCR products with the restriction enzyme *MSP I*. All experiments were repeated three times.

2.7. NEP immunohistochemistry

The immunohistochemical localization of NEP was studied according to the method described by Gurcharan et al. (2002), with some modification. After being anesthetized with pentobarbital sodium (45 mg/kg, intraperitoneal administration), control and isoproterenol rats were perfused with 100 ml of saline, followed by 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer by use of an aortic catheter (at about 100 mmHg pressure). Tissues were kept in the same fixative solution overnight at 4 °C and then cryopreserved in 30% sucrose in phosphate buffer for 24 h at 4 °C. Tissue sections about 10 μm thick were cut by use of a cryostat microtome and mounted onto poly-L-lysine-coated glass slides before being incubated with Tris-buffered saline (TBS) containing 0.1% Triton X-100, 3% normal rabbit serum, and 1% dry milk for 1 h. Endogenous peroxidase activity was quenched by incubating the sections with 3% (v/v) H_2O_2 in 70% methanol for 30 min at room temperature. Indirect immunohistochemical detection of NEP was by use of an avidin-biotin peroxidase (ABC-HRP) immunostaining kit (Vector laboratories, Burlingame, CA, USA). Before staining, goat polyclonal antibody against rat NEP (1:180) was applied in a humidified chamber at room temperature for 1 h then at 4 °C overnight. Immunoreactive sites were revealed by incubation with 0.05% (wt/v) diaminobenzidine, 0.003% (v/v) H_2O_2 in 50 mM Tris–HCl (pH 7.6) for 5 min. Sections were then counterstained with hematoxylin before being dehydrated in ethanol. A blinded judge evaluated sections under light microscopy. The presence of NEP was indicated by a brown color on the plasma membrane or in the cytoplasm. For negative controls, nonimmunized goat serum was substituted for the primary antibody.

2.8. Statistical analysis

Results are shown as mean±S.E.M. Comparisons were preformed using unpaired Student's *t*-test and one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls test; correlation between BNP levels and NEP activity were preformed using linear regression. A P value<0.05 was considered statistically significant.

3. Results

3.1. General characteristics of rats with myocardial necrosis induced by isoproterenol

At the end of the isoproterenol treatment, four rats in isoproterenol group and two in omapatrilat group died of acute myocardial necrosis and severe arrhythmia. As shown in Table 1, in the isoproterenol group, the left ventricular end diastolic pressure and lactic dehydrogenase activity in plasma and malondialdehyde level in the plasma and

Table 1
Weight, hemodynamic parameters, malondialdehyde (MDA) levels in the plasma and myocardium, and lactic dehydrogenase (LDH) activity in the plasma of rats with myocardial necrosis treated with isoproterenol (ISO) and omapatrilat (OMA; mean±S.E.M., Control, n=10; ISO, n=6; and ISO+OMA, n=8)

	Control	ISO	ISO+OMA
Body weight (g)	221±4	229±4	219±5
Heart rate (bpm)	363±5	422±6[a]	438±7[a]
Mean arterial blood pressure (mmHg)	107±2	104±6	81±4[a,b]
Left ventricular end systolic pressure (mmHg)	126±6	92±4[a]	119±7[b]
Left ventricular end diastolic pressure (mmHg)	8.7±0.82	16.4±2.0[a]	10.7±1.4[c]
+Left ventricular dp/dt_{max} (mmHg/s)	3383±58	1773±160[a]	4462±170[a,b]
−Left ventricular dp/dt_{max} (mmHg/s)	3038±148	2093±108[a]	3557±117[b,d]
Plasma MDA content (μM)	1.09±0.053	1.45±0.086	1.22±0.033
Myocardial MDA content (pmol/mg protein)	26.2±1.9	52.1±2.5[a]	31.8±2.4[b]
Activity of plasma LDH (U/l)	224.6±15.6	323.2±22.2[a]	208.4±21.9[b]

ISO: rats administered ISO subcutaneously alone; ISO+OMA: rats administered ISO subcutaneously; and OMA through caudal vein.
[a] P<0.01 vs. the control.
[b] P<0.01 vs. the isoproterenol group.
[c] P<0.05 vs. the isoproterenol group.
[d] P<0.05 vs. the control.

