Remote ischemic post-conditioning protects against myocardial ischemia/reperfusion injury by inhibiting the Rho-kinase signaling pathway

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Abstract. The aim of the present study was to observe the effect of Rho-kinase on remote ischemic post-conditioning (RIPostC) and explore the underlying mechanisms. Male Sprague Dawley rats (n=32) were randomly distributed into four groups: Sham group, ischemia/reperfusion (I/R) group, RIPostC group and I/R with fasudil group (I/R+Fas). Infarction size was detected by triphenyltetrazolium chloride staining. The levels of creatine kinase (CK), lactate dehydrogenase (LDH), superoxide dismutase (SOD), malondialdehyde (MDA) and cardiac troponin I (cTnI) were measured using an ultraviolet spectrophotometer. The mRNA expression levels of Rho-associated coiled-coil containing protein kinase (ROCK)-1 and ROCK2, B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) were detected via reverse transcription-PCR. The protein expression levels of phosphorylated-myosin phosphatase target subunit (p-MYPT1) and phosphorylated-myosin light chain (p-MLC) were assessed by western blotting. The results demonstrated that RIPostC could decrease the infarct size, the levels of CK, LDH, cTnI and MDA and increase the activity of SOD compared with the I/R group. In addition, the mRNA expression of ROCK1 and ROCK2 was downregulated, the protein expression of p-MYPT1 and p-MLC was decreased, and the ratio of Bcl-2/Bax was elevated in the RIPostC groups compared with the I/R group. Notably, the aforementioned index in I/R with Fas group was similar to the RIPostC group and no significant difference was observed between RIPostC and I/R+Fas.

These results revealed that RIPostC could attenuate I/R injury and the underlying mechanisms might be associated with a reduction in myocardial apoptosis and the suppression of the Rho-kinase signaling pathway.

Introduction

Coronary heart disease (CHD) is one of the leading causes of mortality worldwide, accounting for one-third of all mortalities worldwide each year (1). As a severe type of CHD, acute myocardial infarction (AMI) is the most common cause of mortality in China (2). The World Bank estimates that by 2030, 23 million Chinese patients will experience AMI annually (3). The most effective therapeutic measure against myocardial ischemia is to restore heart perfusion. However, the beneficial effects can be compromised by ischemia/reperfusion injury (I/R) (4). Myocardial I/R can induce further damage to the myocardium itself, leading to metabolic and functional disorders (5). The underlying mechanism of I/R injury may be associated with oxidative stress, the inflammatory response, mitochondrial damage and cell apoptosis (6). In view of the dreaded complication of reperfusion and the heavy social burden, it is urgently necessary to further explore the mechanism of I/R pathogenesis and develop effective intervention measures.

Remote ischemic post-conditioning (RIPostC), induced by several episodes of transient I/R intervention on distant tissues and organs away from the heart (such as the limbs, kidneys, small intestine and skeletal muscle), is as an effective strategy for myocardial protection against I/R injury (7). Due to its simple operation and significant curative effects, RIPostC can be performed noninvasively using a blood pressure cuff on the limb, and thus it has gained the attention of numerous researchers (8-10). RIPostC has been verified to have a beneficial effect in animal studies and randomized clinical trials (11-14). However, the underlying mechanism of RIPostC is still poorly understood (15).

Rho-associated coiled-coil containing protein kinase (ROCK), a serine/threonine kinase, has been identified as a downstream effector of RhoA. The ROCK isoforms, ROCK1 and ROCK2, were initially discovered as downstream targets of
Materials and methods

Experimental animals. A total of 32 male Sprague-Dawley (SD) rats (weight, 250-300 g; age, 7-8 weeks), were purchased from the Laboratory Animal Center of Bengbu Medical College. The rats had free access to a normal diet and fresh water. All rats were kept at a constant temperature (22-26°C) and humidity (50%) with a 12-h light/dark cycle and were raised in individual cages. The animal research study protocol was following ‘The Guide for the Care of Use of Laboratory Animals’ published by the National Institute of Health and were approved by the Animal Use and Care Committee of Bengbu Medical College (21).

