

## Original Article

# Rho-kinase inhibition is involved in the activation of PI3-kinase/Akt during ischemic-preconditioning-induced cardiomyocyte apoptosis

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**Abstract:** We and others have reported that Rho-kinase plays an important role in the pathogenesis of heart ischemia/reperfusion (I/R) injury. Studies also have demonstrated that the activation of Rho-kinase was reversed in ischemic preconditioning (IPC). This study aimed to explain the mechanism of Rho-kinase-mediated cardiomyocyte apoptosis increased in I/R and reversed in IPC. Materials and methods: Studies were performed with female Wistar rats. The I/R rats were created by ligating the left anterior descending branch (LAD) for 30 min and releasing the ligature for 180 min. The IPC rats underwent IPC (two cycles of 5 min ligation of the LAD and 5 min reflow) before I/R. Results: Ischemia followed by reperfusion caused a significant increase in Rho-kinase and a decrease in Akt phosphorylation. Administration of fasudil, an inhibitor of Rho-kinase, decreased myocardial infarction size and cardiomyocyte apoptosis and increased Akt activation. IPC also caused the reduced Rho-kinase activity and cardiomyocyte apoptosis and a significant increase in Akt activity ( $P < 0.05$  vs I/R). Conclusion: Rho-kinase inhibition by IPC leads to reduced cardiomyocyte apoptosis may be mediated by activation of PI3-kinase/Akt.

**Keywords:** Rho-kinase, heart ischemia/reperfusion, ischemic preconditioning, apoptosis, PI3-kinase/Akt

## Introduction

The ubiquitously expressed Rho-kinase, a serine/threonine kinase, has been identified as one of the effectors of the small GTP-binding protein Rho. Rho-kinase plays crucial roles in various cellular functions, and mediates cellular events such as changes in cell morphology, cell motility, focal adhesions, and cytokinesis [1, 2]. The myosin phosphatase target subunit (MYPT-1) is the major effector of Rho-kinase [3]. Accumulating evidences have demonstrated that Rho-kinase plays an important role in many major cardiovascular diseases such as hypertension, heart failure, myocardial infarction and atherosclerosis [4-8].

IPC has been exploited as a powerful endogenous form of cardioprotection. IPC was first discovered by Murry and associates, who demonstrated that a brief period of repetitive cardiac

I/R exerts a protective effect against subsequent lethal periods of ischemia [9]. IPC was found to similarly reduce cytosolic and mitochondrial  $Ca^{2+}$  overloading, to augment post-ischemic functional recovery, and to decrease infarct size [10]. And IPC is known to protect cardiomyocyte apoptosis during reperfusion. Previous studies have demonstrated IPC caused a substantial decrease of Rho-kinase activation during sustained ischemia and reduced infarct size [11]. Here, we observed the reduced activity of Rho kinase in IPC. Furthermore, Rho-kinase inhibition may be involved in the activation of PI3-kinase/Akt during IPC-reduced cardiomyocyte apoptosis.

## Materials and methods

All procedures were performed in conformity with Institutional Animal Care and Use Committee and NIH guidelines.

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### *Myocardial I/R and IPC*

Female Wistar rats (250-300 g body weight, from Shandong university, china) were maintained under conditions of standard lighting (alternating 12 h light/dark cycle), temperature ( $22 \pm 0.5^\circ\text{C}$ ) and humidity ( $60\% \pm 10\%$ ) for at least 1 week before the experiments. The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). The trachea was cannulated with a PE-90 catheter and artificial respiration was provided by a respirator with  $\text{FiO}_2$  (fraction of inspired oxygen) of 0.80, a frequency of 100 strokes/min and a tidal volume of 0.8 to 1.2 mL to maintain normal  $\text{PO}_2$  (partial pressure of oxygen),  $\text{PCO}_2$  (partial pressure of carbon dioxide), and pH. A left thoracotomy and pericardiotomy were performed. The left anterior descending branch (LAD) of the left coronary artery was occluded by ligation with a 4-0 silk suture. After 30 min of ischemia, the ligation was loosened and reperfusion occurred. The sham control animals were subjected to the entire surgical procedure and silk suture was passed beneath the coronary artery but the LAD coronary artery was not ligated. Rats were sacrificed at 180 min of reperfusion.

