The protective effect of fasudil pretreatment combined with ischemia postconditioning on myocardial ischemia/reperfusion injury in rats

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Abstract. – OBJECTIVE: Ischemic postconditioning (IPO) and pharmacological pretreatment may reduce myocardial necrosis and apoptosis during ischemia/reperfusion. This study aimed to determine the protective effect of fasudil pretreatment combined with IPO on myocardial ischemia/reperfusion injury in rats and explore the possible mechanisms.

MATERIALS AND METHODS: The SD rats were induced by intraperitoneal injection of fasudil hydrochloride (1 or 10 mg/kg) 60 min before the initiation of ischemia, while the control rats were given the same volume of saline. The hearts were hung on the Langendorff perfusion apparatus and underwent 30 min global ischemia and 120 min reperfusion. The IPO protocol was induced by six cycles of 10 sec ischemia and 10 sec reperfusion at the onset of reperfusion. The hemodynamic changes were measured, myocardial infarct size was determined by triphenyltetrazolium chloride (TTC) staining, cardiomyocyte apoptosis was detected by TUNEL staining, lactate dehydrogenase (LDH) was analyzed from coronary effluents, phosphorylation of Akt and eNOS, as well as expression of Bcl-2 and Bax were measured by western blotting analysis.

RESULTS: The high-dose fasudil (10 mg/kg) pretreatment group and IPO group significantly improved post-ischemia cardiac function, reduced myocardial infarct size, attenuated cardiomyocyte apoptosis, decreased the release of LDH, increased expression of phospho-Akt, phospho-eNOS and Bcl-2, and reduced expression of Bax compared with the control group (p < 0.05). In addition, the high-dose fasudil pretreatment combined with IPO group could further improved post-ischemia cardiac function, reduced myocardial infarct size, attenuated cardiomyocyte apoptosis, decreased the release of LDH, increased expression of phospho-Akt, phospho-eNOS and Bcl-2, and reduced expression of Bax compared with the single treatment groups (p < 0.05).

CONCLUSIONS: The combination of high-dose fasudil pretreatment and IPO had a synergistic protective effect on myocardial ischemia/reperfusion injury, which was mediated via upregulating the PI3K/Akt/eNOS pathway, increasing expression of antiapoptotic Bcl-2, and decreasing expression of proapoptotic Bax.

Key Words: Fasudil hydrochloride, Ischemia/reperfusion injury, Pretreatment, Ischemia postconditioning, PI3K/Akt/eNOS, Bcl-2, Bax.

Introduction

Timely effective reperfusion therapy is the main therapeutic strategy to salvage myocardium from tissue injury after prolonged ischemia; however, the beneficial effects can be compromised by ischemia/reperfusion injury¹². Previous studies have shown that short cycles of ischemia-reperfusion performed at the onset of reperfusion, which is termed “ischemic postconditioning (IPO)” can significantly reduce myocardial ischemia/reperfusion injury³. The protective effect of IPO has been proven in several studies with experimental animals and humans⁴⁵. Rho kinase (ROCK) is a serine/threonine kinase which mediates many important downstream effects of the small GTP-binding protein, RhoA⁷. RhoA/ROCK signaling pathway has been proven to play a deleterious role in myocardial ischemia/reperfusion injury in several models in vivo and in vitro⁸¹⁰. In these models, ROCK inhibition with selective inhibitor achieved smaller infarct size, less inflammation, attenuated apoptosis, and enhanced cardiac contractile function. Fasudil is the only ROCK inhibitor approved for human use, which was initially approved in Japan in 1995 for the prevention and treatment of cerebral vasospasm after surgery for subarachnoid hemorrhage¹⁵. Recent
studies demonstrated that fasudil was effective in treating ischemic heart disease\textsuperscript{16,17}. Meanwhile, study data confirmed that ROCK inhibition with fasudil pretreatment can effectively reduce myocardial ischemia/reperfusion injury\textsuperscript{10-13}.

However, there is a lack of evidence reporting the effect of fasudil pretreatment combined with IPO on myocardial ischemia/reperfusion injury. The purpose of this study, therefore, was to determine whether fasudil pretreatment combined with IPO had a synergistic protective effect on myocardial ischemia/reperfusion injury in isolated hearts, and, if so, to explore the possible protective mechanisms involved.

