Pretreatment with B-type Natriuretic Peptide Protects the Heart From Ischemia-Reperfusion Injury by Inhibiting Myocardial Apoptosis

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The therapy for acute myocardial infarction (AMI) has been improved; yet, AMI remains a major cause of death and heart failure in industrialized countries. B-type natriuretic peptide (BNP), a hormone secreted from the heart, has been shown cardioprotective effects during myocardial ischemia/reperfusion. In the present study, we aimed to examine whether BNP could inhibit myocardial apoptosis during ischemia/reperfusion. Rabbits were randomly divided into three groups (12 animals for each group): sham-operated control and ischemia-reperfusion animals with or without BNP treatment. Occlusion of the left circumflex coronary for 45 min was followed by 3-h reperfusion with infusion of physiological saline (untreated group) or BNP (treated group) starting 5 min before reperfusion and throughout the whole reperfusion. The infarct size, measured by triphenyltetrazolium chloride staining, was reduced by 44% with BNP treatment ($P < 0.01$). Accordingly, serum levels of creatine kinase and lactate dehydrogenase were markedly reduced in BNP-treated group ($P < 0.05$) compared with the untreated group. BNP significantly attenuated apoptotic cells (TUNEL-positive cardiomyocyte nuclei) in the myocardium ($P < 0.01$). The BNP-mediated attenuation of apoptosis was associated with the increased expression of an anti-apoptotic protein Bcl-2 and the reduced expression of a pro-apoptotic protein Bax. Moreover, BNP treatment significantly decreased the magnitude of caspase-3 activation caused by myocardial ischemia-reperfusion. In conclusion, pretreatment with BNP shortly before the onset of reperfusion not only reduces necrosis, but also attenuates myocardial apoptosis. BNP appears to be an ideal pharmacological agent applied as an adjuvant therapy to current myocardial reperfusion strategies. ——— B-type natriuretic peptide; reperfusion injury; apoptosis; Bcl-2 family; caspase-3.

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The therapy for acute myocardial infarction (AMI) has been improved; yet AMI remains a major cause of death and heart failure in industrialized countries (McGovern et al. 1996). Reperfusion is the major treatment for impending acute myocardial infarction by thrombolysis or primary percutaneous coronary angioplasty. Although the restoration of blood flow to the jeopardized myocardial area is a prerequisite for myocardium salvage, reperfusion itself may lead to either the accelerated processes of ischemia or additional injury by ischemia alone. The phenomenon is termed "Ischemia/Reperfusion injury" (Piper et al. 1998; Yellon et al. 2007). Since the reperfusion injury is initiated by the treatment of myocardial infarction, it is important to attenuate the extent of the injury while blood flow is being restored.

Increasing evidence suggests that reperfusion injury possibly consists of two forms of cell death, necrosis and apoptosis (programmed cell death). The apoptotic process

is initiated shortly after the onset of ischemia, and becomes markedly enhanced during reperfusion (Olivetti et al. 1996; Zhao et al. 2000). Therefore, inhibition of the apoptotic process should then attenuate the reperfusion injury (Zhao et al. 2003; Abbate et al. 2008).

B-type natriuretic peptide (BNP), a hormone secreted from the heart, has potent diuretic, natriuretic and vasorelaxant activities, has been used clinically in management of acute decompensated congestive heart failure (Colucci et al. 2000). Recent studies indicate that natriuretic peptide can attenuate the development of irreversible ischemic injury. BNP administration can reduce the infarct size during ischemia and reperfusion in a concentration-dependent fashion in rat heart (Ren et al. 2007). Furthermore, BNP plays a protective role that is mediated in a postconditioning-like manner during reperfusion in isolated rat hearts (Burley and Baxter 2007).

However, the molecular mechanism of BNP-induced

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cardioprotection during ischemia/reperfusion (I/R) is not yet elucidated completely. Therefore, in the present study, we observed the effect of BNP on the infarct size and apoptotic cell death, and discussed the hypothesis that BNP reduces infarct size by attenuating induced apoptotic cell death during reperfusion.

