

Pharmacological preconditioning and postconditioning with nicorandil attenuates ischemia/reperfusion-induced myocardial necrosis and apoptosis in hypercholesterolemic rats

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Abstract. Pharmacological preconditioning and postconditioning may reduce myocardial necrosis and apoptosis during ischemia/reperfusion (I/R), however, hypercholesterolemia interferes with the associated cardioprotective mechanisms. The present study investigated whether pharmacological preconditioning and postconditioning with nicorandil could attenuate myocardial necrosis and apoptosis induced by I/R in hypercholesterolemic rats, and explored the possible mechanisms involved. Male Wistar rats (n=160) were fed normal (normocholesterolemic group, n=10) or high-cholesterol (hypercholesterolemic group, n=150) diets for 8 weeks. Hearts harvested from the normal and hypercholesterolemic rats were subsequently placed on modified Langendorff perfusion apparatus and 30-min global ischemia was performed, followed by 120-min reperfusion. Nicorandil (1, 3, 10, 30, 100 $\mu\text{mol/l}$), and mitochondrial adenosine triphosphate-sensitive potassium (mitoKATP) channel blocker 5-hydroxydecanoic acid sodium salt (5-HD) (100 $\mu\text{mol/l}$) or soluble guanylyl cyclase (sGC) blocker 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 $\mu\text{mol/l}$) were perfused for 10 min, prior to ischemia or at the onset of reperfusion. The myocardial infarct size was determined by triphenyltetrazolium chloride staining, and cardiomyocyte apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling staining. In order to investigate the potential mechanisms, the expression levels of caspase-3, B-cell lymphoma-2 (Bcl-2) proteins and Bcl-2-associated X protein (Bax) were measured using western blot analysis. The present study demonstrated that, in hypercholesterolemic rats, pharmacological preconditioning and postconditioning with nicorandil decreased I/R-induced myocardial necrosis and apoptosis in a concentration-dependent

manner. The optimal preconditioning and postconditioning concentration of nicorandil determined to have anti-infarct and anti-apoptosis effects was 30 $\mu\text{mol/l}$, which significantly ($P<0.05$) reduced the infarct size to $14.88\pm3.25\%$ and $15.96\pm3.29\%$, and attenuated the percentage of cardiomyocyte apoptosis to $25.20\pm3.93\%$ and $26.18\pm4.82\%$, respectively, compared with the I/R group. However, the cardioprotective effects of nicorandil were partially suppressed by cotreatment with 5-HD or ODQ. Western blot analysis demonstrated that pharmacological preconditioning and postconditioning with nicorandil significantly downregulated caspase-3 and Bax expression, and upregulated Bcl-2 expression compared with the I/R group ($P<0.05$). The results of the present study suggest that pharmacological preconditioning and postconditioning with nicorandil may protect hypercholesterolemic hearts against I/R-induced necrosis and apoptosis; and the cardioprotective effects of nicorandil may be due to the dual pharmacological mechanisms of opening the mitoKATP channels and a nitric oxide/sGC-dependent mechanism, and regulation of the expression of caspase-3, Bax and Bcl-2.

Introduction

Ischemic heart disease (IHD) is the most common cause of morbidity and mortality worldwide. Timely effective reperfusion therapy is the major therapeutic strategy to salvage the myocardium from tissue injury following prolonged ischemia; however, the beneficial effects of this treatment can be compromised by ischemia/reperfusion (I/R) injury (1). Previous studies have demonstrated that I/R injury can be suppressed through the application of various mechanical and pharmacological strategies (2-6). For example, brief episodes of sublethal ischemia and reperfusion before sustained ischemia, or at the onset of reperfusion, render the heart resistant to I/R injury. These ischemic conditioning phenomena are termed ischemic preconditioning (IPC) and ischemic postconditioning (IPost), respectively (3,4). Following identification of this phenomena, it became clear that when administered prior to the onset of sustained myocardial ischemia, or at the initial onset of reperfusion, certain pharmacological agents, such as adenosine, opioid agonists and bradykinin, could mimic the cardioprotective phenomena exhibited by IPC and IPost. Such treatment was termed 'pharmacological preconditioning' and 'pharmacological postconditioning' (5,6). This was significant,

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as pharmacological agents can be more readily applied in clinical practice as a means of protecting the heart against I/R injury, rather than inducing ischemia directly.

Although IPC, IPost and their mimetic agents have been shown to reduce I/R injury in animal models, a variety of pharmacological agents have failed to demonstrate cardioprotective effects in human clinical trials (7-9). There may be various reasons for this discrepancy. One important factor is that the animal studies were performed on healthy animals, whereas humans who are treated with cardioprotective agents tend to have various co-morbidities, such as hypercholesterolemia, diabetes, obesity and aging, which may modify the myocardial responses to I/R and cardioprotective agents (7-9). Hypercholesterolemia is commonly found in patients with cardiovascular disease and is considered to be a risk factor (10), as previous studies have shown that patients with a hypercholesterolemic myocardium are vulnerable to I/R injury (10-15). Previous studies have demonstrated that this vulnerability may be associated with the following: Increased expression of proapoptotic proteins, decreased expression of prosurvival proteins, increased myocardial inflammatory responses and oxidative/nitrative stress, inhibition of nitric oxide (NO) synthesis, and the impaired opening of mitochondrial adenosine triphosphate-sensitive potassium (mitoKATP) channels, in response to myocardial I/R (10-15). Furthermore, hypercholesterolemia abrogates the cardioprotection afforded by IPC, IPost and specific pharmacological agents, such as sevoflurane (16-18), however the exact underlying mechanisms are yet to be fully elucidated.