**Materials and Methods**

**Animals**
A total of 60 healthy male Sprague Dawley (SD) rats, weighing at 250-300 g (Department of Experimental Animals, China Medical University, Liaoning Province, China) were studied. The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH). The study protocol was approved by Institutional Ethics Committee.

**Chemicals and Drugs**
Fasudil hydrochloride (Dalian Meilun Biotech Co., Ltd, China); triphenyltetrazolium chloride (TTC) (Sigma, St Louis, MO, USA); lactate dehydrogenase (LDH) assay kit (Nanjing Jiancheng Bioengineering Institute, China); in situ cell death detection kit, POD (Roche, Mannheim, Germany); Akt, phospho-Akt, eNOS, phospho-eNOS antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); Bax, Bcl-2 antibodies (Abcam, Cambridge, MA, USA); β-actin antibody (ZSGB-BIO, Beijing, China).

**Isolated Perfused Heart Preparation**
The SD rats were induced by intraperitoneal injection of fasudil hydrochloride (1 or 10 mg/kg) 60 min before the onset of ischemia, while the control rats were given the same volume of saline. After 30 min of observation, rats were anesthetized by intraperitoneal injection with 4 ml/kg 10% chloral hydrate. Heparin (1,500 IU/kg) was administered intravenously to prevent intracoronary clot formation. The heart was rapidly excised and immediately immersed in Krebs-Henseleit solution (127 mmol/l NaCl, 17.7 mmol/l NaHCO\textsubscript{3}, 5.1 mmol/l KCl, 1.5 mmol/l CaCl\textsubscript{2}, 1.26 mmol/l MgCl\textsubscript{2}, 11 mmol/l D-glucose; pH 7.4) at 4°C for trimming. The heart was hung on the Langendorff perfusion apparatus and retrogradely perfused through the aorta with recirculating Krebs-Henseleit buffer saturated with 95% O\textsubscript{2} and 5% CO\textsubscript{2} at 37°C. The whole procedure took two minutes. The heart was maintained in a thermostatic chamber at 37°C. Perfusion was maintained at a constant pressure of 75 mmHg. A fluid-filled latex balloon was inserted in the left ventricle via the left atrium for pressure measurement. The balloon was connected to a pressure transducer and inflated to an initial left ventricular end-diastolic pressure between 8 and 10 mmHg.

**Experimental Protocol**
The 60 SD rats were randomized into 6 treatment groups (Figure 1). In all groups, the isolated rat hearts were perfused with Krebs-Henseleit solution and allowed for 20 min stabilization. Isolated hearts were then subjected to 30 min global ischemia and 120 min reperfusion.

- **Control group** (n=10): intraperitoneal injection of saline solution 60 min before ischemia, the isolated heart underwent 30 min global ischemia and 120 min reperfusion.
- **Low-dose fasudil pretreatment group (LF)** (n=10): intraperitoneal injection of fasudil hydrochloride 1 mg/kg 60 min before ischemia, the isolated heart underwent 30 min global ischemia and 120 min reperfusion.
- **High-dose fasudil pretreatment group (HF)** (n=10): intraperitoneal injection of fasudil hydrochloride 10 mg/kg 60 min before ischemia, the isolated heart underwent 30 min global ischemia and 120 min reperfusion.
- **Ischemic postconditioning group (IPO)** (n=10): intraperitoneal injection of saline solution 60 min before ischemia, the isolated heart underwent 30 min global ischemia and six cycles of 10 sec ischemia followed by 10 sec reperfusion, and then reperfusion for 120 min.
- **Low-dose fasudil pretreatment and ischemic postconditioning combined group (LF+IPO)** (n=10): intraperitoneal injection of fasudil hydrochloride 1 mg/kg 60 min before ischemia, the isolated heart underwent 30 min global ischemia and six cycles of 10 sec ischemia followed by 10 sec reperfusion, and then reperfusion for 120 min.
- **High-dose fasudil pretreatment and ischemic postconditioning combined group (HF+IPO)** (n=10): intraperitoneal injection of fasudil hy-
Figure 1. Representation of experimental protocol. LF: 1 mg/kg fasudil pretreatment; HF: 10 mg/kg fasudil pretreatment; IPO: ischemic postconditioning; LF+IPO: 1 mg/kg fasudil pretreatment+ischemic postconditioning; HF+IPO: 10 mg/kg fasudil pretreatment+ischemic postconditioning.