Materials and Methods

Animals

Thirty-six healthy male rabbits, weighing between 2.2–2.7 kg, were used in this study. Animals were purchased from the Center of Experimental Animal in Medical College of Wuhan University. This project was approved by the committee of experimental animals of Wuhan University, and the procedures were carried out according to the routine animal-care guidelines. All experimental procedures complied with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Surgical procedures and experimental design

All the animals were anaesthetized by injecting 3% pentobarbital sodium (30 mg/kg) via an ear vein. After insertion of an endotracheal tube, the animals were placed on positive pressure ventilation with room air. A polyethylene catheter was inserted into the aorta through the right carotid artery for continuously monitoring aorta blood pressure via a transducer attached to a multichannel recorder. Limb leads were used for electrocardiogram monitoring. The chest was opened via a left thoracotomy in the fourth intercostal space and the heart was exposed after pericardiotomy. A 5.0-silk suture was passed through the myocardium around the major marginal branch of the left circumflex coronary artery 7–8 mm from its origin and then ligated. Myocardium ischemia was confirmed by regional cyanosis and electrocardiographic change (Over two ST-segment elevation ≥ 0.1 mV). After 45 min, the ligature was released and the myocardium was reperfused for 180 min.

The animals were randomly divided into three groups ($n = 12$ per group): sham-operated control, untreated reperfusion, and treated with BNP. The sham-operated control group received a left thoracotomy without ligation of the left circumflex coronary artery. The reperfusion group and BNP group received physiological saline and BNP (0.01 $\mu\text{g/kg/min}$, recombinant human brain natriuretic peptide, Tibet Rhodiola Pharmaceutical Holding Company, Chendu, China) treatments respectively 5 min before reperfusion. Physiological saline and BNP were intravenously infused at the rate of 1 ml/kg/hour throughout the reperfusion period.

Serum assays

Blood samples were taken four times, namely, before ligation, 45 min after ligation, 60 min and 180 min after reperfusion. Blood was centrifuged at 3000 rpm, for 10 min at 4°C and the serum was separated and kept in microtubes and stored at –20°C until assay. Plasma creatine kinase (CK) and lactate dehydrogenase (LDH) activities were measured by using commercial kits (Beijing Kemeidongya Biotechnology Ltd., Beijing, China). All these enzymes were expressed as U/L and were assayed in duplicates in a blinded manner.

Hemodynamic monitoring assessment

The hemodynamic assessment included heart rate (HR) and mean arterial blood pressure (MABP). These parameters were contin-

uously monitored throughout the entire experimental protocol. The HR and MABP were sampled and digitally processed via a hemodynamic analyzing system (LEAD2000B; Jinjiang Ltd., Chengdu, China).

Preservation of hearts

After the experiment, the hearts were excised rapidly and were washed with 0.01 M phosphate buffered saline (PBS). Then, the hearts were fixed with 10% phosphate-buffered formalin and paraffin embedded. For Western blot analysis, the risk area was separated. After being weighed, the tissues were stored at –70°C until assay.

Measurement of infarct and risk area

In each group, six rabbits were used for measuring the infarct and risk area. At the end of each experiment, the coronary artery was religated and 2% Evans blue (Sigma, St Louis, MO, USA) solution 1 ml/kg was injected via the right jugular vein until the eyes turned blue. The hearts were then excised, and washed with PBS. The presence of Evans blue was used to identify the left ventricular tissue that was not subjected to regional ischemia (perfused area). For measuring the infarct size, the fresh hearts (risk area) were cut into 2-mm-thick transverse slices and were incubated in 1% triphenyltetrazolium chloride (TTC; Sigma, St Louis, MO, USA) for 40 min at 37°C. TTC demarcates the non-infarcted myocardium within the risk area in a brick red color, and the irreversibly injured tissue in pale white (infarct area). Then the slices were immersed in 10% formalin. The samples were weighed in a blinded manner from three portions (perfused area, risk area, and infarct area) in the left ventricular cardiac tissue. The percentage of infarcted myocardial tissue and risk tissue in the left ventricular cardiac tissue was calculated.