IPC was introduced by two cycles of 5 min ischemia, followed by 5 min reperfusion. The rats were then subjected to 30 min LAD occlusion followed by 180 min reperfusion similar to I/R rats.

### *Experimental groups*

48 Wistar rats were randomly assigned to the following experimental groups:

1. Sham operation. (n=12) (Control).
2. LAD was occluded for 30 min and reperfused for 180 min. [1 ml normal saline were injected i.v. 1 hour before operation] (n=12) (I/R).
3. LAD was occluded for 30 min and reperfused for 180 min plus administration of fasudil, an inhibitor of Rho kinase [12]. [10 mg/kg i.v., 1 hour before operation. Fasudil was dissolved in 1 ml normal saline] (n=12) (I/R + fasudil).
4. Two cycles of 5 min ischemia followed by 5 min reperfusion before sustained ischemia. (n=12) (IPC).

### *Determination of myocardial infarct size*

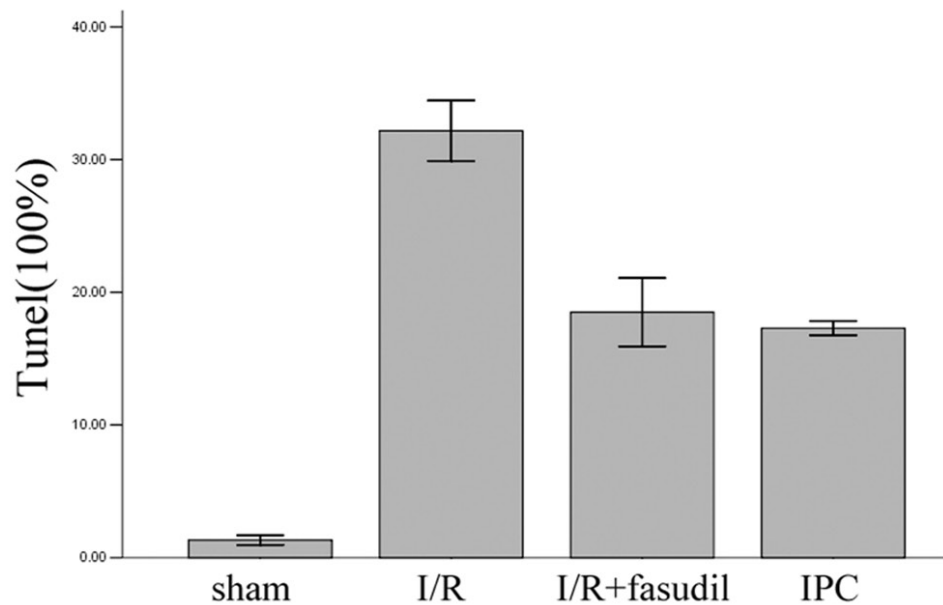
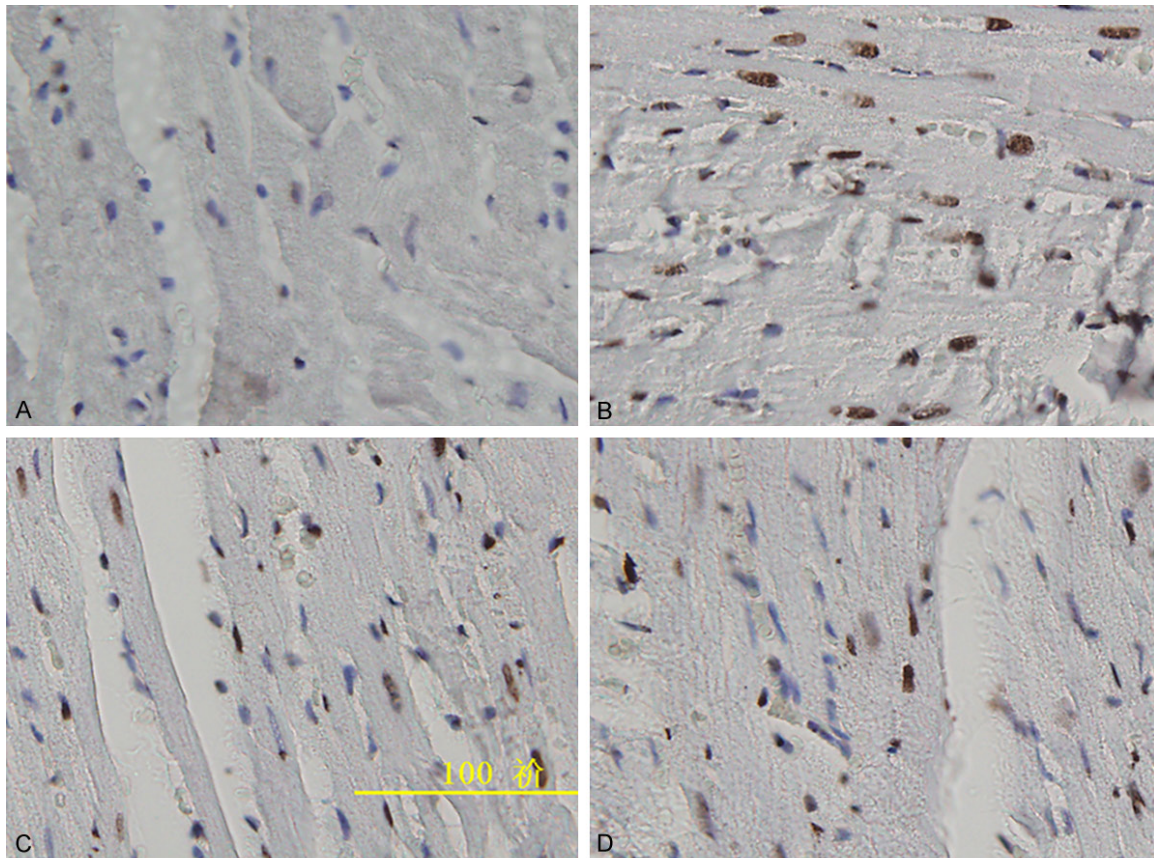
At the end of reperfusion, the coronary artery was re-occluded and the heart was perfused with 3 ml of Evans blue to delineate myocardial area at risk (AAR). Then the hearts were harvested and rinsed in normal saline. The atria, right ventricle and great vessels were removed. The tissues were semi-frozen for 30 min in a  $-20^\circ\text{C}$  freezer in order to be cut more easily. After that, the left ventricle was surgically isolated and cut into slices (1 mm thick). Nitro blue tetrazolium was obtained in powder form (Sigma, USA). The tetrazolium powder was diluted in a phosphate buffer. We used a two part buffer system consisting of  $\text{NaH}_2\text{PO}_4$  (0.1 M) and  $\text{Na}_2\text{HPO}_4$  (0.1 M). The ratio of the volume of  $\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$  in the buffer was 77.4% and 22.6%. The pH of the buffer was 7.4. The slices were incubated in the solution at a temperature of  $37^\circ\text{C}$  for 5 min. After evaluating the entire surface area, segments with blue staining were designated as viable, and those without staining were designated as non-viable (infarcted). Finally the different areas of the ventricle were weighed separately. Myocardial ischemic area (AAR) was expressed as the percentage of the left ventricle (LV). Infarct size was expressed as the percentage of the AAR [13].