Nicorandil, a hybrid KATP channel opener and nitrate compound, is used clinically for the treatment of angina pectoris (19). A previous randomized and placebo-controlled trial, termed the 'Impact Of Nicorandil in Angina' (20), demonstrated that nicorandil reduced the incidence of major cardiovascular events in patients with stable angina. Nicorandil is not only an antianginal; previous studies have demonstrated that it may also exert potentially cardioprotective effects on I/R myocardium, some of which are likely due to its ability to mimic IPC by opening mitoKATP channels (21-23). However, previous studies have shown that nicorandil, as a NO donor, may inhibit oxidative stress- or hypoxia-induced apoptosis in cardiomyocytes, through the activation of mitoKATP channels and a NO/soluble guanylyl cyclase (sGC)-dependent mechanism (24,25). Furthermore, nicorandil has been shown to be associated with the regulation of apoptosis-related proteins (25,26).

Although nicorandil has been demonstrated to reduce I/R injury in healthy animals and cardiomyocytes, whether nicorandil has a cardioprotective effect on hypercholesterolemic animals during I/R remains unknown. Therefore, the aim of the present study was to determine whether pharmacological preconditioning and postconditioning with nicorandil could attenuate myocardial necrosis and apoptosis induced by I/R in the isolated hypercholesterolemic hearts of rats, and, if so, to explore the possible protective mechanisms involved.

Materials and methods

Ethics statement. The present study conformed to the Guide for the Care and Use of Laboratory Animals published by

the National Institutes of Health (NIH publication no. 85-23, revised 1996), and the protocol was approved by the China Medical University (Liaoning, China) institutional Ethics Committee.

Induction of experimental hypercholesterolemia. A total of 160 healthy male Wistar rats (6 weeks old, ~100-120 g) were obtained from the Department of Experimental Animals, China Medical University). The rats were housed in polypropylene cages with a 12-h light-dark cycle at 22±1°C. The rats were divided into two groups, the normocholesterolemic group (n=10), fed on a normal diet for 8 weeks, and the hypercholesterolemic group (n=150), fed on a high-cholesterol diet for 8 weeks. The high-cholesterol diet consisted of 1.5% cholesterol, 5% egg yolk powder, 10% lard, 0.5% sodium cholate, 3% sugar and 80% normal feed (Beijing Keao Xieli Feed Co., Ltd., Beijing, China). Wistar rats were selected for the present study because they had previously demonstrated a moderate increase in serum cholesterol level after receiving a high-cholesterol diet, without developing substantial atherosclerosis (18). The normocholesterolemic group contained fewer animals as it was set up to determine that the hypercholesterolemia model was successfully established. Following the 8-week feeding period, blood samples were collected via the caudal vein for serum lipid analysis in order to determine the success of the hypercholesterolemic models.

Chemicals and therapeutic agents. Nicorandil, 5-hydroxydecanoic acid sodium salt (5-HD), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, MO, USA); *in situ* cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany); rabbit monoclonal caspase-3 (ab32351), rabbit polyclonal B-cell lymphoma-2 (Bcl-2; ab7973), and rabbit polyclonal Bcl-2-associated X protein (Bax; ab7977) antibodies (Abcam, Cambridge, MA, USA); mouse monoclonal β -actin (TA-09), goat anti-rabbit IgG (ZB-2301) and goat anti-mouse IgG (ZB-2305) antibodies (ZSGB-BIO, Beijing, China).

Isolated perfused heart preparation. The rats were anesthetized immediately following the 8-week feeding period by intraperitoneal injection with 10% chloral hydrate (4 ml/kg; Sinopharm Chemical Reagent Co., Ltd., Shenyang, China) and 1,500 U/kg heparin was intravenously administered in order to prevent the formation of intracoronary clots. The hearts were rapidly excised and immediately immersed in ice-cold heparinized, modified Krebs-Henseleit (K-H) solution (NaCl, 127 mmol/l; NaHCO₃, 17.7 mmol/l; KCl, 5.1 mmol/l; CaCl₂, 1.5 mmol/l; MgCl₂, 1.26 mmol/l; D-glucose, 11 mmol/l; pH 7.4) at 4°C for trimming. The hearts were subsequently mounted on Langendorff perfusion apparatus and retrogradely perfused, via the aorta, with recirculating K-H solution saturated with 95% O₂ and 5% CO₂ at 37°C. The hearts were maintained in a thermostatic chamber at 37°C, with perfusion maintained at a constant pressure of 75 mmHg. In order to measure cardiac pressure changes, a fluid-filled latex balloon was connected to a pressure transducer, inserted into the left ventricle (LV) via the left atrium, and inflated to an initial LV end-diastolic pressure of 8-10 mmHg.

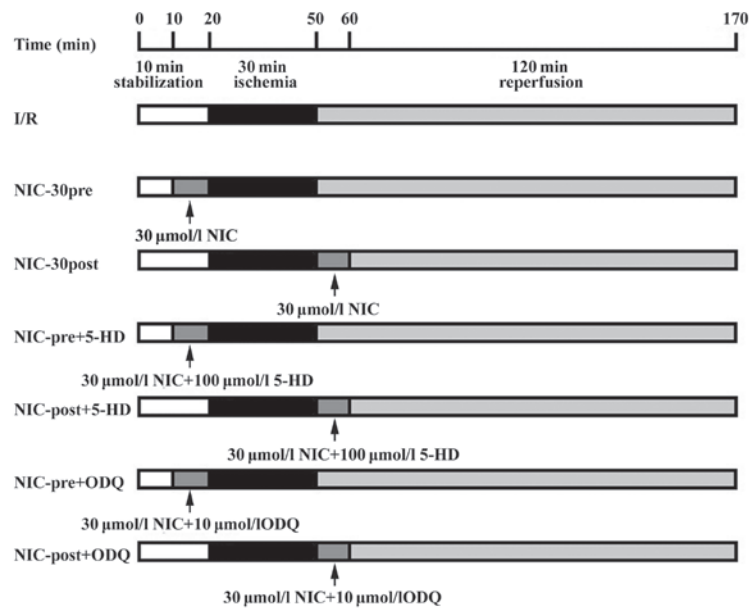


Figure 1. Representation of experimental protocol. I/R, ischemia/reperfusion; NIC, nicorandil; pre, preconditioning; post, postconditioning; HD, 5-hydroxydecanoic acid sodium salt; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one.