Apoptosis in myocardial cells detected by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end-labeling (TUNEL)

Four longitudinal sections of ischemic regions were cut and fixed in 4% paraformaldehyde for 20 hrs at room temperature. Apoptotic cardiomyocytes were detected using immunohistochemical method (Roche Diagnostics GmbH, Mannheim, Germany). The digoxigenin-conjugated dUTP was incorporated to the ends of DNA fragments by terminal deoxynucleotidyl transferase (TdT). The signal of TdT-mediated dUTP nick end labeling was then detected by an anti-fluorescein antibody conjugation with alkaline phosphatase, a reporter enzyme that catalytically generates a red-colored product from vector red substrate. For each section, 10 microscopic fields were randomly chosen by using an eyepiece grid ($\times 200$ magnification). In each field, cells were calculated and the percentage of apoptotic cardiomyocytes was counted. The apoptotic index was determined (*i.e.*, number of positively stained apoptotic myocytes / total number of myocytes counted $\times 100\%$). The assays were performed in a blinded manner.

Western blot analysis of Bcl-2 and Bax proteins

Total proteins were extracted from normal and ischemic zones of left ventricle (LV). The myocardium sample (100 mg) was placed in 500- μl lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS] in 1 $\times$ PBS) containing inhibitors (Roche Diagnostics GmbH, Mannheim, Germany), and homogenized at 4°C for 20 seconds, and incubated on ice for 2–3 hrs. After that, the sample was centrifuged twice at 16,000 g for 20 min. Protein concentration was measured by Bicinchoninic Acid assay (Beyotime Institute

of Biotechnology, Shanghai, China). Total proteins (80 μ g) were mixed with loading buffer (5% beta-mercaptoethanol, 0.05% bromophenol blue, 75 mM Tris-HCl, pH 6.8, 2% SDS and 10% glycerol), boiled for 2 min, and loaded onto 4-20% gradient SDS-polyacrylamide gel using Mini Protean II Dual Stab Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins were transferred to nitrocellulose filters in the presence of glycine/methanol transfer buffer (20 mM Tris base, 0.15 M glycine, 20% methanol) in Mini Protean II Transfer system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Nitrocellulose filters were blocked with 5% milk in 1 $\times$ TBS-T buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween-20) for 1 h at room temperature. Membrane was subsequently exposed to polyclonal anti-rabbit Bcl-2 and Bax (1 : 1,000, Cell Signaling Technology, Beverly, MA, USA) in 5% milk with TBS-T for 1 h, respectively, followed by a secondary antibody conjugated with horseradish peroxidase (1 : 4,000, Jackson Immuno Research Laboratories, West Grove, PA, USA) and visualize peroxidase reaction products. Bcl-2 protein was detected as a 28-kDa band, and Bax protein was detected as a 20-kDa band using molecular weight marker bands. The intensities of bands were determined with the NIH image program.

Caspase-3 assay

Caspase-3 activity assays were measured by using commercialized caspase-3 activity kit (Beyotime Institute of Biotechnology, Shanghai, China). Tissues from the risk area of the heart were homogenized in lysis buffer. The lysates were centrifuged at 16,000 rpm at 4°C for 15 min, and protein concentrations in the supernatants were obtained. Immediately after the extracts were used, the protein concentrations were stored at -70°C. Aliquots of protein (10 μ l) were incubated with 10- μ l caspase-3 substrate (2 mM Ac-DEVD-pNA; Calbiochem, La Jolla, CA, USA) in a total volume of 100 μ l at 37°C for 2 hrs. The colorimetric release of *p*-nitroaniline from the Ac-DEVD-pNA substrate was recorded at 405 nm.

Data analysis and statistics

All data are expressed as means $\pm$ standard deviation (S.D.). The Kolmogorov-Smirnov test was applied to test for a normal distribution. Differences between control and experimental groups were determined by using a one-way or two-way ANOVA for repeated measures. Differences between groups were determined by using Bonferroni's post hoc test. A value of $P < 0.05$ was considered significant differences.

Results

Changes of hemodynamics

HR and MABP are shown in Table 1. There was no significant difference in the baseline levels of these parameters in the three groups. Compared with the baseline, HR and MABP decreased significantly during myocardial ischemia, and reperfusion. HR and MABP remained relatively stable during the reperfusion period. There was no significant difference in the HR and MABP among three groups during the I/R period.

