

# Protective effects of fasudil hydrochloride post-conditioning on acute myocardial ischemia/reperfusion injury in rats

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## Abstract

**Background:** *In recent years, the alleviation of acute myocardial ischemia and reperfusion injury (MI/RI) during myocardial reperfusion has presented a significant clinical challenge. This study was performed to investigate the effects of fasudil hydrochloride (FH) post-conditioning on MI/RI and the underlying mechanism.*

**Methods:** *Seventy-two rats were randomly divided into four groups: a Sham group, an ischemia/reperfusion (I/R) group, a fasudil hydrochloride (FH) group, and a fasudil hydrochloride+PI3K inhibitor (FH+I) group. Myocardial infarct size, cell apoptotic index (AI), and myocardial tissue expression of Rho-associated coiled-coil containing protein kinase 1 (ROCK1), Bcl-2, Bcl-2 associated X protein (Bax), caspase-3, Akt and phosphorylated Akt (P-Akt) were detected.*

**Results:** *All these parameters, except Akt expression, were higher in the I/R group than in the Sham group ( $p < 0.05$ ). Compared to the I/R group, myocardial infarct size, AI, Bax and caspase-3 expression were significantly reduced in the FH group ( $p < 0.05$ ), while Bcl-2 expression was increased ( $p < 0.05$ ). However, the myocardial infarct size and AI of the FH+I group were similar to those of the I/R group ( $p > 0.05$ ). Compared to the FH group, Bcl-2 expression was reduced in the FH+I group, while Bax and caspase-3 expression was increased ( $p < 0.05$ ). Furthermore, P-Akt expression in the FH group was significantly higher than that of the I/R group ( $p < 0.05$ ).*

**Conclusions:** *FH post-conditioning alleviated MI/RI, with narrowing of the infarct size and decreased apoptosis of ischemic cardiocytes. The mechanism was associated with activation of the PI3K-Akt signaling pathway. (Cardiol J 2013; 20, 2: 197–202)*

**Key words:** fasudil hydrochloride (FH), ischemia/reperfusion, post-conditioning

## Introduction

Acute myocardial infarction (AMI) is a common acute disease in cardiology with a high mortality rate [1, 2]. Clearing obstructed vessels rapidly and effectively is critical for the treatment of AMI [3].

However, reperfusion is closely associated with metabolic dysfunction and structural damage to the cardiocytes, which is known as myocardial ischemia/reperfusion injury (MI/RI) [4]. Therefore, alleviating MI/RI during myocardial reperfusion represents a significant clinical challenge. Re-

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searchers [5] have proposed ischemic pre-conditioning (IPC) to address this issue. But the application value of IPC is rare in AMI patients. Ischemic post-conditioning (IPost) was first demonstrated in 2003 in a study of ischemia/reperfusion in dogs [6] and its clinical value in protecting the myocardium and improving prognosis was subsequently verified [7, 8]. However, the application of IPost is also limited as it is time-consuming and carries numerous serious complications. In recent years, a variety of agents such as adenosine, bradykinin (BK) and erythropoietin have been shown to activate the same signaling pathways that IPC and IPost mediate, mitigating MI/RI [9, 10]. This is known as pharmacological post-conditioning (PPost) [11]. PPost has become a significant focus of clinical research, with encouraging application prospects for its convenience and effectiveness.

The mechanism underlying MI/RI includes oxygen free radical injury, calcium overload, endothelial dysfunction and cell apoptosis, which is an important contributing factor. Decreased Bcl-2 and increased Bcl-2 associated X protein (Bax) induces opening of the mitochondrial permeability transition pore (mPTP), resulting in the release of effectors such as cytochrome c from mitochondria, initiating the caspase cascade, leading to cell apoptosis [12–15]. However, reperfusion injury salvage kinase (RISK) inhibits apoptosis by blocking the process [15–17]. During early reperfusion, Rho associated coiled-coil forming protein kinase (ROCK) was activated, suppressing the RISK pathway [18, 19].