### *Terminal Deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL)*

To evaluate apoptotic activity, the TUNEL technique was used. Each section was deparaffinized and rehydrated with serial changes of xylene and ethanol. Proteinase K (20 mg/L) was applied to the section for 30 min with the intention of producing optimal proteolysis. The endogenous peroxidase was inhibited with 3% hydrogen peroxide for 10 min. A commercial apoptosis detection kit (Roche, Germany) was used. The TdT reaction was carried out for 1 h at  $37^\circ\text{C}$  in a humidified chamber, and then DAB chromogen was applied. Hematoxylin was used as a counterstain. TUNEL-positive cells were determined by randomly counting 10 fields of the section and were expressed as a percentage of normal nuclei [14].

### *Immunohistochemistry staining for p-Akt*

The 5- $\mu\text{m}$ -thick, formalin-fixed, paraffin-embedded sections were cut, deparaffinized, and

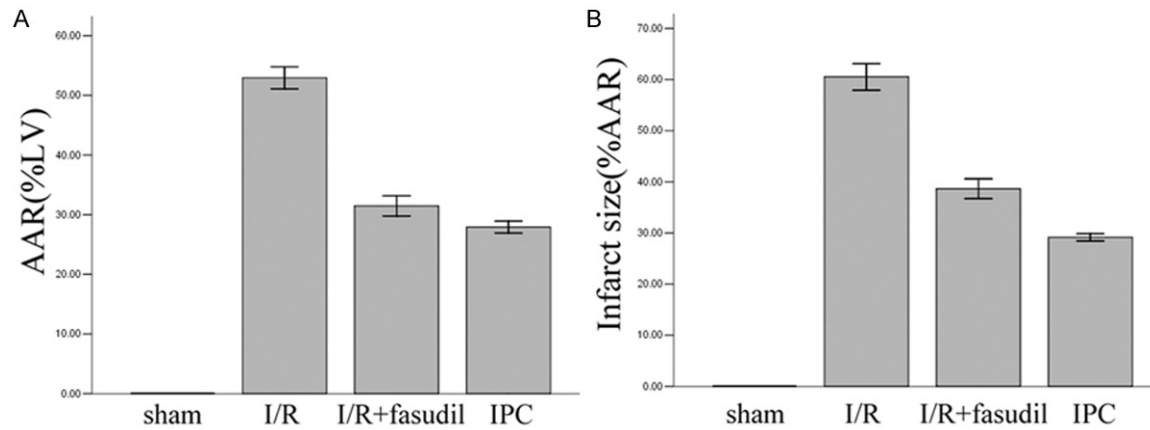


**Figure 1.** Representative photomicrographs of ventricular tissue stained for TUNEL for DNA breaks. A: Sham group; B: I/R group; C: IPC + fasudil group; D: IPC group. In the I/R group a large number of TUNEL positive cells was observed. After administration of fasudil, the percentage of TUNEL positive cells was significantly reduced (\* $P < 0.05$  vs I/R group). The percentage of TUNEL positive cells of IPC group was significantly reduced compared with I/R group (# $P < 0.05$ ).

rehydrated with graded alcohol and xylene. Endogenous peroxidase was blocked using 3%

hydrogen peroxide for 10 minutes, followed by a brief wash in Tris buffer (pH, 7.2). Sections

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**Figure 2.** Changes in infarct size of different groups (sham, I/R, IPC + fasudil, IPC) in rats' hearts. The myocardial infarct size was similar between IPC and IPC + DMSO groups. After administration of fasudil, the infarct size were reduced (\* $P < 0.05$  vs I/R group). A significant reduction in infarct size was observed in IPC ( $\#P < 0.05$  vs I/R group).

were boiled in citrate buffer (pH, 6.0) with microwaves at 92°C-98°C for 10 minutes to retrieve the antigen. After blocking nonspecific binding with 5% bovine serum albumin, the slides were incubated with primary antibody overnight at 4°C (p-Akt 1:100, Bipec Biopharma Corporation, USA). Sections were thoroughly washed in phosphate buffer saline (PBS) and incubated with a peroxidase-conjugated polymer which carries antibodies to rabbit (1:200) immunoglobulin for 30 min. After rinsing with PBS, the sections were exposed for 5-10 min to DAB. The slides were rinsed in water and counterstained with hematoxylin & eosin. Sections incubated with PBS, instead of the primary anti-serum, served as the negative controls. The sections were examined using light microscopy and analyzed with a computer-assisted color image analysis system (Image-ProPlus 5.0, Media Cybernetics, MD).

### Western blot

Proteins were extracted using the kit from Bipec according to the manufacturer's instructions (Bipec Biopharma Corporation, USA). We used a Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) to determine each sample's protein concentration. Equal amounts of protein (50  $\mu$ g) were fractionated on 14% SDS-polyacrylamide gels in running buffer [25 mM Tris, 192 mM glycine, 0.1% (wt/vol) SDS, pH 8.3] at 90 V and then electro-transferred to nitrocellulose membranes. Membranes were blocked at room temperature with 5% nonfat milk in Tris-buffered saline (25 mM Tris, 137

mM NaCl, and 2.7 mM KCl) containing 0.05% Tween-20 (TBS-T) and then incubated overnight at 4°C with the following primary antibodies:  $\beta$ -actin (Santa Cruz, USA. Dilutions: 1:1000, Weight molecular of  $\beta$ -actin: 43 kD), rabbit polyclonal anti-rat p-MYPT-1 (Thr850) antibody (Upstate, USA. Dilutions: 1:1500. Weight molecular of p-MYPT-1: 80 kD), rabbit polyclonal anti-rat p-Akt antibody (Bipec Biopharma Corporation, USA. Dilutions: 1:1000. Weight molecular of p-Akt: 60 kD). Then the membranes were washed three times in TBS-T and incubated with the corresponding secondary antibody (Santa Cruz, USA. Dilutions: 1:1000) conjugated to horseradish peroxidase at room temperature. Immunoreactive bands were visualized with the SuperSignal West Pico enhanced chemoluminescence kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Band intensities were quantified using a densitometer analysis system Quantity One (Bio-Rad, USA).