Measurement of myocardial injury in I/R

Measurement of serum levels of CK and LDH during I/R was shown in Fig. 1. In untreated reperfusion group and BNP group, the level of serum CK and LDH slightly increased during ischemia and increased dramatically after reperfusion ($P < 0.01$). Compared with the reperfusion group, these reperfusion-related CK and LDH elevations were markedly attenuated when treated with BNP. At the end of 3-h reperfusion, serum CK and LDH levels of treated with BNP group decreased by 23.3% ($P < 0.05$) and 20.7% ($P < 0.05$) compared with untreated reperfusion group.

There was no significant difference in the ratio of risk areas between untreated reperfusion group and treated with BNP group ($P > 0.05$). The ratio of I/R-induced infarct size was significantly reduced in treated with BNP group ($9.0 \pm 2.2\%$ in BNP group vs $16.2 \pm 3.1\%$ in reperfusion group; $P < 0.01$). Treatment with BNP induced a 44% relative reduction in infarct size in our experiment. (Fig. 2).

Changes of TUNEL

Quantitative analysis of DNA fragmentation was performed by using the TUNEL method at the single-cell level. TUNEL-positive cardiomyocyte nuclei are shown in Fig. 3. No TUNEL-positive cardiomyocyte was detected in sham-operated control group. I/R induced percentage of TUNEL-positive cardiomyocytes was $12.9 \pm 2.5\%$ in untreated reperfusion group. Treatment with BNP, significantly decreased the percentage of apoptotic cells to $4.7 \pm 1.9\%$

Table 1. Hemodynamic parameters (Heart rate and Mean arterial pressure) before and during myocardial I/R.

Time (min)	Baseline	I-45	R-60	R-120	R-180
Heart rate (bpm)					
Sham-operated	257 $\pm$ 10				
reperfusion	255 $\pm$ 10	236 $\pm$ 8*	228 $\pm$ 13*#	214 $\pm$ 11*#	210 $\pm$ 11*#
BNP	250 $\pm$ 9	224 $\pm$ 9*	221 $\pm$ 12*#	208 $\pm$ 13*#	217 $\pm$ 15*#
Mean arterial blood pressure (mmHg)					
Sham-operated	103 $\pm$ 7				
reperfusion	106 $\pm$ 8	94 $\pm$ 6*	83 $\pm$ 6*#	82 $\pm$ 5*#	79 $\pm$ 5*#
BNP	110 $\pm$ 10	98 $\pm$ 7*	80 $\pm$ 6*#	78 $\pm$ 5*#	76 $\pm$ 4*#

Sham-operated, sham-operated control group; reperfusion, untreated reperfusion group; BNP, treated with BNP group. I-45, ischemia 45 min; R-60, reperfusion 60 min; R-120, reperfusion 120 min; and R-180, reperfusion 180 min. $N = 12$ /group. * $P < 0.01$, vs. baseline; # $P < 0.01$, vs. I45 within same group.

Fig. 1. Changes of CK and LDH serum levels in different experimental groups.
 (a) Serum levels of CK in pre-ischemia (Pre-I), ischemia 45 min (I-45), reperfusion 60 min and 180 min (R-60, R-180) in three groups.
 (b) Serum levels of LDH in pre-ischemia (Pre-I), ischemia 45 min (I-45), reperfusion 60 min and 180 min (R-60, R-180) in three groups.
 Sham-operated, sham-operated control group; reperfusion, untreated reperfusion group; and BNP, treated with BNP group. Bars represent mean values $\pm$ s.d. ($n = 12$).
 # $P < 0.01$ vs. Sham-operated control group; * $P < 0.05$ vs. untreated reperfusion group.

Fig. 2. Tissue wet weight of risk area and infarct size as a percentage of the left ventricle (% of LV).
 No significant difference in the ratio of risk areas between untreated reperfusion group and treated with BNP group.
 Administration of BNP at reperfusion significantly reduced infarct size compared with untreated reperfusion group.
 Reperfusion, untreated reperfusion group; and BNP, treated with BNP group. Bars represent mean values $\pm$ s.d. ($n = 6$);
 * $P < 0.01$ vs. untreated reperfusion group.