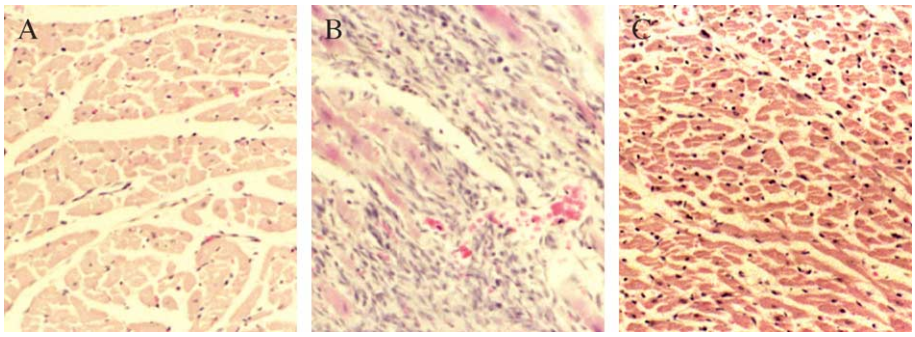

Fig. 1. Damaged myocardium in rat cardiac apex induced by isoproterenol (ISO; hematoxylin–eosin stain, all sections: ×200). (A) Control rats; (B) Rats administrated ISO alone; (C) Rats administrated ISO and omapatrilat.

myocardium were higher, by 88.5%, 43.9%, 33.0%, and 98.9%, respectively, than in controls (all $P<0.01$). The left ventricular end systolic pressure, +LV dp/dt_{max} and −LV dp/dt_{max} were lower than those of controls, by 27.0%, 47.6%, and 31.1%, respectively (all $P<0.01$), but mean arterial blood pressure did not significantly differ ($P>0.05$). However, treatment with omapatrilat ameliorated the isoproterenol-induced disturbance. Compared with the isoproterenol group, the omapatrilat group had higher left ventricular end systolic pressure, +LV dp/dt_{max} and −LV dp/dt_{max}, by 29.3%, 151.7%, and 69.9%, respectively (all $P<0.01$), and lower left ventricular end diastolic pressure, mean arterial blood pressure, plasma lactic dehydrogenase activity, and malondialdehyde levels in the plasma and myocardium, by 34.8% ($P<0.05$), 22.1% ($P<0.01$), 35.5% ($P<0.01$), 15.9% ($P<0.05$), and 39.0% ($P<0.01$), respectively. Pathological sections showed that myocardium from the isoproterenol group (Fig. 1B) had considerable microfocal necrosis infiltrated with neutrophils, and necrotic myocardial cells lost their nuclei and appeared eosinophilic in contrast to controls (Fig. 1A). However, the omapatrilat group (Fig. 1C) showed markedly fewer lesions and necrotic myocardial cells than the isoproterenol group.

3.2. ir-BNP content

Compared with that in controls, *ir*-BNP levels in the plasma (Table 2) and myocardium (Table 3) of the isoproterenol group were higher, by 36.9% and 53.4%, respectively (both $P<0.01$). Compared with those in the isoproterenol group, *ir*-BNP levels in rats treated with omapatrilat were 12.1% and 17.1% higher, respectively (both $P<0.05$).

3.3. NEP activity

NEP activity in the isoproterenol group was lower in the plasma (Table 2) and myocardium (Table 3), by 62.8% and 63.3%, respectively, than in controls (both $P<0.01$). NEP activity was lower in the plasma and myocardium in rats treated with omapatrilat, by 42.4% and 49.2%, respectively (both $P<0.05$), than in the isoproterenol group.

3.4. NEP immunohistochemistry

All myocytes of controls (Fig. 2A) showed intense, positive NEP staining along the plasma membrane and in the cytoplasm. Compared with controls, the isoproterenol group showed weaker myocardial NEP staining (Fig. 2B).