Experimental protocol. As outlined in Fig. 1, all hypercholesterolemic rats were randomized into seven study groups. In all groups, the isolated hearts were perfused with K-H solution and stabilized for 10 min. The I/R control group (n=10) underwent 30 min global ischemia, followed by 120 min reperfusion with no pharmacological intervention. To determine the optimal concentration for pharmacological preconditioning, the nicorandil preconditioning group (NIC-pre, n=50) were perfused with five different concentrations of nicorandil (1, 3, 10, 30, 100 $\mu\text{mol/l}$; n=10 per subgroup) prior to global ischemia for 10 min. The nicorandil postconditioning group (NIC-post, n=50) were perfused with five different concentrations of nicorandil (1, 3, 10, 30, 100 $\mu\text{mol/l}$; n=10 per subgroup) for 10 min at the onset of reperfusion, in order to determine the optimal concentration for pharmacological postconditioning. To further examine the pharmacological mechanisms of nicorandil in hypercholesterolemic hearts, four additional groups underwent cotreatment with 100 $\mu\text{mol/l}$ of the mitoKATP channel blocker, 5-HD (NIC-pre + 5-HD, n=10; NIC-post + 5-HD, n=10), or 10 $\mu\text{mol/l}$ of the sGC blocker, ODQ (NIC-pre + ODQ, n=10; NIC-post + ODQ, n=10). The control I/R group were perfused with K-H solution prior to global ischemia for 10 min and at the onset of reperfusion for 10 min to match the corresponding time in the other groups.

Measurement of infarct size. The infarct size was determined as previously described (17). Briefly, after 120 min of reperfusion, the hearts were harvested. The hearts were partially frozen for 60 min at -20°C , sectioned from apex to base into 3 mm sections, incubated in 1% TTC solution (TTC dissolved in $\text{NaH}_2\text{PO}_4/\text{NaHPO}_4$ buffer, pH 7.4) for 5 min at 37°C , and unstained tissue was subsequently separated from stained tissue by an independent observer. Successful staining of the tissue indicated that the cells were still viable, whereas the unstained tissue contained the dead cells. Therefore, the unstained mass was expressed as a percentage of the total left

ventricular mass, which was defined as the risk area since a global ischemia was induced.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay for apoptosis. Apoptotic cardiomyocytes were detected using an *in situ* cell death detection kit (Roche Diagnostics GmbH), according to the manufacturer's instructions. Following 30 min of reperfusion, the hearts were removed and cut into 4 μm thick, formalin-fixed, paraffin-embedded sections. The sections were subsequently deparaffinized with xylene and rehydrated with graded alcohol (Sinopharm Chemical Reagent Co., Ltd.). A total of 20 mg/l proteinase K (Roche Diagnostics GmbH) was applied to the section for 10 min to ensure optimal proteolysis, prior to supplementation with 3% hydrogen peroxide in methanol for 30 min to inhibit the endogenous peroxidase. The tissue sections were incubated with terminal deoxynucleotidyl transferase enzyme in a humidified chamber at 37°C for 60 min. Finally, streptavidin horseradish peroxidase was bound to the biotinylated nucleotides and peroxidase activity was revealed in each section by the application of a stable chromogen diaminobenzidine. This technique caused the apoptotic nuclei to be stained dark brown, whereas the total nuclei were counterstained with hematoxylin. Three sections from each myocardial sample were randomly selected and 10 microscopic fields (magnification, x400; BX51 microscope, Olympus Tokyo, Japan) per section were evaluated by two independent blind observers. The percentage of cardiomyocyte apoptosis was calculated as follows: (Number of apoptotic cardiomyocytes / total number of cardiomyocytes counted) \times 100%.

Western blot analysis. Following 30 min of reperfusion, the hearts were homogenized in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) prior to protein quantification using the BCA method (11). Equal quantities of protein from each sample were

then separated by SDS-PAGE and transferred onto polyvinylidene difluoride-plus membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After blocking with 5% bovine serum albumin, the membranes were incubated overnight at 4°C with the following primary antibodies: Caspase-3 (1:5,000), Bax (1:1,500), Bcl-2 (1:1,500) and β -actin (1:2,000). The membranes were subsequently washed three times with Tris-buffered saline and Tween-20 (TBST; Beyotime Institute of Biotechnology) and incubated with the corresponding goat anti-rabbit IgG and goat anti-mouse IgG (1:5,000), prior to conjugation to horseradish peroxidase at room temperature for 2 h. The membranes were once again washed three times with TBST. Signals were detected using an enhanced chemiluminescence kit (Beyotime institute of biotechnology) and relative densitometry was performed using a computerized software package (Image J, version 1.63; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. The quantitative data are expressed as the mean \pm standard deviation. One-way analysis of variance was applied to analyze the differences between the groups. If the difference was deemed statistically significant, a Student-Newman-Keuls post hoc test was applied in a further pairwise comparison. Statistical analyses were performed using SPSS statistical software (version 17.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of a high-cholesterol diet on serum lipid levels. As outlined in Table I, the levels of total cholesterol (TC; 2.11 ± 0.30 vs. 1.21 ± 0.31 mmol/l, $P < 0.05$) and low density lipoprotein cholesterol (LDL-C; 0.84 ± 0.28 vs. 0.34 ± 0.14 mmol/l, $P < 0.05$) were significantly increased in rats fed with high-cholesterol diet, compared with those fed a normal diet; whereas the levels of high density lipoprotein cholesterol (HDL-C; 0.99 ± 0.23 vs. 1.02 ± 0.19 , $P = 0.715$) were not significantly different between the two groups.