Fig. 3. Detection of apoptotic myocytes using the terminal transferase UTP nick end labeling (TUNEL) techniques. TUNEL detection in untreated reperfusion group (a); treated with BNP group (b); and TUNEL positive cells ratio (c). Treatment with BNP at reperfusion significantly decreased the number of TUNEL positive cells vs untreated reperfusion group. Bars represent mean values $\pm$ S.D. ($n = 6$). $P < 0.05$. Arrow indicates TUNEL positive cells (red-staining). Magnification $\times 200$, Bar = $100 \mu\text{m}$.

Fig. 4. Western blot analysis of Bcl-2 and Bax proteins after I/R.

Original representative western blots are reported in the upper panels and relative levels of Bcl-2 and Bax in the lower panels. Immunodetection of β -actin with monoclonal anti-actin antibody was performed as an internal control. BNP significantly increased expression of Bcl-2 and decreased expression of Bax proteins, respectively. Bars represent mean $\pm$ S.D. ($n = 6$); * $P < 0.05$ vs. sham-operated group, # $P < 0.05$ vs. untreated reperfusion group.

($P < 0.01$), and exerted the antiapoptotic effect.

Expression of Bcl-2 and Bax proteins

Expression of Bcl-2 and Bax proteins was visualized by Western blot analysis (Fig. 4). Bcl-2 was clearly expressed in sham-operated control group. I/R significantly decreased Bcl-2 level and increased Bax level in untreated

reperfusion group compared with sham-operated control group, respectively ($P < 0.05$). However, the degree of decrease in Bcl-2 level was attenuated in the BNP-treated group ($P < 0.05$), although the Bcl-2 level in the BNP group was still lower than the sham control. In contrast, upregulated Bax level in the reperfusion group was significantly decreased in the BNP group ($P < 0.05$), suggesting that

Fig. 5. Caspase-3 activity.

Ischemia-reperfusion increased caspase-3 activity by 3 folds compared with sham-operated control group. BNP treatment reduced caspase-3 activity significantly. Bars represent mean values $\pm$ S.D. ($n = 6$); * $P < 0.05$ vs. sham-operated control group, # $P < 0.05$ vs. untreated reperfusion group.

inhibition of apoptosis by BNP was related to the modulation of the expression of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax.

Assay of Caspase-3 activity

Caspase-3 activation is unique to apoptosis as it does not occur in other forms of cell death and provides strong evidence for the presence of apoptosis. I/R increased caspase-3 activity by about 3 folds compared with sham-operated control group (Fig. 5). Increased caspase-3 activity was significantly attenuated in the BNP group, suggesting the inhibition of apoptosis by attenuated caspase-3 activity.

Discussion

The principal findings of present study were that (1) BNP infused shortly before the onset of reperfusion inhibited the increased levels of serum CK and LDH and reduced infarct size in *in vivo* rabbit hearts. (2) BNP administration decreased TUNEL-positive ratio, caspase-3 activity and increased the expression of Bcl-2, downregulated the Bax level. Our data showed that the cardioprotective effects of BNP were associated with attenuated myocardial apoptosis after I/R in the heart of rabbits.

Paradoxical results in myocyte death as the act of reperfusion itself have been well documented in recent years. Pharmacological agents administered at the time of reperfusion aim to attenuate reperfusion injury as an adjunct to current reperfusion therapy strategies, should lead to improved reperfusion benefit.

Increased levels of CK and LDH are well-known diagnostic markers of myocardial injury. A significant correlation between increased levels of cytosolic enzymes and the extent of myocardial damage after acute myocardial infarction has been studied (Roe et al. 1977). In our experiment, BNP induced a 44% relative reduction in infarct size (TTC staining) confirmed by reduced CK and LDH activity.

Marked elevations in the levels of these enzymes in the serum of the reperfusion group indicated severe myocardial cell membrane damage. Treatment with BNP significantly reduced the levels of these enzymes compared with the reperfusion group, suggesting that BNP can protect myocardial cells.