### Statistics

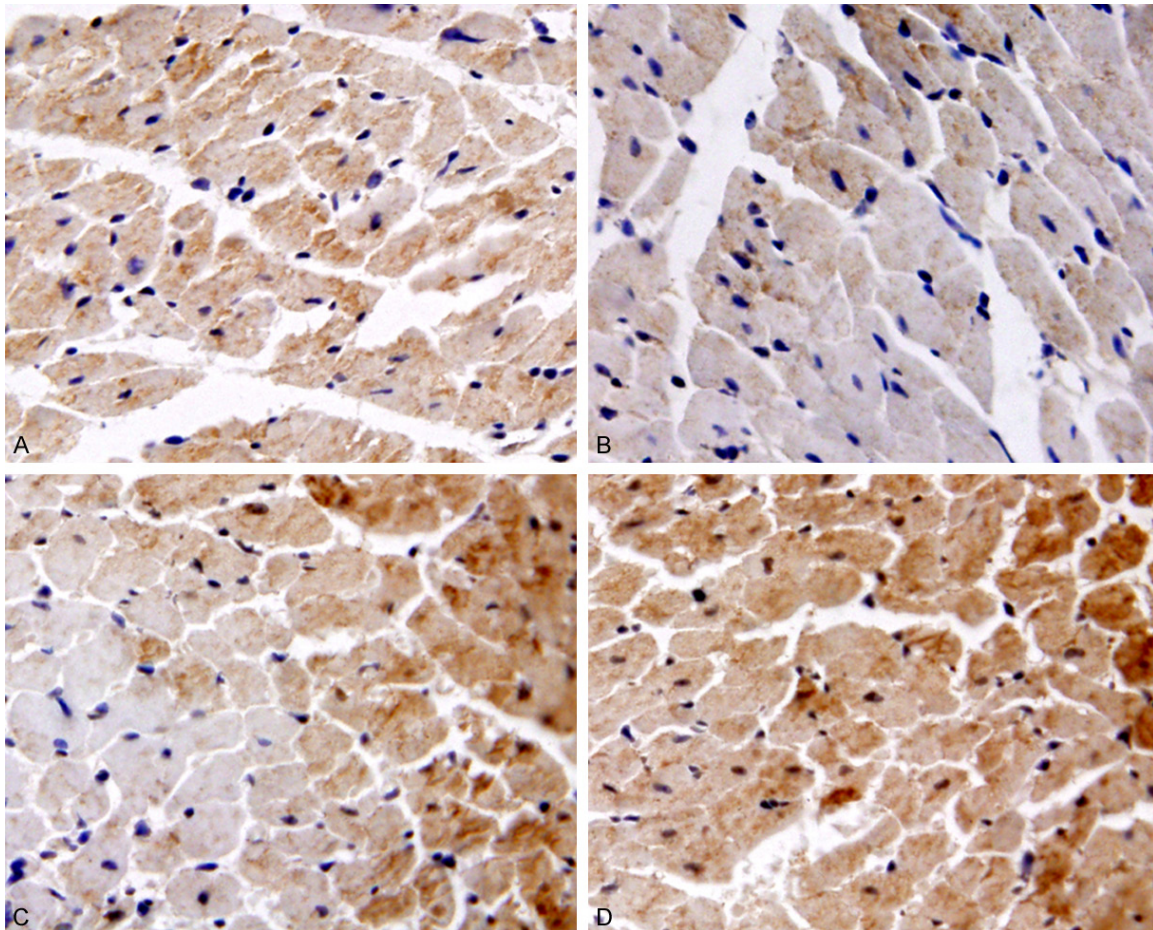
Values were expressed as means  $\pm$  SD. Statistical analysis of the results was carried out by one-way analysis of the variance followed by the Newman-Keuls test. Significance was defined at  $P < 0.05$ .