Effects of preconditioning and postconditioning with nicorandil on myocardial infarct size. Staining with TTC revealed the infarct size of the hearts 120 min after reperfusion. As demonstrated in Fig. 2A, preconditioning with nicorandil (1, 3, 10, 30, 100 μ mol/l) reduced the infarct size in a concentration-dependent manner in the hypercholesterolemic hearts. In particular, preconditioning with 30 μ mol/l nicorandil significantly decreased the infarct size to $14.88 \pm 3.25\%$ compared with $44.04 \pm 2.70\%$ in the I/R group ($P < 0.05$), whereas 100 μ mol/l nicorandil preconditioning did not reduce the infarct size further. Similarly, as shown in Fig. 2B, nicorandil (1, 3, 10, 30, 100 μ mol/l) postconditioning also reduced the infarct size in a concentration-dependent manner in the hypercholesterolemic hearts, and postconditioning with 30 μ mol/l nicorandil significantly reduced the infarct size to $15.96 \pm 3.29\%$, with 100 μ mol/l nicorandil unable to further reduce the infarct size. Therefore, these results suggested that the optimal preconditioning and postconditioning concentration of nicorandil to reduce infarct size is 30 μ mol/l.

Table I. Effects of a high-cholesterol diet on serum lipid level.

Diet	TC (mmol/l)	LDL-C (mmol/l)	HDL-C (mmol/l)
Normal	1.21 ± 0.31	0.34 ± 0.14	1.02 ± 0.19
High-cholesterol	2.11 ± 0.30^a	0.84 ± 0.28^a	0.99 ± 0.23

Data are expressed as the mean \pm standard deviation. Normal diet, $n = 10$; High-cholesterol diet, $n = 150$. $^aP < 0.05$ vs. normal diet group. TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol.

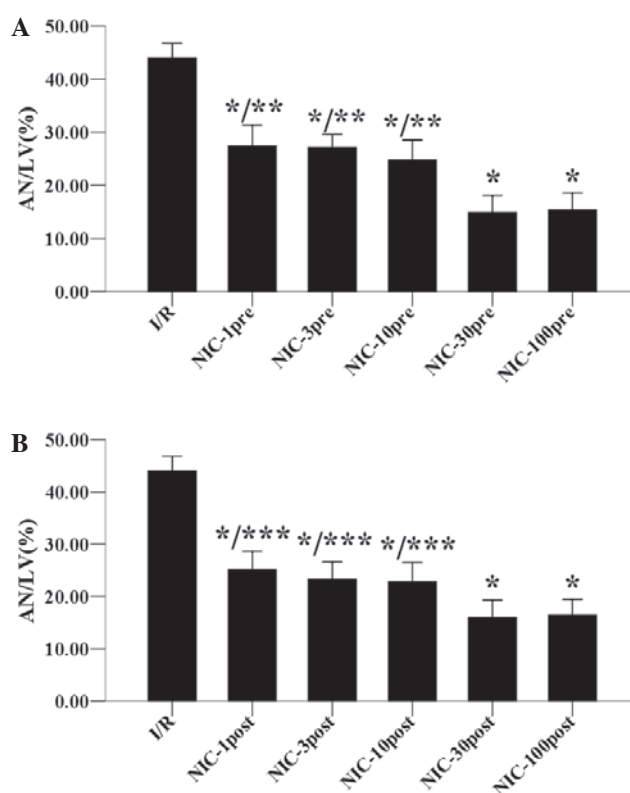


Figure 2. Concentration-response relationships for (A) preconditioning and (B) postconditioning with different concentrations of NIC (1, 3, 10, 30, 100 μ mol/l) on anti-infarct effects in hypercholesterolemic rat hearts. Data are expressed as the mean \pm standard deviation, $n = 5$ for each. $^*P < 0.05$ vs. I/R group; $^{**}P < 0.05$ vs. NIC-30pre group; $^{***}P < 0.05$ vs. NIC-30post group. AN/LV, area of necrosis/left ventricle; I/R, ischemia/reperfusion; NIC, nicorandil; pre, preconditioning; post, postconditioning.

Effects of preconditioning and postconditioning with nicorandil on cardiomyocyte apoptosis. TUNEL staining was used to measure the cardiomyocyte apoptosis, another form of I/R injury, by revealing apoptotic cardiomyocytes 30 min after reperfusion. As demonstrated in Fig. 3A, in the hypercholesterolemic hearts, nicorandil (1, 3, 10, 30, 100 μ mol/l) preconditioning decreased the percentage of apoptotic cardiomyocytes in a concentration-dependent manner. Furthermore, preconditioning with 30 μ mol/l nicorandil significantly reduced the percentage of apoptotic cardiomyocytes to $25.20 \pm 3.93\%$, and 100 μ mol/l nicorandil preconditioning did not reduce the percentage of apoptotic cardiomyocytes any further. Similarly, as outlined in Fig. 3B,