Fasudil hydrochloride (FH), which is the first specific ROCK inhibitor that has been applied in clinical practice, functions by competing with ATP for the ATP binding site in the Rho kinase catalytic domain [20]. Ichinomiya et al. [21] have shown that high-dose FH preserves post-conditioning against myocardial infarction via the activation of m-KATP channels [21]. However, many other specific mechanisms about FH postconditioning are still unclear. So the discovery of more details, for example the relationship between FH and ROCK expression, FH and apoptosis, would be valuable. Previously, intravenous injection was the most commonly adopted route of administration, while intracoronary injection was rare. Researchers [22] have hypothesized that intracoronary injection might improve the efficiency of PPost, based on the observation that intracoronary administration of edaravone was more effective in the protection of the myocardium during MI/RI. Increasing experience of percutaneous interventions in clinical practice makes intracoronary injection possible. Furthermore, it is thought

that direct application of the drug to the target organ will limit the reduction in the drug concentration due to distribution to various organs, thus greatly improving the utilization rate of the drug.

In this study, an *in vivo* myocardial ischemia and reperfusion model was established in rats by ligating the coronary artery with a balloon. Intracoronary injection was adopted to test the effectiveness of FH post-conditioning and to explore the possible apoptosis mechanism.

## Methods

Animals in this work were treated in accordance with all guiding principles in the care and use of animals. All procedures were approved by the local ethical committee.

## Reagents

Fasudil hydrochloride (HAI077, Chuan Wei, lot-number 1011051, Tianjin Chase Sun Pharmaceutical Corporation, China), nitrotetrazolium blue chloride (NBT, Shanghai Haoran Biotechnologies Corporation, Ltd., China), Rho-associated coiled-coil containing protein kinase 1 (ROCK1) antibody, Bcl-2 antibody, Bax antibody and caspase-3 antibody (Abcam Corporation, Ltd., UK), Akt and phosphorylated Akt (P-Akt) antibodies (Cell Signal Corporation, Ltd., US).

## Myocardial ischemia and reperfusion model

Healthy and specific pathogen free male Sprague Dawley (SD) rats ( $n = 72$ ,  $250 \pm 10$  g) were selected. The animals were provided by the Experimental Animal Center of the Second Military Medical University and maintained in 12 cages (six per cage) at  $23 \pm 2^\circ\text{C}$ , and 60–65% relative humidity and given free access to food and water.

The *in vivo* myocardial ischemia and reperfusion model was established in rats by the ligating the coronary artery with a balloon. Animals were anesthetized and respiration was assisted with a ventilator. A polyethylene plastic catheter (0.8 mm internal diameter) connected to the Power Lab physiological experimental system was inserted into the right common carotid artery until a characteristic left ventricular pressure wave was observed. The chest was opened via the fourth or fifth left lateral thoracotomy. The pericardium was opened and the heart was exposed. A high-pressure balloon was placed on the left anterior descending artery (LAD). The myocardial ischemia was performed by ligating the LAD with the balloon ( $3.0 \times 10$  mm, 12 atm) with a 6-0 silk suture around the

LAD at 1 mm distal to the left atrial appendage. The reperfusion was performed by emptying the balloon (0 atm) to re-open the LAD.

SD rats ( $n = 72$ ) were randomly divided into four groups (18 per group) as follows: (1) The Sham group: the LAD was encircled by a 6-0 silk suture without ligation for 180 min; (2) Ischemia/reperfusion (I/R) group: after ligation for 60 min, the pressure was adjusted to 0 atm to restore blood flow for 120 min; (3) Fasudil hydrochloride (FH) group: after ligation for 60 min, the pressure pump was adjusted to 0 atm. A micro-injection pump was connected to the polyethylene plastic catheter in the right common carotid artery. The catheter was pulled from the left ventricle to the aortic root, where the left ventricular pressure wave disappeared. FH (500  $\mu\text{g}/\text{mL}$ , dissolved in normal saline) was pumped into the aortic root (500  $\mu\text{g}/\text{kg}/\text{min} \times 5$  min), followed by reperfusion for 115 min; (4) Fasudil hydrochloride + PI3K inhibitor (FH+I) group: at 10 min before ligation, the PI3K inhibitor LY-294002 (75  $\mu\text{g}/\text{mL}$ , dissolved in normal saline) was administered (dose, 1 mL/kg). Subsequently, animals received identical treatment to the FH group.