Dichloride 10 mg/kg 60 min before ischemia, the isolated heart underwent 30 min global ischemia and six cycles of 10 sec ischemia followed by 10 sec reperfusion, and then reperfusion for 120 min.

Hemodynamic Monitoring

The hemodynamic assessment included heart rate (HR), left ventricular developed pressure (LVDP), maximal ascending rate of left ventricular pressure (+dp/dt), maximal descending rate of left ventricular pressure (-dp/dt). These parameters were continuously monitored throughout the experimental protocol. The HR, LVDP, +dp/dt and -dp/dt were sampled and digitally processed via a hemodynamic system (MP150; Biopac Systems, Inc., Goleta, CA, USA).

Measurement of Infarct Size

Infarct size was determined as previously described. Briefly, after 120 min of reperfusion, the hearts were harvested. The hearts were semifrozen for 60 min in a -20°C freezer, and were sectioned from apex to base into 2-3 mm sections. The slices were incubated in 1% TTC solution (TTC dissolved in pH 7.4 Na₂HP₀₄/NaH₂PO₄ buffer) for 5 min at 37°C, and unstained tissue was carefully separated from stained tissue by an independent observer. The unstained tissue represented the dead cells, while the stained tissue represented viable cells. The unstained mass was expressed as a percentage of total left ventricular mass. The total left ventricle mass also represented the risk area since global ischemia was induced.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay for Apoptosis

Apoptotic cardiomyocyte was detected by in situ cell death detection kit according to the manufacturer’s instructions. After 30 min of reperfusion, the hearts were removed. The 4-μm-thick, formalin-fixed, paraffin embedded sections were cut, deparaffinized with xylene and then rehydrated with graded alcohol. Proteinase K (20 mg/L) was applied to the section for 10 min to produce optimal proteolysis. The endogenous peroxidase was inhibited with 3% hydrogen peroxide for 30 min. 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 demonstrated in each section by the application of a stable chromogen.
diaminobenzidine. In this technique, apoptotic nuclei were stained dark brown. The sections were counterstained with haematoxylin for total nuclei. Three sections from each myocardial sample were randomly selected and 10 microscopic fields (×400) (Olympus BX51 microscope, Tokyo, Japan) per section were evaluated by two independent blind observers. The percentage of TUNEL staining positive cells was calculated as follows: number of apoptotic cells/total number counted×100%.

**Assessment of Myocardial Injury**

To determine the extent of myocardial injury, the release of lactate dehydrogenase (LDH) in coronary effluents at 30 min of reperfusion was measured using commercially available kits and spectrophotometer. Values were expressed in international units (IU) per liter.

**Western Bloting**

At the end of 30 min reperfusion, the hearts were homogenized in a lysis buffer (10 mmol/l Tris HCl, 20 mmol/l orthophosphate, 1 mmol/l EGTA, 1 mmol/l EDTA, 2 mmol/l Na3VO4 and 1 mmol/l phenylmethylsulfonyl fluoride; pH 7.4). After homogenization and protein quantification, equal amounts of protein from each sample were separated by electrophoresis on SDS-PAGE, and transferred onto polyvinylidene difluoride (PVDF)-plus membranes. After blocking with 5% albumin bovine serum (BSA), the membranes were incubated overnight at 4°C with the following primary antibodies: Akt antibody (1:1000), phospho-Akt antibody (Ser473, 1:1000), eNOS antibody (1:1000), phospho-eNOS antibody (Ser1177, 1:1000), Bax antibody (1:1500), Bel-2 antibody (1:1500), and β-actin antibody (1:2000). Then, the membranes were washed three times in TBST and incubated with the corresponding secondary antibody (1:5000) conjugated to horseradish peroxidase at room temperature for 2 h. Then the membranes were washed three times in TBST. Relative densitometry was performed using a computerized software package (NIH Image 1.63 software).