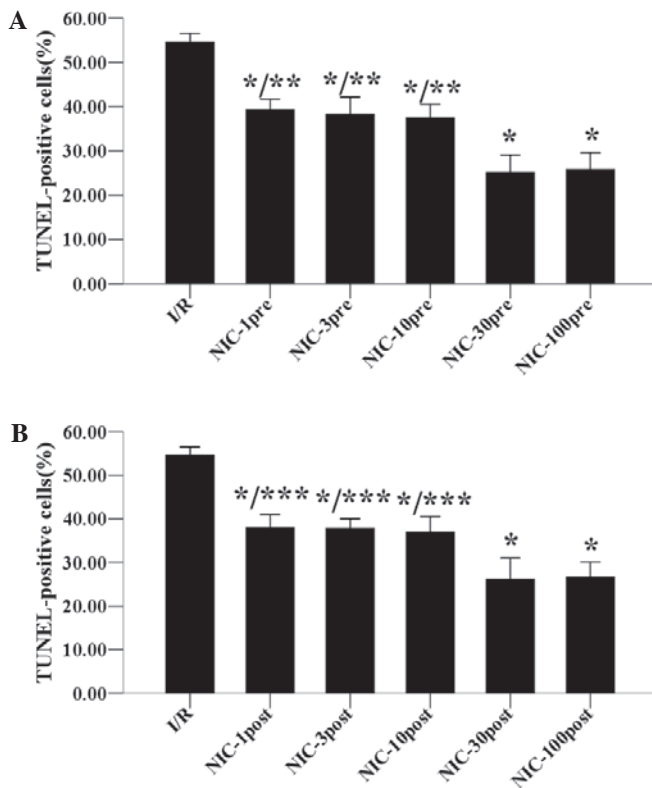


Figure 3. Concentration-response relationships for (A) preconditioning and (B) postconditioning with different concentrations of NIC (1, 3, 10, 30, 100 $\mu\text{mol/l}$) on anti-apoptotic effects in hypercholesterolemic hearts. Data are expressed as the mean \pm standard deviation, $n=5$. * $P<0.05$ vs. I/R group; ** $P<0.05$ vs. the NIC-30pre group; *** $P<0.05$ vs. the NIC-30post group. TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; I/R, ischemia/reperfusion; NIC, nicorandil; pre, preconditioning; post, post-conditioning.

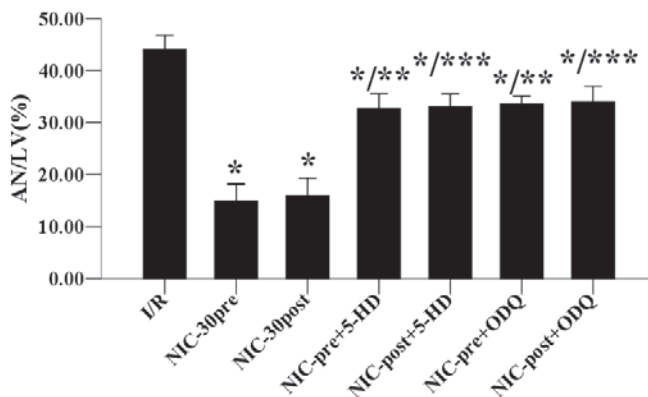


Figure 4. Effects of 5-HD and ODQ on NIC-induced (30 $\mu\text{mol/l}$) inhibition of infarct size in hypercholesterolemic hearts. Data are expressed as the mean \pm standard deviation, $n=5$. * $P<0.05$ vs. I/R group; ** $P<0.05$ vs. NIC-30pre group; *** $P<0.05$ vs. NIC-30post group. AN/LV, area of necrosis/left ventricle; I/R, ischemia/reperfusion; NIC, nicorandil; pre, preconditioning; post, post-conditioning; HD, 5-hydroxydecanoic acid sodium salt; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one.

postconditioning with nicorandil (1, 3, 10, 30, 100 $\mu\text{mol/l}$) also reduced the percentage of apoptotic cardiomyocytes in a concentration-dependent manner in the hypercholesterolemic hearts. Nicorandil (30 $\mu\text{mol/l}$) postconditioning significantly decreased the percentage of apoptotic cardiomyocytes to

26.18 \pm 4.82%, whereas 100 $\mu\text{mol/l}$ nicorandil postconditioning was unable to reduce this percentage any further. Therefore, these results suggest that 30 $\mu\text{mol/l}$ may be the optimal concentration of preconditioning and postconditioning with nicorandil to cause an anti-apoptotic effect.

Effects of 5-HD and ODQ on nicorandil-induced inhibition of infarct size and apoptosis. As shown in Fig. 4, the anti-infarct effects of preconditioning and postconditioning with nicorandil (30 $\mu\text{mol/l}$) were significantly inhibited by cotreatment with 100 $\mu\text{mol/l}$ 5-HD (32.68 \pm 2.90 vs. 14.88 \pm 3.25%; and 33.00 \pm 2.57 vs. 15.96 \pm 3.29%) or 10 $\mu\text{mol/l}$ ODQ (33.54 \pm 1.56 vs. 14.88 \pm 3.25%; and 34.04 \pm 2.89 vs. 15.96 \pm 3.29%) respectively, as compared with untreated samples. Furthermore, as outlined in Fig. 5, the anti-apoptotic effects of respective preconditioning and postconditioning with nicorandil (30 $\mu\text{mol/l}$) were also significantly inhibited by cotreatment with 100 $\mu\text{mol/l}$ 5-HD (42.22 \pm 2.13 vs. 25.20 \pm 3.93%; and 41.80 \pm 3.05 vs. 26.18 \pm 4.82%) or 10 $\mu\text{mol/l}$ ODQ (43.94 \pm 3.17 vs. 25.20 \pm 3.93%; and 43.20 \pm 1.78 vs. 26.18 \pm 4.82%) respectively, as compared with untreated samples. Together, these results suggested that nicorandil preconditioning and postconditioning may protect hypercholesterolemic hearts against I/R-induced infarction and apoptosis, which are both stimulated by the opening of mitoKATP channels and a NO/sGC dependent mechanism.

Preconditioning and postconditioning with nicorandil inhibits expression of caspase-3 protein. As a common factor in caspase-dependent apoptosis, the protein expression levels of caspase-3 were examined. As outlined in Fig. 6, respective pre- and post-conditioning with 30 $\mu\text{mol/l}$ nicorandil, significantly inhibited the expression of caspase-3, as compared with the I/R group (0.72 \pm 0.14 and 0.70 \pm 0.14 vs. 1.58 \pm 0.28, $P<0.05$).

Preconditioning and postconditioning with nicorandil regulates the expression of Bax and Bcl-2 proteins. Finally, the expression levels of Bax and Bcl-2 proteins were determined. Preconditioning and postconditioning with nicorandil (30 $\mu\text{mol/l}$) significantly suppressed the expression of Bax (0.65 \pm 0.16 and 0.74 \pm 0.17 vs. 1.64 \pm 0.35, $P<0.05$) (Fig. 7) and upregulated Bcl-2 expression (1.13 \pm 0.03 and 1.20 \pm 0.05 vs. 0.70 \pm 0.07, $P<0.05$) (Fig. 8), as compared with the I/R group.