### Immunohistochemical analysis

At the end of reperfusion, six SD rats randomly selected from each group were sacrificed by cervical vertebra dislocation. Hearts were removed and washed with normal saline. After being dried with filter paper, the total ventricle was weighed. The ventricle was cut into five slices of equal thickness from the cardiac apex to the cardiac base, in parallel with the atrioventricular groove. Tissue slices were stained with 0.1% NBT at 37°C (10–20 min) and excess dye was washed off with normal saline. Infarcted myocardium was not stained, while non-infarcted myocardium was stained black. After acquisition of digital photographs, the stained myocardium was removed and the unstained myocardium was weighed. The weight ratio of the infarcted myocardium to the total ventricle was calculated to determine the myocardial infarct size.

### Terminal deoxy-nucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay

Another six SD rats were randomly selected from each group. Myocardial blocks from anterior wall of left ventricle were cut off, fixed with neutral formalin for 24 hours and embedded in paraffin. Apoptotic cells were detected using terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay according to the in-

structions provided by the manufacturer. Normal myocardial nuclei were stained blue, while apoptotic nuclei were brown as visualized by general microscopy (400  $\times$  magnification). Five fields were randomly selected in each section for enumeration of TUNEL-positive myocytes and total myocytes. The percentage (%) of TUNEL-positive myocytes was calculated to represent the apoptotic index.

### Western blot analysis

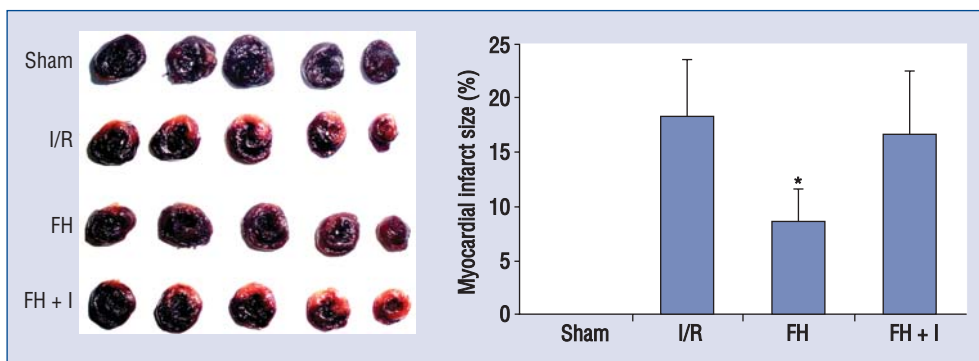
A further six SD rats in each group were randomly selected. Hearts were removed. Total cellular protein isolated from the myocardium was separated by SDS-PAGE, and transferred to a nitrocellulose membrane. Target bands were detected by immunoblotting with various primary antibodies (ROCK1, Bcl-2, Bax, caspase-3, Akt, P-Akt). The membranes were probed with horseradish peroxidase conjugated secondary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence and quantified by densitometry.