3.5. proBNP, corin, and NEP gene expression

In the isoproterenol group, in the cardiac ventricle, proBNP, and corin mRNA levels were significantly more intense than those of controls, by 168.6% and 156.8% (both

Table 2
Brain natriuretic peptide (BNP) contents and neutral endopeptidase (NEP) activity in the plasma of rats with myocardial necrosis treated with isoproterenol (ISO) and omapatrilat (OMA; mean±S.E.M., Control, n=10; ISO, n=6; and ISO+OMA, n=8)

	BNP contents (pM)	NEP activity (U/ml)
Control	2.60±0.070	3.57±0.21
ISO	3.56±0.17[a]	1.33±0.11[a]
ISO+OMA	3.99±0.11[a,b]	0.77±0.070[a,b]

1U=1 nmol/min. ISO: rats administered ISO subcutaneously alone; ISO+OMA: rats administered ISO subcutaneously; and OMA through caudal vein.

[a] $P<0.01$ vs. the control.

[b] $P<0.05$ vs. the isoproterenol group.

Table 3
Brain natriuretic peptide (BNP) contents and neutral endopeptidase (NEP) activity in the tissue of left ventricles of rats with myocardial necrosis treated with isoproterenol (ISO) and omapatrilat (OMA; mean±S.E.M., Control, n=10; ISO, n=6; and ISO+OMA, n=8)

	BNP contents (pmol/g w.w.)	NEP activity (U/mg protein)
Control	3.38±0.17	0.37±0.024
ISO	6.35±0.23[a]	0.13±0.012[a]
ISO+OMA	7.43±0.23[a,b]	0.068±0.0081[a,c]

1U=1 nmol/min. ISO: rats administered ISO subcutaneously alone; ISO+OMA: rats administered ISO subcutaneously; and OMA through caudal vein.

[a] $P<0.01$ vs. the control.

[b] $P<0.01$ vs. the isoproterenol group.

[c] $P<0.05$.

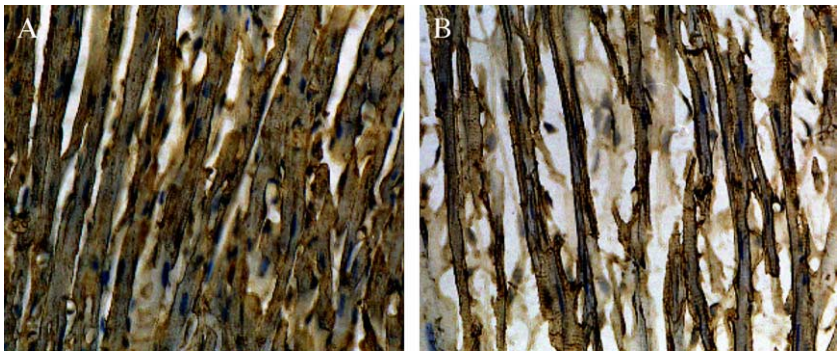

Fig. 2. The immunoreactivity of neutral endopeptidase (NEP) in left ventricles of rats with myocardial necrosis (n=3/group, all sections: ×400). (A) Ventricle of controls; (B) ventricle of rats subcutaneously administered isoproterenol alone.

P<0.01; Fig. 3), respectively, and the NEP mRNA level was significantly lower than that in the controls, by 75.0% (P<0.01; Fig. 3). Compared with the isoproterenol group, the omapatrilat group had 11.2% (P<0.05) and 24.5% (P<0.01) lower in mRNA level of proBNP and corin, respectively, but a 35.9% (P<0.05) more intense NEP mRNA expression (Fig. 3).