Increasing studies have demonstrated that reperfusion induced or accelerated myocardial apoptosis in different animal species and human, especially during early stage of myocardial infarction (Saraste et al. 1997; McCully et al. 2004). Both necrosis and apoptosis have been linked with I/R and may contribute independently to infarct size. Recent studies have shown that inhibition of apoptosis, as a therapeutic target, may reduce infarct size and improve regional contractile dysfunction during reperfusion (Wang et al. 2004; Kin et al. 2006; Li et al. 2007). Consistent with these previous reports, in the present study, we found that treatment with BNP shortly before the onset of reperfusion significantly reduced infarct size at 3 hours of reperfusion and reduced the number of apoptotic cells in the perinecrotic myocardium, providing direct evidence that limitation in extension of infarct size can be achieved by anti-apoptotic effect.

The mitochondrial permeability transition pore (mPTP), a protein complex that spans both membranes, known to play a central role in mediating myocyte apoptosis following I/R injury (Adrain and Martin 2001; Crompton et al. 2002). The opening of mPTP during reperfusion, resulted in the releases of intermembrane proteins that facilitate the apoptotic signal, most notably was cytochrome *c*. Once released, cytochrome *c* leads to the formation of the apoptosome, a complex comprised of apoptotic protease-activating factor-1 (Apaf-1), procaspase-9 and ATP, which permits the autoactivation of procaspase-9 that in turn facilitates caspase-3 activation. Active caspase-3 activates the caspase activated DNase, leading to oligonucleosomal DNA frag-

mentation (Baliga and Kumar 2003).

It has been demonstrated that the Bcl-2 family proteins play a key role in regulating apoptotic cell death. In response to an apoptotic stimulus, the pro-apoptosis protein Bax translocate to the mitochondria (Tsuruta et al. 2002), where it induces mitochondrial cytochrome *c* release (Marzo et al. 1998). The anti-apoptosis protein Bcl-2 may directly or indirectly promote mPTP closure and abrogate the mitochondrial translocation of Bax (Shimizu et al. 2000; Murphy et al. 2000).

The balance of pro- to antiapoptotic Bcl proteins has been shown to shift toward proapoptosis in various pathological processes. In our study, I/R downregulated Bcl-2 and upregulated Bax proteins expression, in agreement with previous report (Zhao et al. 2003). Treatment with BNP at early reperfusion was related to reciprocal alterations in upregulated level of the anti-apoptotic protein Bcl-2 and downregulated pro-apoptotic protein Bax, which inhibit mPTP opening, and decreases the release of cytochrome *c*, thus attenuates the downstream caspase-3 activation. As far as we know, our results showed, for the first time, that BNP significantly inhibited apoptosis caused by I/R injury, which was proved by reduced caspase-3 activation and TUNEL positive cells.

BNP binds to its receptor, natriuretic peptide receptor-A (NPR-A), which is a membrane-associated particulate guanylate cyclase receptor (pGC). pGC catalyzes the formation of cyclic guanosine 3,5-monophosphate (cGMP), cGMP-dependent protein kinase (PKG) is a major intracellular signaling target of cGMP (Potter et al. 2006). Activation of cGMP/PKG signaling pathway has been proposed to be important in inhibition of apoptosis. For example, nitric oxide (NO) increased expression of the anti-apoptotic protein bcl-2, and inhibited mPTP formation through the cGMP/PKG signalling cascade (Razavi et al. 2005). Kim et al also showed that NO inhibited caspase-3 activation via cGMP-dependent mechanism (Kim et al. 1997a, 1997b). NPR-A/cGMP/PKG signaling pathway appear to be the upstream signal transduction that BNP affects Bax, Bcl-2 and caspase-3.

In summary, our data contributed to a better understanding that BNP administered shortly before the onset of reperfusion can reduce cardiomyocyte necrosis and also inhibit I/R-induced myocardial apoptosis. BNP may be an ideal pharmacological agent applied as adjuvant therapy to current myocardial reperfusion strategies for patients presenting with an acute myocardial infarction.

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