### Calculations and statistics

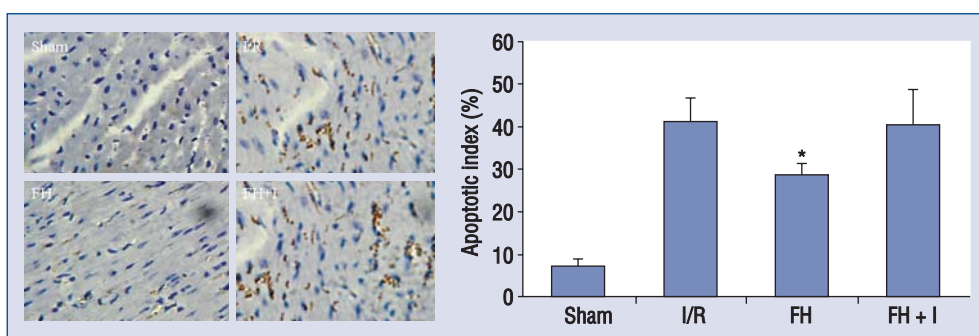
Data is presented as the mean  $\pm$  SD from at least three independent experiments. Statistical analysis was performed with Student's t-test by SPSS 17.0 software. A value of  $p < 0.05$  was considered statistically significant.

## Results

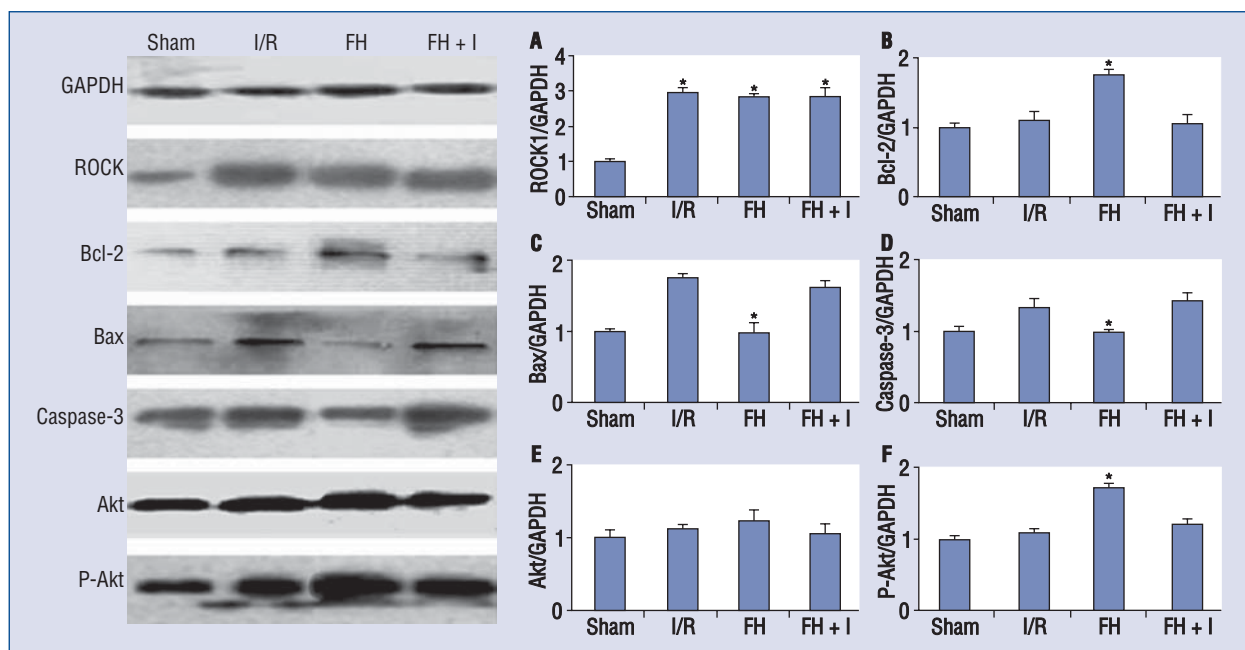
1. FH reduced the myocardial infarct size in the acute myocardial ischemia-reperfusion model (Fig. 1). In the Sham group, the myocardial infarct size was 0. The myocardial infarct sizes in the FH group ( $8.62\% \pm 3.04\%$ ) were reduced significantly compared to those of the I/R group ( $18.25\% \pm 5.19\%$ ;  $p < 0.05$ ). There was no difference in myocardial infarct sizes between the I/R group and the FH+I group ( $16.70\% \pm 5.80\%$ ;  $p > 0.05$ ).
2. FH decreased apoptosis in cardiocytes following reperfusion (Fig. 2). Few apoptotic cells ( $7.11\% \pm 1.24\%$ ) were detected in the Sham group. However, the number of apoptotic cells significantly increased in the other three groups ( $p < 0.05$ ). Compared to the I/R group ( $41.12\% \pm 5.47\%$ ), significantly fewer apoptotic cells were detected in the FH group ( $28.56\% \pm 2.87\%$ ;  $p < 0.05$ ), although there was no significant difference between the I/R group and the FH+I group ( $40.40\% \pm 7.89\%$ ;  $p > 0.05$ ).
3. ROCK1 expression in myocardial tissues was not influenced by FH (Fig. 3A). Compared to