Discussion

Hypercholesterolemia is a major risk factor for the induction and progression of IHD, and the incidence of myocardial infarction is higher in patients with chronic hypercholesterolemia, as hypercholesterolemia modifies the responses of the myocardium to I/R and cardioprotective interventions (7-9). Various studies have demonstrated that the myocardia of patients with hypercholesterolemia are more vulnerable to I/R-induced myocardial injury (10-15). In the present study, rats fed a high cholesterol diet for 8 weeks demonstrated significantly increased levels of TC and LDL-C. Hypercholesterolemia significantly exacerbated myocardial I/R injury by increasing the myocardial infarct size (44.04 \pm 2.70 vs. 39.04 \pm 1.90%, $P<0.05$) and the percentage of apoptotic cardiomyocytes (54.64 \pm 1.88 vs. 46.06 \pm 2.74%, $P<0.05$), as compared with the normocholesterolemic control

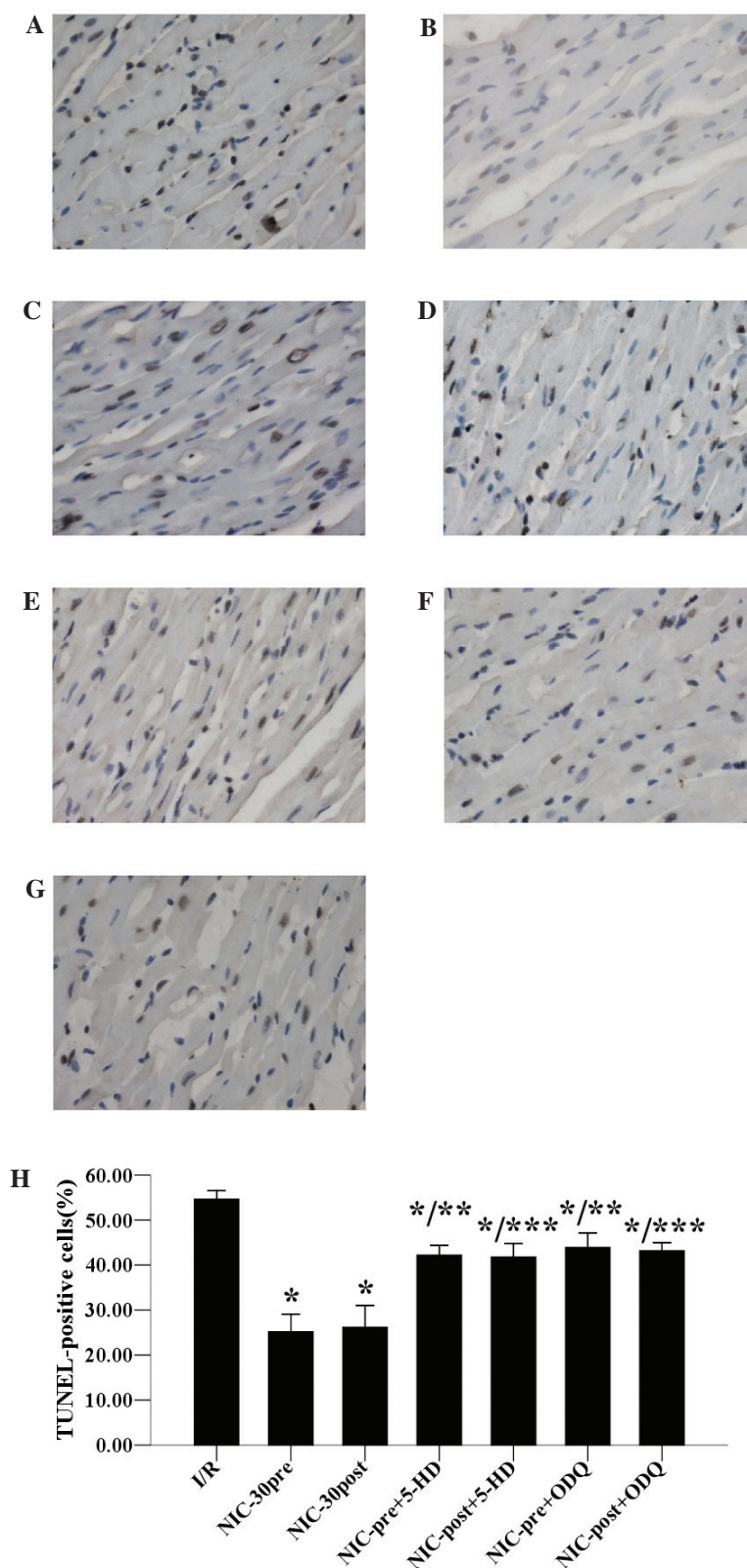


Figure 5. Effects of 5-HD and ODQ on NIC-induced inhibition of apoptosis in hypercholesterolemic hearts (diaminobenzidine and hematoxylin staining; magnification, x400). Data are expressed as the mean \pm standard deviation, $n=5$ for each. * $P<0.05$ vs. I/R group; ** $P<0.05$ vs. NIC-30pre group; *** $P<0.05$ vs. NIC-30post group. (A) I/R; (B) NIC-30pre; (C) NIC-30post; (D) NIC-pre+5-HD; (E) NIC-post+5-HD; (F) NIC-pre+ODQ; and (G) NIC-post+ODQ groups; (H) Statistical histogram of apoptosis in the seven groups. TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; I/R, ischemia/reperfusion; NIC, nicorandil; pre, preconditioning; post, postconditioning; HD, 5-hydroxydecanoic acid sodium salt; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one.

rats subjected to I/R (data not shown). The results of the present study are consistent with previous studies conducted on Yucatan pigs (10) and New Zealand rabbits (11).