**Statistical Analysis**

The data were expressed as mean ± SD values. Statistical analysis was performed by using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). One-way ANOVA was applied in analyzing the difference between the groups. If the difference was statistically significant, Student-Newman-Keuls post hoc test (SNK) was applied in a further pairwise comparisons. All p values < 0.05 were considered statistically significant.

**Results**

**Hemodynamic Changes**

As shown in Table I, there were no significant differences in hemodynamic parameters among the groups at the baseline before ischemia (p > 0.05). During reperfusion, the HF, IPO, LF+IPO, and HF+IPO groups showed significantly increased in HR, LVDP and ± dp/dt compared with the control group (p < 0.05). In addition, the HF+IPO group could further increase the HR, LVDP and ± dp/dt during reperfusion compared with the single treatment groups (HF group and IPO group) (p < 0.05).

**Infarct Size Measurement.**

As shown in Figure 2, the infarct size were significantly reduced in HF, IPO, LF+IPO, and HF+IPO groups compared with the control group (36.67 ± 2.94%, 35.83 ± 3.19%, 35.50 ± 2.88%, 28.83 ± 2.79% vs. 46.83 ± 2.86%; p < 0.05). However, the LF group did not reduce infarct size compared with the control group (45.17 ± 2.71% vs. 46.83 ± 2.86%; p > 0.05). In addition, the HF+IPO group could further reduce the infarct size compared with the single treatment groups (HF group and IPO group) (28.83 ± 2.79% vs. 36.67 ± 2.94%, 35.83 ± 3.19%; p < 0.05).

**TUNEL Staining for Apoptosis**

As shown in Figure 3, the TUNEL-positive cells were significantly reduced in HF, IPO, LF+IPO, and HF+IPO groups compared with the control group (44.83 ± 2.86%, 46.33 ± 2.50%, 44.83 ± 2.04%, 34.67 ± 2.94% vs. 54.33 ± 2.88%; p < 0.05). However, the LF group did not reduce TUNEL-positive cells compared with the control group (52.50 ± 2.07% vs. 54.33 ± 2.88%; p > 0.05). In addition, the HF+IPO group could further reduce the TUNEL-positive cells compared with the single treatment groups (HF group and IPO group) (34.67 ± 2.94% vs. 44.83 ± 2.86%, 46.33 ± 2.50%; p < 0.05).

**LDH Level in Coronary Effluents**

As shown in Figure 4, the level of LDH were significantly reduced in HF, IPO, LF+IPO, and HF+IPO groups compared with the control group (229.17 ± 12.41, 239.83 ± 14.19, 231.50 ± 17.89,
However, the LF group did not reduce the level of LDH compared with the control group (179.83 ± 18.32 vs. 309.17 ± 14.41; p < 0.05). In addition, the HF+IPO group could further reduce the level of LDH compared with the single treatment groups (HF group and IPO group) (179.83 ± 18.32 vs. 229.17 ± 12.41, 239.83 ± 14.19; p < 0.05).

**Expression of Akt and eNOS Proteins**

As shown in Figure 5 and 6, Akt phosphorylation and eNOS phosphorylation were significantly enhanced in HF, IPO, and HF+IPO groups compared with the control group (1.12 ± 0.19, 1.08 ± 0.14, 4.95 ± 0.37 vs. 0.42 ± 0.10; p < 0.05). In addition, Akt and eNOS phosphorylation in HF+IPO group were further increased compared with the single treatment groups (HF group and IPO group) (4.95 ± 0.37 vs. 1.12 ± 0.19, 1.08 ± 0.14; p < 0.05) (3.35 ± 0.18 vs. 1.16 ± 0.13, or 1.14 ± 0.18; p < 0.05).