3.6. Correlation between BNP level and NEP activity in plasma and myocardium

Compared with controls, the isoproterenol group showed increased BNP level and marked decreased NEP activity in the plasma and myocardium. In the omapatrilat group, the BNP content was higher and NEP activity lower in the plasma

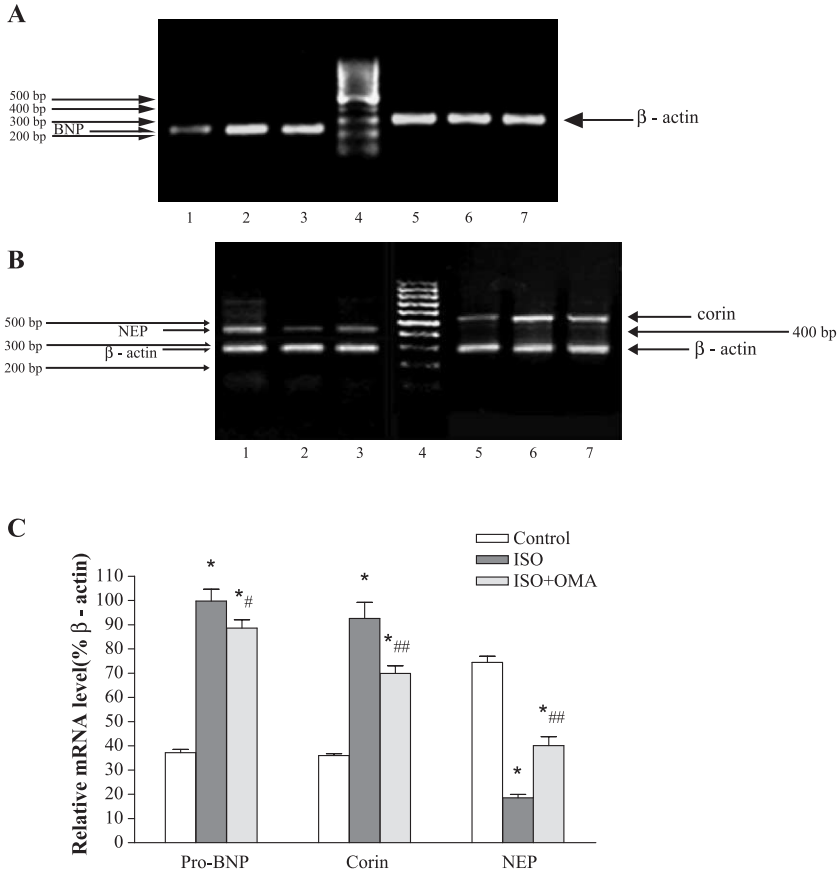

Fig. 3. Gene expression of pro-brain natriuretic peptide (proBNP), corin, neutral endopeptidase (NEP) and β-actin gene in left ventricles of rats with myocardial necrosis (n=3/group). (A) Lanes 1, 2, and 3, proBNP mRNA expression of the control, ISO, and ISO+OMA, respectively; lane 4, the marker; lanes 5, 6, and 7, β-actin mRNA expression of the control, ISO, and ISO+OMA, respectively. (B) Lanes 1, 2, and 3, NEP mRNA expression of the control, ISO, and ISO+OMA, respectively; lane 4, the marker; lanes 5, 6, and 7, corin mRNA expression of the control, ISO, and ISO+OMA, respectively. (C) Changes of pro-brain natriuretic peptide (proBNP), corin, and neutral endopeptidase (NEP) gene in rat ventricles. The data (n=9/group; expressed as the ratio of target gene to β-actin gene) are mean±S.E.M. and compared by one-way ANOVA. ISO: rats subcutaneously administered isoproterenol (ISO) alone; ISO+OMA: rats administered ISO subcutaneously and omapatrilat (OMA) through caudal vein. *P<0.01 vs. the control; $^{\#}P$<0.05 and $^{\#\#}P$<0.01 vs. isoproterenol-group.