Materials and reagents. Fasudil was purchased from Tianjin Red Sun Pharmaceutical Co., Ltd., (cat. no. 1604071). Creatine kinase (CK; cat. no. 20170626), lactate dehydrogenase (LDH; cat. no. 20160606), superoxide dismutase (SOD; cat. no. 20160608) and malondialdehyde (MDA; cat. no. 20160620) assay kits were purchased from Nanjing Jiancheng Institute of Biotechnology. The cardiac troponin I (cTnI) ELISA kit was purchased from Calvin Biotechnology Co., Ltd. (cat. no. CK-E30258R). TRizol® reagent was purchased from Invitrogen; Thermo Fisher Scientific, Inc. (cat. no. 15596026). PCR master mix was purchased from Invitrogen; Thermo Fisher Scientific, Inc. (cat. no. CK-E30258R). TRIzol (~1.5 ml per rat) were collected with disposable heparin blood collection tubes (1604071). The cTnI content was measured by an ELISA kit at a wavelength of 450 nm. The absorbances were measured and the contents were calculated according to the manufacturer's protocol.

Measurement of plasma CK, LDH, MDA and SOD. At the end of the reperfusion period, carotid artery blood samples (~1.5 ml per rat) were collected with disposable heparin blood vessels and centrifuged at 1,500 g for 15 min at 4°C. The supernatant fluid was collected and stored at -80°C. The MDA contents and LDH, CK and SOD activities were measured by spectrophotometer at wavelengths of 532, 440, 660 and 550 nm, respectively according to the manufacturer's protocol.

Assessment of myocardial infarct size. Infarct size was established by Evans blue and triphenyltetrazolium chloride (TTC) staining as described previously (21,24). After the rats were sacrificed, the hearts were removed immediately and washed 2-3 times with PBS. Using re-occlusion of LAD in isolated Langendorff-perfused equipment, the hearts were perfused with 0.3 ml 1% Evans blue dye to delineate the risk area. Following freezing at -80°C, the hearts were cut into 2 mm thick sections along the left ventricular transverse section and incubated with 1% TTC at 37°C for 20 min. Subsequently, the
sections were fixed in 4% paraformaldehyde solution at 37°C for 30 min. The gray area indicated the percentage infarct size (IS) and the red area represented the area at risk (AAR) was; these were quantified by computerized planimetry using ImageJ software (version 1.40; National Institutes of Health). Infarct size was calculated as the percentage of IS to AAR.

RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay detecting ROCK1, ROCK2, Bax and Bcl-2 mRNA expression. Total RNA was extracted from the ischemic myocardial tissue using TRIzol® reagent. Total RNA (3 µg) was reverse transcribed into cDNA at 42°C and 1.5 µl cDNA was utilized for PCR amplification with the following temperature protocol: Denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing (ROCK1, 58°C; ROCK2, 62.5°C; Bax, 62.5°C; Bcl-2, 62.5°C; and β-actin, 62.5°C) for 30 sec, and extension at 72°C for 30 sec, then 72°C for 10 min and held at 4°C. The primer sequences are presented in Table I. The PCR products were analyzed on a 1.2% agarose gel. The densitometry results for the ROCK1, ROCK2, Bcl-2 and Bax genes were compared with the corresponding β-actin levels to analyze the relative expression, and the Bcl-2/Bax ratio was calculated using a Tanon-3500 Gel imaging system (GIS 4.1.2).

Western blot analysis of the protein expression of MLC, p-MLC, MYPT1 and p-MYPT1. Left anterior myocardium tissues from each group were collected and homogenized in a lysis buffer, which contained 20 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Triton X-100, 0.1% SDS and 1% protease inhibitor cocktail. The homogenates were sonicated by 3-5 cycles (30 sec sonication and 10 sec rest) at a frequency of 25 kHz and 60% amplitude before they were centrifuged at 12,000 x g for 30 min at 4°C. The protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) (24). Total protein (45 µg) was separated by SDS-PAGE using a 10% separation gel and a 5% concentrated gel, and transferred to PVDF membranes. The membranes were blocked with QuickBlock™ Blocking solution at 37°C for 2 h and then were incubated with the primary antibodies at 4°C overnight. Immunoblots were performed using the following antibodies: Rabbit GAPDH antibody (1:4,000), rabbit MYPT antibody (1:500), rabbit p-MYPT1 antibody (1:2,000), rabbit MLC antibody (1:500) and mouse p-MLC antibody (1:2,000). All membranes were incubated for 1 h with the corresponding HRP-linked anti-rabbit IgG (1:8,000) or HRP-linked anti-mouse IgG (1:4,000) secondary antibodies. The membranes were analyzed by an ECL system. Gray values of the target bands were analyzed using ImageJ software.