**Figure 1.** Myocardial infarct size in the acute myocardial ischemia-reperfusion model in rats in each group. FH narrowed the myocardial infarct size. Compared to I/R group; \* $p < 0.05$ .



**Figure 2.** Apoptotic index of cardiocytes in each group. FH decreased apoptosis in ischemic cardiocytes. Compared to I/R group; \* $p < 0.05$ .



**Figure 3.** Expression of ROCK1, Bcl-2, Bax, Caspase-3, Akt and P-Akt in myocardial tissues in each group by Western blot. Compared to the Sham group, ROCK1 expression was increased by FH; \* $p < 0.05$  (A). Compared to the I/R group, FH increased the expression of Bcl-2 and P-Akt in the acute myocardial ischemia reperfusion model (B, F), while it decreased the expression of Bax and Caspase-3 (C, D), \* $p < 0.05$ . Compared to the I/R group, Akt expressions were not influenced by FH, \* $p > 0.05$  (E)

the Sham group, the ROCK1 protein expression in the other groups increased significantly ( $p < 0.05$ ), although there was no statistically significant difference between these groups ( $p > 0.05$ ).

4. FH increased the expression of Bcl-2 in the acute myocardial ischemia-reperfusion model (Fig. 3B). There was no significant difference in Bcl-2 expression between the Sham group and the I/R group ( $1.11 \pm 0.12$ ;  $p > 0.05$ ). Compared to the I/R group, Bcl-2 expression was increased in the FH group ( $1.76 \pm 0.08$ ;  $p < 0.05$ ). Furthermore, no difference was detected between the I/R group and the FH+I group ( $1.05 \pm 0.12$ ;  $p > 0.05$ ).
5. FH decreased Bax expression in the acute myocardial ischemia-reperfusion model (Fig. 3C). Compared to the Sham group, Bax expression was significantly increased in the I/R group ( $1.75 \pm 0.06$ ;  $p < 0.05$ ). Compared to the I/R group, Bax expression was decreased in the FH group ( $0.98 \pm 0.14$ ;  $p < 0.05$ ). There was no difference between the I/R group and the FH+I ( $1.61 \pm 0.11$ ) group ( $p > 0.05$ ).
6. FH decreased the expression of caspase-3 in the acute myocardial ischemia-reperfusion model (Fig. 3D). Compared to the Sham group, caspase-3 expression was significantly increased in the I/R group ( $1.32 \pm 0.12$ ;  $p < 0.05$ ). Compared to the I/R group, caspase-3 expression was decreased in the FH group ( $0.97 \pm 0.06$ ;  $p < 0.05$ ). No significant difference was found between the I/R group and the FH+I group ( $1.42 \pm 0.11$ ;  $p > 0.05$ ).
7. Akt expression in myocardial tissues was not influenced by FH (Fig. 3E). There were no significant differences in Akt expression between the groups.
8. FH promoted the expression of P-Akt in the acute myocardial ischemia-reperfusion model (Fig. 3F). Compared to the Sham group, no significant differences in P-Akt expression were detected between the I/R group ( $1.09 \pm 0.06$ ), and the FH+I group ( $1.21 \pm 0.06$ ;  $p > 0.05$ ), while P-Akt expression increased significantly in the FH group ( $1.73 \pm 0.05$ ;  $p < 0.05$ ).