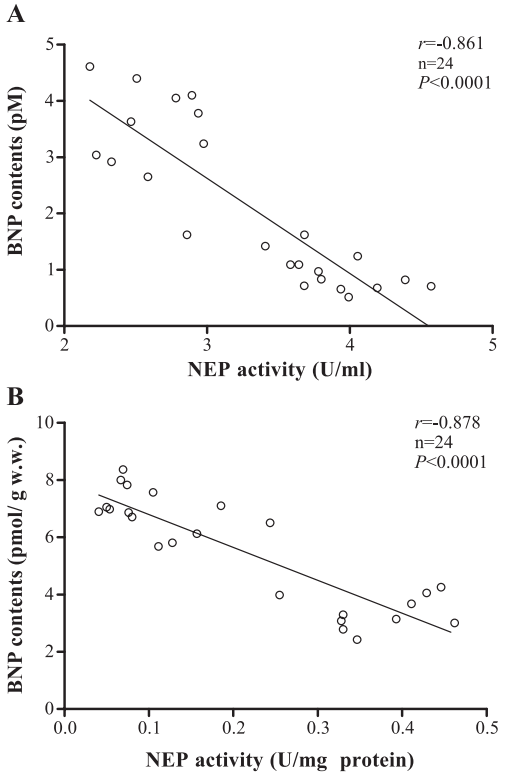

Fig. 4. Linear regression analysis of neutral endopeptidase (NEP) activity versus the brain natriuretic peptide (BNP) contents in the plasma and left ventricular myocardium of rats with myocardial necrosis treated with isoproterenol (ISO) and omapatrilat (OMA), all data from the three groups—control, isoproterenol, and omapatrilat—were analyzed (n=24/group). The x-axis represents NEP activity; the y-axis represents the contents of BNP. Solid line represents the linear regression line. (A) Linear regression analysis of NEP activity and BNP contents in the plasma, line equation is $y=-0.44x+4.233$. (B) Linear regression analysis of NEP activity and BNP contents in the myocardium, line equation is $y=-11.47x+7.937$.

and myocardium than those in isoproterenol group. Linear regression analysis of all data in the three groups indicated a strong negative correlation between BNP level and NEP activity in the plasma (Fig. 4A) and myocardium (Fig. 4B; $r=-0.861$ and -0.878, respectively; both $P<0.01$, $n=24$).

4. Discussion

BNP is an important cardiac hormone to induce natriuresis, vasodilation and inhibition of aldosterone secretion and renin actions (Stein and Levin, 1998). BNP affects cardiovascular function by several mechanisms, including modifying renal function and vascular tone, counteracting the renin–angiotensin–aldosterone system and acting on brain regulatory sites. These systems maintain a balance that ensures relative constancy of body electrolytes, water content, and circulatory homeostasis (Maisel, 2001). proBNP mRNA is highly expressed in cardiac ventricles (Stein and Levin, 1998). The BNP-gene, also present as a single-copy gene, is organized into three exons and two introns (He et al., 2001). Recently, a 1.8-kb 5′ flanking region of the human BNP gene has been cloned and analyzed; it contains an AP-1 binding site and several copies of acute-phase regulatory elements. These specific DNA sequences are relevant to the upregulation of the BNP gene in diseased ventricles (Nakao et al., 1992). BNP is synthesized as a precursor peptide and stored in human cardiac tissue mainly in a low molecular weight form, BNP-32, with a lesser amount of the precursor peptide proBNP (1–108; He et al., 2001), then secreted predominantly from the ventricle. The circulating plasma forms of BNP are BNP-32 and the amino-terminal portion of proBNP (1–108) [i.e., N-terminal proBNP (NT-proBNP)] (Shimizu et al., 2003). Results of many studies have shown that plasma BNP levels increase markedly in myocardial necrosis and heart failure. Multiple factors involved in the progression of these diseases, such as ventricular overloading, increased tension in the ventricular wall, and catecholamine and cytokines, including endothelin, angiotensin, and cardiotrophin, may induce BNP gene expression through multiple mechanisms (Magga et al., 1998; Nakao et al., 1992). The increased BNP production and circulating BNP levels are closely correlated with the dilatation of the left ventricle, decrease in ventricular contractility and improvement of ventricular compliance (Doggrell, 2001). The upregulation of BNP is not only an important biological attempt to compensate for cardiac and renal damage, but also with potent therapeutic effect, and circulating plasma levels of BNP-32 are good markers for the evaluation of condition and prognosis in patients with cardiovascular diseases (Maisel, 2001).