Nicorandil is a hybrid agent with two distinct mechanisms of pharmacological action; it opens KATP channels, thereby dilating peripheral and coronary resistance arterioles,

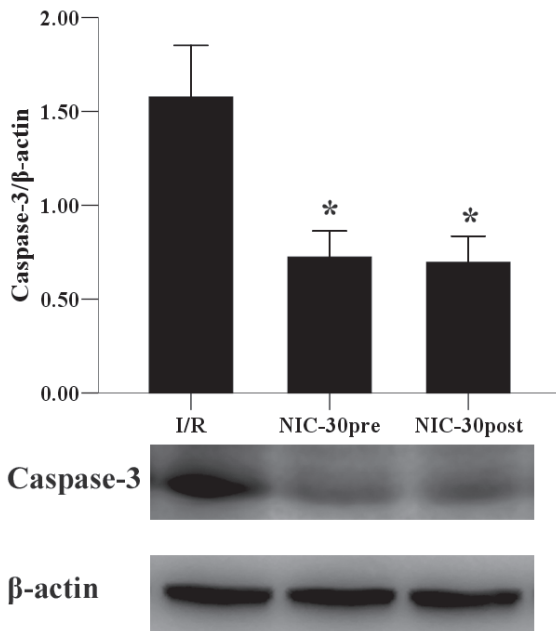


Figure 6. Expression levels of caspase-3 protein following preconditioning and postconditioning with 30 μ mol/l NIC. Data are expressed as the mean \pm standard deviation, n=3 for each. *P<0.05 vs. I/R group. I/R, ischemia/reperfusion; NIC, nicorandil; pre, preconditioning; post, postconditioning.

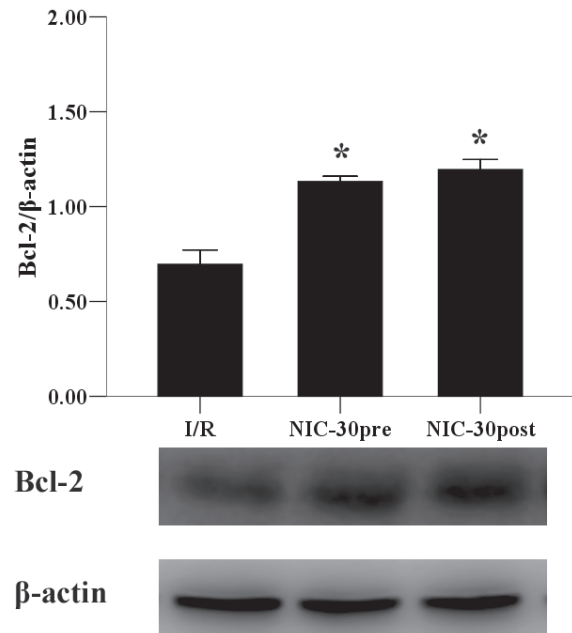


Figure 8. Expression levels of Bcl-2 protein following preconditioning and postconditioning with 30 μ mol/l NIC. Data are expressed as the mean \pm standard deviation, n=3 for each. *P<0.05 vs. I/R group. I/R, ischemia/reperfusion; NIC, nicorandil; pre, preconditioning; post, postconditioning; Bcl-2, B-cell lymphoma 2

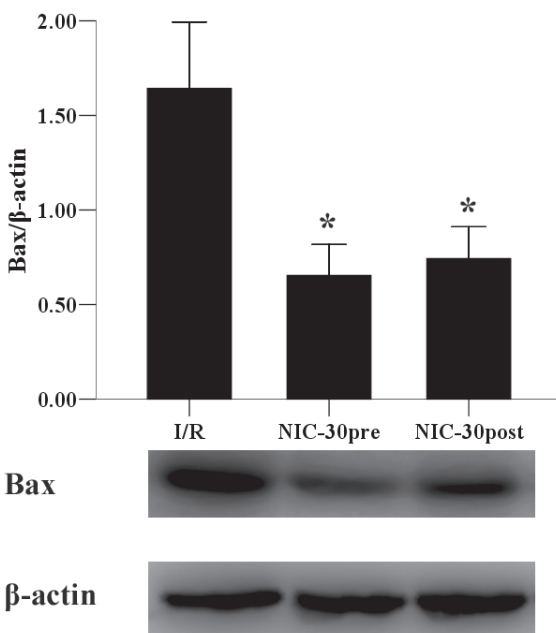


Figure 7. Expression levels of Bax protein following preconditioning and postconditioning with 30 μ mol/l NIC. Data are expressed as the mean \pm standard deviation, n=3 for each. *P<0.05 vs. I/R group. I/R, ischemia/reperfusion; NIC, nicorandil; pre, preconditioning; post, postconditioning; Bax, Bcl-2-associated X protein.

in addition to activating sGC through its nitrate-like effect, which increases cyclic guanosine monophosphate (cGMP) levels and subsequently dilates the systemic veins and epicardial coronary arteries. Therefore, nicorandil increases coronary blood flow, reduces preload and afterload, and exerts an anti-anginal effect (19). Although perfusion with nicorandil had previously been demonstrated to produce

pharmacological preconditioning-induced cardioprotection in normal hearts (21,23), few studies have been conducted on whether it still exerts cardioprotective effects on hypercholesterolemic hearts, particularly when administrated at the onset of reperfusion or reoxygenation. In the present study, five different concentrations of nicorandil were administrated either before ischemia, or at the onset of reperfusion, in order to induce pharmacological preconditioning and postconditioning, respectively. To the best of our knowledge, the present study has demonstrated for the first time that pharmacological preconditioning and postconditioning with nicorandil (1-100 μ mol/l) reduced myocardial necrosis and apoptosis induced by I/R, in a concentration-dependent manner in hypercholesterolemic rats. The present study also demonstrated that, for anti-infarct and anti-apoptosis, the optimal concentration of respective nicorandil preconditioning and postconditioning was 30 μ mol/l, as no further improvements were determined at 100 μ mol/l.