### Results

#### Effect of IPC on the activation of Rho-kinase

Rho-kinase activity was assessed by examining phosphorylation of MYPT-1, a well established





**Figure 3.** Representative immunohistochemical staining for p-Akt in the myocardium of different groups of rats (A: Sham group; B: I/R group; C: IPC + fasudil group; D: IPC group).

Rho-kinase specific substrate. Western blot analysis was performed to evaluate Rho-kinase activity. As shown in **Figure 4**, p-MYPT-1 increased during the I/R protocol. This increase in MYPT-1 phosphorylation was reversed in IPC, demonstrating that Rho-kinase activity decreased in IPC.

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**Figure 1** showed the TUNEL positive cells. TUNEL positive cells were expressed as a percentage of normal nuclei. The number of TUNEL positive cells was significantly increased in I/R group ( $32.78 \pm 5.1\%$ ). The TUNEL positive cells were significantly reduced to  $17.05 \pm 4.2\%$  in I/R + fasudil group and  $17.29 \pm 0.84\%$  in IPC group ( $P < 0.05$  vs I/R group). These data suggest that inhibition of Rho-kinase activity in I/R reduces cardiomyocyte apoptosis and cell apoptosis was also attenuated in IPC.

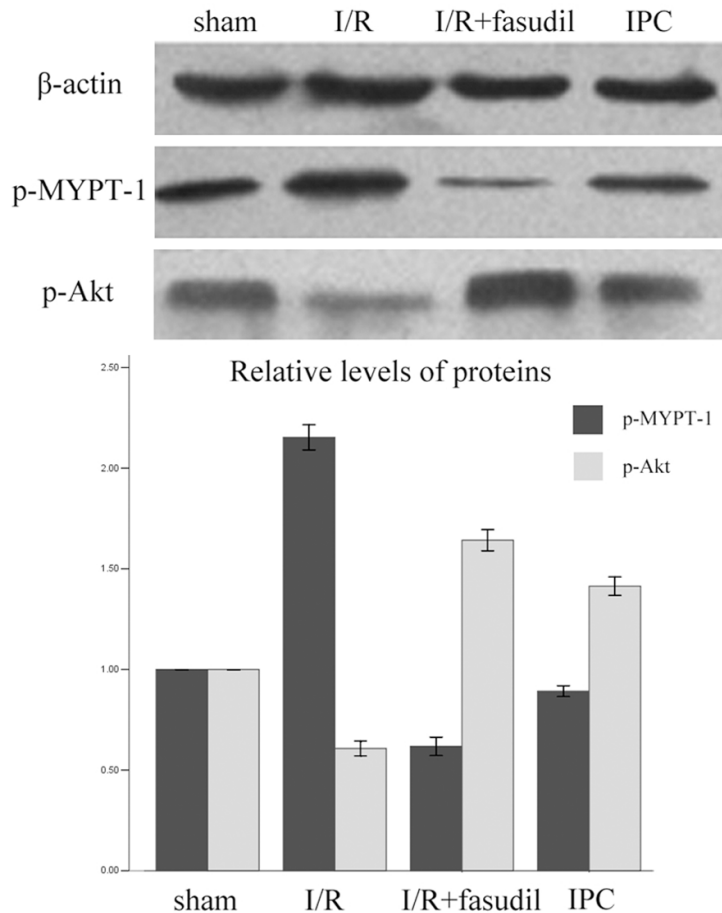
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As shown in **Figure 2**, the AAR and infarct size of the heart were  $52.62 \pm 2.73\%$  and  $59.89 \pm 3.83\%$ , respectively in I/R group. Administration of fasudil caused significant reduction of AAR and infarct size. The AAR and infarct size of the heart were  $32.07 \pm 3.0\%$  and  $38.62 \pm 2.66\%$ , respectively in I/R + fasudil group. These data suggest that inhibition of Rho-kinase activity reduces myocardial infarct size in I/R injury. In IPC, the AAR and infarct size were  $27.92 \pm 1.54\%$  and  $29.16 \pm 1.08\%$  ( $P < 0.05$  vs I/R group). These data suggest that AAR and infarct size were attenuated in IPC.