Cleavage is required to produce the mature, active peptide for BNP (Shimizu et al., 2003). The expression of the BNP gene increases in cardiovascular diseases, as does the production of proBNP (1–108; Hunt et al., 1997). Currently, besides furin, corin is identified as another proBNP-converting enzyme in myocardium for the activation of proBNP through hydrolysis to produce BNP-32 (amino acids 77–108) and amino terminal proBNP (1–76), with the specific cleavage sequence being Leu-Arg-Ala-Pro-Arg-Ser (residues 72–77; Yan et al., 2000). As a short peptide with low molecular weight (about 3.45 kDa), the circulating half-life of BNP is only 4.6 ± 0.1 min in rats (Roques et al., 1993). Two clearance pathways of tissue BNP have been recognized: binding to BNP clearance receptors and protease hydrolysis (Smith et al., 2000). NEP is the major enzyme pathway for BNP degradation, at an optimal action pH of about 7, its zinc-binding action center contributes to the cleavage of BNP at Phe^{5}-Ser^{6} in hydrophobic residues (Roques et al., 1993). BNP contents in plasma and tissues are regulated by the balance between its production and degradation (Smith et al., 2000). However, it is still not clear whether NEP and corin play important roles in regulating the BNP metabolism in cardiovascular disease, which might represent new therapeutic pathways to potentiate endogenous BNP activity.

The myocardial lesion induced by catecholamine excess is one of the major pathogenesis factors in myocardial ischemia and congestive heart failure and relevant to the induction of intracellular Ca^{2+} overload (Rona, 1985) and oxygen-derived free radical production (Singal et al., 2000). In the present study, as has also been reported previously (Rona, 1985), the administration of high doses of isoproterenol induced myocardial ischemia and necrosis in rats, the plasma lactic dehydrogenase activity and malondialdehyde levels in the plasma and myocardium increased significantly, cardiac function was inhibited, left ventricular end diastolic pressure increased and (±) LV dP/dt_{max} declined, and myocardial tissue sections showed diffused focal necroses. The BNP contents in the plasma and myocardium and its mRNA expression in the myocardium were elevated, as was found by He et al. (2000). In our study, the ventricular myocardial corin mRNA level in rats treated with isoproterenol was more intense as those reported by Tran et al. (2004) in hypertrophic cardiomyocytes and failing myocardium induced by ligation of the left coronary artery, and the NEP activity in the plasma and myocardium and myocardial NEP expression were markedly decreased. Linear regression analysis indicated a negative correlation between BNP level and NEP activity in the plasma and myocardium. The results suggested that the increased BNP levels in the plasma and myocardium in rats with myocardial necrosis could depend on increased BNP production and its conversion as well as decreased degradation.