**Expression of Bcl-2 and Bax Proteins**

As shown in Figure 7 and 8, the Bcl-2 expression were significantly increased and the Bax expression were significantly reduced in all treatment groups compared with the control group (p < 0.05). In addition, the HF+IPO group could further reduce the level of Bax expression compared with the single treatment groups (HF group and IPO group) (p < 0.05).
The protective effect of fasudil pretreatment

Bax expression were significantly decreased in HF, IPO, and HF+IPO groups compared with the control group (1.02 ± 0.13, 0.99 ± 0.09, 1.43 ± 0.10 vs. 0.57 ± 0.13; \( p < 0.05 \)) (0.98 ± 0.11, 1.05 ± 0.06, 0.48 ± 0.13 vs. 1.37 ± 0.08; \( p < 0.05 \)). In addition, the Bcl-2 expression were further increased and the Bax expression were further decreased in HF+IPO group compared with the control group (1.43 ± 0.10 vs. 1.02 ± 0.13, 0.99 ± 0.09; \( p < 0.05 \)) (0.48 ± 0.13 vs. 0.98 ± 0.11, 1.05 ± 0.06; \( p < 0.05 \)).

Figure 3. Myocyte apoptosis. A, Control group. B, LF group. C, HF group. D, IPO group. E, LF+IPO group. F, HF+IPO group. G, TUNEL-positive cells. Data were presented as means ± SD and \( n=5 \) for each. \(^a p < 0.05 \) vs. Control. \(^b p < 0.05 \) vs. HF. \(^c p < 0.05 \) vs. IPO.

Discussion

Brief episodes of nonlethal ischemia and reperfusion before sustained ischemia or at the onset of reperfusion can reduce ischemia/reperfusion injury. These ischemic conditioning phenomena are termed “ischemic preconditioning (IPC)” and “ischemic postconditioning (IPO)”, respectively\(^{3,19} \). The IPO procedure is carried out after ischemia, therefore it may be more clinically applicable than IPC. Increasing number of
studies have shown the protective effect of IPO in heart. In line with these studies, our study reconfirmed that IPO significantly improved post-ischemia cardiac function, reduced infarct size, attenuated cardiomyocyte apoptosis, and decreased LDH release in coronary effluents compared with the control group.

Following the initial discovery of IPC, it became clear that some pharmacological agents such as adenosine, bradykinin, opioid agonists, and others could also mimic the cardioprotection by IPC when administered prior to the onset of sustained myocardial ischemia, which is called "pharmacological preconditioning." This is significant since pharmacological agents could be more readily applied to clinical practice as a means of protecting the heart against ischemia/reperfusion.
Although there have been numerous studies reported the cardioprotective effects of IPO and fasudil pretreatment respectively, whether fasudil pretreatment combined with IPO had a synergistic protective effect on myocardial ischemia/reperfusion injury remained unknown. This present study for the first time demonstrated that high-dose fasudil (10 mg/kg) pretreatment combined with IPO could significantly improve post-ischemia cardiac function, reduce infarct size, attenuate cardiomyocyte apoptosis, and decrease LDH release in coronary effluents compared with single treatment group, suggesting that high-dose fasudil pretreatment combined with IPO might have a synergistic protective effect against myocardial ischemia/reperfusion injury. However, the low-dose fasudil (1 mg/kg) pretreatment combined with IPO failed to offer further protective effect than single IPO treatment, suggesting that the protective effect of the low-dose fasudil combined with IPO group should be completely attributed to the effect of IPO, which further confirmed that the cardioprotective effect of fasudil pretreatment might be dose-dependent.

The ischemic and pharmacological pre/post-conditioning have been proposed to act via similar signaling pathways and target similar end points. They both have been shown to exert their cardioprotective effects by upregulating the prosurvival kinases named “reperfusion injury salvage kinase (RISK)”31. The RISK pathway consists of phosphatidylinositol 3-kinase (PI3K)/Akt pathway and extracellular signal-regulated kinases 1/2 (ERK1/2) pathway. Tsang et al20 showed for the first time that IPO protected the myocardium by activating the prosurvival kinase PI3K/Akt and its downstream target eNOS in isolated rat heart. Wolfrum et al10 also reported that acute inhibition of ROCK by fasudil (10 mg/kg) pretreatment led cardiovascular protection. And this cardioprotective effect of fasudil was completely blocked after pretreatment with the NOS inhibitor, L-NAME or the PI3K inhibitor, wortmannin, which confirmed that the protective mechanism of fasudil pretreatment involved activation of PI3K/Akt/eNOS pathway. Moreover, Hamid et al9 demonstrated that fasudil given 10 min before ischemia until 10 min after reperfusion reduced infarct size. They also speculated that the balance of activities of injurious factors, such as ROCK, and RISK signals, such as PI3K/Akt, is a crucial determinant of tissue survival in ischemia/reperfusion. Consistent with