Statistical analysis. All values were expressed as the mean ± standard deviation (n=8). Statistical comparisons were performed by one-way analysis of variance and the Newman-Keuls test using SPSS 16.0 software (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Changes in plasma CK, LDH, MDA and SOD levels in each group. Compared with the Sham group, the activities of CK and LDH, and MDA contents were significantly increased in the I/R group (P<0.01), while SOD activities were significantly decreased (P<0.01). Compared with the I/R group, the activities of CK and LDH, and MDA contents were significantly decreased (P<0.01), and the SOD activities were significantly elevated in the RIPostC and I/R+Fas groups (P<0.01). However, the levels of CK, LDH, MDA and SOD were no significant differences between the RIPostC and I/R+Fas groups (P>0.05; Fig. 1).

Changes in plasma cTnI levels in each group. Compared with the Sham group, the contents of cTnI were significantly increased in the I/R group (P<0.01). Compared with the I/R group, the contents of cTnI were significantly decreased in the RIPostC and I/R+Fas groups (P<0.01). However, there were no significant differences in cTnI levels between the RIPostC and I/R+Fas groups (P>0.05; Fig. 2).

| Gene            | Primer     | Sequence               | Product (bp) |
|-----------------|------------|------------------------|--------------|
| ROCK1           | Forward    | 5'-GCA AAT GCG GGA GTT ACA AG-3' | 314          |
|                 | Reverse    | 5'-CAA GCC GAC TAA CGG TAT GAT C-3' |              |
| ROCK2           | Forward    | 5'-AGA ACC TGT CAA GCG TGG TAG TG-3' | 339          |
|                 | Reverse    | 5'-GAC AGC CAT CCT TCT ATT CGT GA-3' |              |
| Bax             | Forward    | 5'-GGA TCG AGC AGA GAG GAT GG-3' | 464          |
|                 | Reverse    | 5'-TGG TGA GTG AGG CAG TGA GG-3' |              |
| Bcl-2           | Forward    | 5'-CTG GTG GAC AAC ATC GCT CTG-3' | 228          |
|                 | Reverse    | 5'-GGT CTG CTG ACC TCA CTT GTG-3' |              |
| β-actin         | Forward    | 5'-CTG TAT GCC TCT GGT CGT AC-3' | 214          |
|                 | Reverse    | 5'-TGA TGT CAC GCA CTA TTG CC-3' |              |

ROCK1, Rho-associated coiled-coil containing protein kinase 1; ROCK2, Rho-associated coiled-coil containing protein kinase 2; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2.
Changes in myocardial infarct size in each group. Compared with the I/R group, the myocardial infarct size was significantly decreased in the RIPostC and I/R+Fas groups (P<0.01). There were no significant differences between the RIPostC and I/R+Fas groups (P>0.05; Fig. 3).

Expression of ROCK1, ROCK2, Bax and Bcl-2 mRNA in the myocardium. The RT-PCR results revealed that, compared with those in the Sham group, the mRNA expression of ROCK1 and ROCK2 was significantly increased (P<0.01), whereas the ratio of Bcl-2/Bax was significantly decreased in the I/R group (P<0.01). However, compared with the I/R group, the expression of ROCK1 and ROCK2 was significantly decreased, and the ratio of Bcl-2/Bax was increased in the RIPostC and I/R+Fas groups (P<0.01). In contrast to RIPostC group, the expression of ROCK1 and ROCK2, and the ratio of Bcl-2/Bax exhibited no statistical differences in the I/R+Fas group (P>0.05; Fig. 4).

Protein expression of p-MLC, MLC, p-MYPT1 and MYPT1. Compared with the Sham group, the protein expression of p-MLC and p-MYPT1 was significantly increased in the I/R group (P<0.01). By contrast, the protein expression of p-MLC and p-MYPT1 in the RIPostC and I/R+Fas groups was decreased compared with the I/R group (P<0.01; Fig. 5). In contrast to those of the RIPostC group, there were no statistical differences in the I/R+Fas group (P>0.05; Fig. 4).
Discussion

In the present study, it was demonstrated that RIPostC could provide protection against myocardial I/R injury. Rho-kinase pathway served an important role in myocardial I/R injury. The present study also demonstrated that the Rho-kinase inhibitor fasudil produced attenuation of the myocardial infarction and myocardial apoptosis associated with I/R. It was further confirmed that RIPostC had a cardioprotective and anti-apoptotic effect through inhibition of the Rho-kinase signaling pathway.

RIPostC, which is induced by several episodes of brief I/R in distant organs from the heart, has been developed as
an effective strategy to protect against the harmful effects of I/R injury (26-29). Although the protective role has been widely recognized, the mechanism of the cardioprotective effect induced by RIPostC remains to be fully elucidated (15). Myocardial I/R injury can cause the generation of reactive oxygen species, calcium overload, the inflammatory response, mitochondrial damage, lipid peroxidation and further damage to the myocardial tissue (30). Myocardial infarction and the levels of LDH, CK and cTnI are frequently used to quantify the amount of myocardial damage (31,32). In addition, measuring MDA content and SOD activity can reflect the degree of myocardial cell damage and the production of oxygen free radicals (33).