Corin is a precursor-converting enzyme of the natriuretic peptide family and has specific high expression in cardiac myocytes of both the atrium and ventricle. It has a predicted structure of a type II transmembrane protein and contains two frizzled-like cysteine-rich motifs, seven low-density-lipoprotein receptor repeats, a macrophage scavenger receptor-like domain, and a trypsin-like protease domain in the extracellular region (Yan et al., 1999). Currently, little is known about the mechanisms involved in the regulation of the expression and activity of corin (Yan et al., 1999). Protease inhibitors such as aprotinin, leupeptin, and trypsin can inhibit the activity of corin, but what factors activate its transcription and expression is still unclear (Yan et al., 2000). Further study is required on the mechanism in the upregulation of corin mRNA in the ischemic myocardium. Recently, the changes of corin expression in cardiovascular diseases have attracted the interest of a few research groups. Our findings are coincident with those reported by Tran et al. (2004). Interestingly, these results are also parallel to the changes of furin in the research of Sawada et al. (1997), in which the expression and activity of furin increased significantly in the same pattern as that of BNP in a rat myocardial infarction model induced by ligation of the coronary arteries. Furin is a membrane-associated endoprotease that efficiently cleaves precursor proteins including proBNP on the C-terminal side of the consensus sequence, Arg (P4)-X-Lys/Arg-Arg (P1) (Molloy et al., 1994). Corin and furin are both transmembrane serine proteases, while corin uniquely distributes in myocardium but furin ubiquitously in various tissues (Yan et al., 1999; Hay and Docherty, 2003). Although now there are still no reports available to have investigated whether corin and furin are parts of a cascade in the procedure of certain signal transduction, the relation between them needs advanced study. Similarly, little is known about how NEP is regulated during cardiovascular disease (Roques et al., 1993). NEP is a membrane-bound metalloprotease of 87–96 kDa. The human NEP gene is located at chromosome 3, which spans more than 80 kb and contains 24 miniexons (Roques et al., 1993). The overexpression of angiotensin-converting enzyme significantly decreases NEP activity (Oliveri et al., 2001); phorbol 12-myristate 13-acetate and diacyl-glycerol, the activators of protein kinase C (PKC), induce the internalization of NEP and decrease NEP activity (Gonzalez et al., 1998). The activators of adenylate cyclase or protein kinase A, glucocorticoid, thrombin, calcitonin, and cytokines such as interleukin-1β, tumor necrosis factor-á and granulocyte–macrophage colony-stimulating factor, can increase NEP mRNA expression and activity (Roques et al., 1993). In our study, we observed that in the ischemic and necrotic myocardium, BNP and corin are upregulated, but NEP is downregulated, which might represent a new compensatory mechanism for BNP-induced cardiac protection during myocardial necrosis and indicates that the upregulation of corin might be a new therapeutic pathway.

Vasopeptidase inhibition is an effective therapeutic strategy for cardiovascular diseases and may ameliorate myocardial infarction and heart failure (Seymour et al., 1992). The primary consequence of NEP inhibition is the potentiation of endogenous vasodilatory and natriuretic systems such as atrial natriuretic polypeptide, BNP, adrenomedullin, and bradykinin, which results in natriuresis, diuresis, and vasodilatation (Lapointe and Rouleau, 2002). Clinical and experimental data increasingly show that inhibitors of NEP potently enhance the effects of natriuretic peptides. After combined inhibition of NEP and angiotensin-converting enzyme in congestive heart failure, myocardial necrosis, and cardiovascular remodeling, hemodynamic improvement and cardiorenal protection are more significant than with selective inhibitors of either enzyme alone (Lapointe et al., 2003). Omapatrilat, a novel compound that can inhibit both NEP and angiotensin-converting enzyme, is a 7, 6-fused bicyclic thiazepinone with a molecular weight of 408.5 (Nawarskas and Anderson, 2000). Rats with isoproterenol-induced myocardial necrosis, pro-administered with omapatrilat, had higher BNP content and lower NEP activity in the plasma and myocardium. Interestingly, treatment with omapatrilat decreased the BNP and corin mRNA level and increased NEP mRNA expression in the myocardium, which suggested feedback mechanisms of gene transcription. The administration of omapatrilat effectively attenuated the isoproterenol-induced myocardial injury, with the plasma

lactic dehydrogenase activity and malondialdehyde level being lower in the plasma and myocardium. In addition, omapatrilat significantly improved isoproterenol-induced heart failure, with lower left ventricular end diastolic pressure and higher LV dP/dt_{max} values than those in the isoproterenol alone group. These results confirm that omapatrilat had a beneficial effect on myocardial necrosis and heart failure. Because corin acts at the top of the natriuretic peptide-mediated molecular pathway to control cardiovascular function indirectly by regulating natriuretic peptide contents (Yan et al., 2000), our results also suggest that increase in the activity of corin associated with the inhibition of NEP could be a new optimized therapeutic strategy for the treatment of cardiovascular diseases.

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