The KATP channels, including the mitoKATP and sarcolemmal KATP (sarcKATP) channels, are important in cardioprotection; and the mitoKATP channel in particular has been demonstrated to be the final effector of cardioprotection (21). The opening of the mitoKATP channels ensures the preservation of mitochondrial integrity and protects cellular function, which subsequently mediates the cardioprotective effects of ischemic and pharmacological preconditioning and postconditioning (5,6). Accordingly, pharmacological mitoKATP opening may be a putative therapeutic strategy to reduce I/R injury, particularly in hypercholesterolemic hearts with impaired KATP channels (15). Nicorandil is a KATP channel opener, and a previous study by Sato *et al* (27) demonstrated that nicorandil concentrations as low as 10 μ mol/l may open mitoKATP channels, whereas sarcKATP activation requires exposure to concentrations as high as 1 mmol/l. In

previous studies, nicorandil has limited the infarct size (21), blunted the rate of cardiomyocyte death (27), and reduced oxidative stress-induced cellular apoptosis (22) by opening mitoKATP channels. In the present study, the cardioprotective effects of nicorandil preconditioning and postconditioning were partially but significantly ameliorated by the mitoKATP channel blocker 5-HD, indicating that the cardioprotective effects of nicorandil in hypercholesterolemic hearts were partially mediated by the selective activation of mitoKATP channels.

In cardiomyocytes, NO is the major activator of sGC, which results in the generation of cGMP-induced cardioprotection against I/R injury (28). However, previous studies have demonstrated that the myocardial NO/sGC pathway may be impaired in hypercholesterolemia. For example, Prasan *et al* (14) demonstrated that NO production is decreased in the hearts of hypercholesterolemic rabbits during I/R, and Schwemmer *et al* (29) detected decreased cGMP levels in the hearts of hypercholesterolemic guinea pigs. Furthermore, previous studies have also demonstrated that cardioprotective mechanisms, such as preconditioning and postconditioning, in which the NO/sGC pathway has an important role, are abolished in hyperlipidemia (16-18). Therefore, we hypothesize that NO donors may have induced cardioprotection against I/R injury in hypercholesterolemic hearts. Giricz *et al* (30) and Tang *et al* (31) have previously reported that administration of an NO donor failed to induce cardioprotective effects in hypercholesterolemic animals. However, in the present study, as an NO donor, nicorandil preconditioning and postconditioning protected the hypercholesterolemic hearts against I/R-induced necrosis and apoptosis. Furthermore, the cardioprotective effects associated with nicorandil were significantly suppressed by cotreatment with OGD, an sGC inhibitor, suggesting that the cardioprotective effects of nicorandil in hypercholesterolemic hearts may also be associated with its NO/sGC dependent mechanism. The discrepancy between the present and previous studies may be due to the dual effects of nicorandil, particularly considering that the NO signaling pathway has been associated with the opening of mitoKATP channels (32,33) and that the NO released from nicorandil activates mitoKATP channels (34), thus making it difficult to separate the dual mechanisms of nicorandil action.

Myocardial injury during I/R implicates two morphologically and biologically distinct pathways, necrosis and apoptosis. Apoptosis is a fundamental process of cell death, which is mediated by a family of aspartate-specific cysteine proteases, known as caspases. Of the 14 caspases characterized to date, caspase-3 plays a critical role in the apoptosis of cardiomyocytes and thus represents the final common pathway of the caspase cascade (35). Early studies demonstrated that nicorandil inhibited hypoxia-induced apoptosis in cardiomyocytes and human pulmonary arterial endothelial cells, by reducing caspase-3 activation (25,36). Similarly, the present study demonstrated that nicorandil preconditioning and postconditioning also suppressed cardiomyocyte apoptosis, by downregulating the expression of caspase-3 in hypercholesterolemic hearts.

The apoptosis pathway is also regulated by members of the Bcl-2 family, which are associated with cell survival by regulating the permeability of mitochondria (37). The Bcl-2

family is composed of anti-apoptotic members, such as Bcl-2 and Bcl-extra large, and pro-apoptotic members, such as Bax, Bcl-2-associated death promoter and Bcl-2 homologous antagonist/killer. It has been suggested that protein interactions between Bcl-2 family members may play an important pathophysiological role in the control of apoptotic processes in cardiomyocytes. Nishikawa *et al* (25) demonstrated that nicorandil inhibited hypoxia-induced Bcl-2 downregulation and Bax upregulation in mitochondria, and thus protected cardiomyocytes. Furthermore, Wang *et al* (26) also found that, when administered before/during ischemia or at the onset of reperfusion, nicorandil increased Bcl-2 expression and reduced Bax expression in normal Sprague-Dawley rats. In agreement with these observations, the present study demonstrated that respective nicorandil preconditioning and postconditioning significantly upregulated the expression of Bcl-2 and downregulated the expression of Bax in hypercholesterolemic hearts, suggesting that the cardioprotective effects of nicorandil may be mediated by regulation of the Bcl-2 family.

In conclusion, the present study demonstrated that pharmacological preconditioning and postconditioning with nicorandil can protect hypercholesterolemic hearts against I/R-induced necrosis and apoptosis, in a concentration-dependent manner. Furthermore, the cardioprotective effects of nicorandil may be due to the dual pharmacological mechanisms of mitoKATP channel opening and a NO/sGC dependent mechanism, in addition to the regulation of the following apoptosis-related proteins: Caspase-3, Bax and Bcl-2. Therefore, nicorandil may be of potential clinical benefit to patients suffering from both IHD and hypercholesterolemia. The nicorandil conditioning strategy may be capable of reducing myocardial injury in patients presenting with acute myocardial infarction, cardiac arrest, undergoing percutaneous coronary intervention or cardiac surgery such as coronary artery bypass grafting.

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