## Discussion

Ischemia-reperfusion injury is a major obstacle to the therapy of AMI. The ability to reduce ischemia-reperfusion injury represents a clinical challenge [4]. PPost has gradually become an important way of alleviating MI/RI, replacing IPC and IPost

with their limited clinical value and numerous complications.

FH is an effective drug to reduce MI/RI. In this study, we detected that FH significantly narrowed the myocardial infarct size when ischemic myocardium of rats received reperfusion (Fig. 1). The result was consistent with that of previous studies concerning fasudil post-conditioning [21], verifying that FH preserved post-conditioning against myocardial infarction.

Myocardial ischemia and reperfusion is a complicated pathological process [23–25]. Although FH has been proved to be able to activate m-KATP channels to induce PPost [21], many other mechanisms are still valuable. As the inhibitor of ROCK, FH plays a heart protection role via inhibiting the activity of ROCK by competing for the ATP binding site in the ROCK catalytic domain [20]. ROCK, which is also known as Rho kinase and Rho-associated kinase, is the most extensively studied downstream target of Rho [26]. Rho/ROCK is widely distributed in mammalian tissues and cells, and performs molecular switch and signal transduction functions. Hamid et al. [18] showed that ROCK was activated during early reperfusion. Inhibition of ROCK at reperfusion onset limited infarct size through an Akt/eNOS-dependent mechanism, suggesting that ROCK action at reperfusion may be deleterious through suppression of the RISK pathway [18].

In our study, we found that ROCK expression was upregulated in the acute myocardial ischemia-reperfusion models, although it was not influenced by FH (Fig. 3A). So we speculated that FH PPost was solely associated with the activity of ROCK, not the expression.

Reperfusion injury salvage kinase (RISK) is a protein kinase involved in the maintenance of cell survival. In the early stages of myocardial reperfusion, RISK is activated by a kinase cascade and plays an important role in myocardial protection by inhibiting apoptosis [27]. Apoptosis is an important mechanism for MI/RI [23–25]. So we studied the relationship between FH and apoptosis. We found that FH significantly reduced cell apoptosis in ischemia-reperfusion myocardium (Fig. 2). Bcl-2 expression was increased (Fig. 3B), while Bax and Caspase-3 expression was decreased (Fig. 3C, D). However, these were abrogated following treatment with the PI3K inhibitor LY-294002 (Fig. 2, 3B–D), demonstrating that FH PPost was associated with the activation of PI3K-Akt. PI3K-Akt is an important part of the RISK signaling pathway, playing a critical role in heart protection induced by

ROCK inhibition [18]. Our study showed that the expression of Akt in ischemia-reperfusion myocardium was not influenced by FH (Fig. 3E), but the phosphorylation of Akt was promoted by it (Fig. 3F). This indicated that FH was able to inhibit cell apoptosis and protect myocardium against MI/RI via the activation of PI3K-Akt. And it was thought to be associated with the suppression of the activity of ROCK.

FH is a novel calcium antagonistic vasodilator marketed by Asahi Chemical Company in 1994 under the trade name Erill. Currently, it is mainly used as a vasodilator to treat cerebral vasospasm induced by subarachnoid hemorrhage, pulmonary hypertension, and cognition and memory dysfunction in Alzheimer's disease.

In this study, we have confirmed the post-conditioning effect of FH and found it was possibly due to the reduced incidence of apoptosis following reperfusion of cardiomyocytes. And the specific molecular mechanism may involve activation of PI3K-Akt signaling pathway. Therefore, FH may provide a novel agent for the protection of myocardium in myocardial ischemia-reperfusion.

### Conclusions

FH post-conditioning was able to alleviate MI/RI, with narrowing of the infarct size and decreased apoptosis of ischemic cardiocytes. The mechanism was associated with activation of the PI3K-Akt signaling pathway.

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**Conflict of interest:** none declared

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