Figure 8. Myocardial Bax expression. Data are presented as means ± SD and n=3 for each. *p < 0.05 vs. Control. †p < 0.05 vs. HF. ‡p < 0.05 vs. IPO.
previous studies, our study showed that the high-dose fasudil (10 mg/kg) pretreatment group and IPO group markedly increase the phosphorylation of Akt and eNOS compared with the control group. In addition, we firstly revealed that high-dose fasudil (10 mg/kg) pretreatment combined with IPO can significantly increase the phosphorylation of Akt and eNOS compared with single treatment group, suggesting that the synergistic cardioprotective effect of high-dose fasudil pretreatment and IPO was mediated via upregulation of PI3K/Akt/eNOS pathway.

Myocardial injury during ischemia/reperfusion implicates two morphologically distinct pathways: necrosis and apoptosis. Apoptosis is highly regulated, energy-dependent suicide program, which is mediated by two different evolutionarily conserved pathways: the extrinsic and intrinsic cell death pathway32. The intrinsic pathway of apoptosis is regulated by members of the Bcl-2 family, which are known to affect cell survival by regulating the permeability of mitochondria33. Bcl-2 family proteins can be divided into anti-apoptotic members (e.g. Bcl-2 and Bcl-xL) and proapoptotic members (e.g. Bax, Bak). Activation of proapoptotic Bax, which occurs in response to apoptotic stimuli such as ischemia/hypoxia, leads to its oligomerization and translocation to mitochondria, where it induces cytochrome c release and activates the downstream caspases. While, the antiapoptotic Bcl-2 bind to and sequester proapoptotic factors, such as Bax, preventing their ability to increase mitochondrial permeability. Therefore, the ratio of Bcl-2/Bax is the key to decide cell apoptosis. Sun et al34 reported that the cardioprotective effect of IPO was achieved via increasing Bcl-2 expression and decreasing Bax expression. In addition, Bao et al35 demonstrated that another selective ROCK inhibitor Y-27632 could enhance post-ischemia cardiac function, reduce ischemia/reperfusion induced myocardial apoptosis by upregulation of Bcl-2 in myocardium. Del et al36 also showed that Y-27632 could attenuate the upregulation of Bax induced by ROCK activation during ischemia/reperfusion and prevent cardiomyocyte apoptosis. However, there were rare studies concerning the effect of fasudil in apoptotic pathway. Especially, the effect of fasudil pretreatment combined with IPO in apoptotic pathway remained unknown. In this study, we found that the antiapoptotic Bcl-2 expression were significantly increased and the proapoptotic Bax expression were significantly decreased in high-dose fasudil (10 mg/kg) group and IPO group compared with the control group. In addition, we reported for the first time that high-dose fasudil (10 mg/kg) pretreatment combined with IPO could significantly increase expression of Bcl-2 and decrease expression of Bax in comparison to the single treatment groups, suggesting that the synergistic cardioprotective effect of high-dose fasudil pretreatment and IPO was mediated via regulation of mitochondrial death pathway and cardiomyocyte apoptosis.

Conclusions

High-dose fasudil pretreatment combined with IPO had a synergistic protective effect on myocardial ischemia/reperfusion injury, which including significantly improved post-ischemia cardiac function, reduced myocardial infarction, attenuated cardiomyocyte apoptosis, and decreased the release of LDH. In addition, the synergistic cardioprotective effect of high-dose fasudil pretreatment and IPO could be attributed to the upregulation of PI3K/Akt/eNOS pathway, increasing expression of antiapoptotic Bcl-2, and decreasing expression of proapoptotic Bax. However, the clinical therapeutic effect of the combination and their deep mechanisms of cardioprotective effect still need to be further studied.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References