The present study showed that RIPostC reduced the myocardial infarct size and inhibited the release of LDH, CK, cTnI and MDA, while increasing the activity of SOD. These results suggested that the RIPostC could effectively alleviate myocardial I/R injury. It was also demonstrated that the Rho-kinase inhibitor fasudil reduced infarct size, attenuated the increased levels of LDH, cTnI, CK and MDA, and reduced the activity of SOD, which had the cardioprotective effect similar to RIPostC. These observations indicated that inhibition of Rho-kinase and RIPostC could alleviate myocardial I/R injury by reducing the production of free radicals.

Rho-kinase is a 160 kDa serine/threonine protein kinase that acts as a downstream effector of the small G protein RhoA. Rho-kinase has two highly homomeric isoforms, ROCK1 and ROCK2, which regulate cell formation, migration, proliferation and apoptosis (17). Both ROCK1 and ROCK2 are expressed in vascular smooth muscle and the heart (17,18). An increasing body of evidence has demonstrated that the Rho-kinase pathway serves an important role in myocardial I/R damage and inhibition of the Rho-kinase signaling pathway has beneficial effects on cardiac functions and apoptosis (20,34). Apoptosis is an essential contributor to cardiac dysfunction (35). Bcl-2 family members serve important roles in regulating apoptotic signaling. The balance in the expression levels of the anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins play a major role in the regulation of myocardial apoptotic cell death (24). Moreover, activation of Rho-kinase enhances the contractions of vascular smooth muscle cells, leading to coronary artery spasm and aggravating myocardial injury (17,18). Certain studies have demonstrated that MLC and MYPT1 acted as the principal downstream effector protein of Rho-kinase, and their phosphorylation level could indirectly reflect the activity of Rho-kinase (36). Rho-kinase can increase MLC phosphorylation through direct effect on MLC or indirectly by inactivating MLC phosphatase (MYPT1) (37,38). Specifically, activation of Rho-kinase may increase calmodulin formation, upregulate the concentration of intracellular Ca\(^{2+}\) and induce the phosphorylation of MYPT1, which inhibits the activity of MLC phosphatase (MYPT1), causing MLC phosphorylation (37,39). In the present study, it was demonstrated that the expression of ROCK1 and ROCK2 were upregulated, the level of p-MLC and p-MYPT1 was increased, and the ratio of Bcl-2/Bax was decreased in the I/R group. However, the expression of ROCK1 and ROCK2...
were downregulated, the level of p-MLC and p-MYPT1 was decreased, and the ratio of Bcl-2/Bax was increased in the RIPostC group compared with the I/R group. Interestingly, the aforementioned index in the I/R with fasudil group was similar to RIPostC group and no significant difference was observed between RIPostC and I/R+Fas. The results revealed that RIPostC could downregulate the activity of Rho-kinase as well as inhibited the occurrence of myocardial apoptosis. These observations suggested that RIPostC could attenuate the I/R-induced injury in the myocardium by inhibiting the Rho-kinase signaling pathway through its anti-apoptotic effect.

There were several limitations in this study that are important to note. In the present study, it was only shown that the inhibition of the Rho-kinase was associated with RIPostC, but it was not investigated how RIPostC inhibits the Rho-kinase signaling pathway. Furthermore, the present results confirmed that RIPostC had a cardioprotective and anti-apoptotic effect through inhibition of the Rho-kinase signaling pathway, however, the study was not able to examine the Rho-kinase changes with pharmacological intervention. In addition, further analysis of cardiac function measurements and the direct evaluation of apoptosis are required to verify the cardioprotective effect of RIPostC by inhibiting the Rho-kinase pathway.

In conclusion, the present study demonstrated that RIPostC could reduce heart infarct size and cardiomyocyte apoptosis by reducing lipid peroxidation, suppressing oxidative stress and inhibiting Rho-kinase activity. Thus, the Rho-kinase signaling pathway may be an important mediator of RIPostC against myocardial I/R injury in vivo.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FM, QG and YY designed the study, performed the experiments and drafted the manuscript. FN, ZYH, YLH and HJS performed the experiments and collated the experimental data. XJJ contributed to experiments, data interpretation and statistical analysis. All authors revised the manuscript critically for important intellectual content and gave the final approval of the version to be published.

Ethics approval and consent to participate

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Bengbu Medical College of China (Bengbu, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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