*IPC and Rho-kinase inhibition during I/R increased the activation of Akt*

Representative pictures of p-Akt expression in the myocardium of different groups of rats are

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**Figure 4.** Western blot analysis of p-MYPT-1 and p-Akt in rats hearts of different groups (sham, I/R, IPC + fasudil, IPC). After administration of fasudil, the amount of p-MYPT-1 was significantly attenuated ( $*P<0.05$  vs I/R) and the amount of p-Akt increased ( $^{\#}P<0.05$  vs I/R). The amount of p-Akt increased in IPC group ( $^{##}P<0.05$  vs I/R), while the amount of p-MYPT-1 attenuated in IPC group ( $^{**}P<0.05$  vs I/R).

shown in **Figure 3**. Western blot analysis for the phosphorylation of Akt also served to determine the activity of Akt in IPC. As shown in **Figure 3**, p-Akt expression was clearly increased in the heart in IPC group and I/R + fasudil group. As shown in **Figure 4**, p-Akt increased in rat I/R treated with the Rho-kinase inhibitor and was markedly increased in IPC group. These data suggest that Akt was strongly activated in IPC.

### Discussion

This study shows that IPC attenuates myocardial infarct size and cardiomyocyte apoptosis during reperfusion, and the activation of PI3-kinase/Akt pathway may be involved via inhibition of Rho-kinase.

Previously, we have reported that activation of Rho-kinase by heart I/R increased myocardial infarct size and cell apoptosis. The inhibition of Rho-kinase was able to reduce the heart infarct size and cardiomyocyte apoptosis in heart I/R injury in vivo [15]. Previous studies have demonstrated that I/R up-regulated expression of RhoA in ischemic myocardium and subsequently increased Rho-kinase activity [16]. In this study, we found that the activation of Rho-kinase increased in the myocardium following I/R and the activation was reversed in IPC. In the present study, the activity of Rho-kinase was assessed by examining phosphorylation of MYPT-1. IPC resulted in the decrease in the amount of p-MYPT-1, indicating the activation of Rho-kinase was reduced in the myocardium following IPC. We also demonstrated that IPC produced an attenuation of myocardial infarction and myocardial apoptosis. In this study, we examined apoptotic myocardial cell death with TUNEL assay. The percentage of TUNEL-positive cardiomyocytes after 30 min ischemia and 180 min reperfusion was  $32.17 \pm 3.6\%$ . IPC resulted in a 46% reduction in apoptotic cardiomyocytes. These results suggest that Rho-kinase activity and cardiomyocyte apoptosis were reduced by IPC.

Recent studies have shown that inhibition of Rho-kinase leads to the activation of the PI3-kinase/Akt pathway and cardiovascular protection [17]. Inhibition of Rho-kinase protects against I/R injury exclusively during reperfusion through a mechanism dependent on constitutive PI3K/Akt signaling [18]. Rho-kinase activation could represent a reperfusion injury kinase by contributing to negative regulation of the PI3K/Akt RISK pathway. In the present study, we found that the inhibition of Rho-kinase was able to increase the phospho-Akt (p-Akt) level (**Figure 4**) in I/R. Then we attempted to find whether the phosphorylation of Akt was different in IPC. These data show that Akt was

strongly activated in IPC, assessed by immunoblotting and immunohistochemistry. Rho-kinase is a negative regulator of the pro-survival PI3-kinase/Akt pathway [19].

The mechanism can be proposed which the effects of Rho-kinase inhibition on PI3-kinase/Akt and apoptosis in IPC. Rho-kinase may regulate p-Akt levels by regulating phosphatase and tensin homologue (PTEN) activity. PTEN is in inactive state in the cytosol and translocates to the membrane upon RhoA or Rho kinase-induced activation, where it can inhibit the phosphorylation of Akt via PI3-kinase [20]. Rho-kinase inhibition by IPC or fasudil may prevent the translocation and activation of PTEN, thereby preventing its inhibitory effect on Akt phosphorylation.

In conclusion, we have shown that Rho-kinase inhibition by IPC is a major mechanism in reduced cardiomyocyte apoptosis. The inhibition of Rho-kinase leads to cardiovascular protection might be mediated by the activation of PI3-kinase/Akt during IPC. This may provide new treatment possibilities for patients suffering coronary heart diseases in the future.

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### Disclosure of conflict of interest

None.

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