ORIGINAL RESEARCH

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PI3K-Akt/eNOS in remote postconditioning induced by brief pulmonary ischemia

Abstract

Purpose: Postconditioning, a series of brief ischemia-reperfusion sequences given before an ischemic heart undergoes sustained reperfusion, has been shown to lessen ischemia/reperfusion injury. The current study establishes a rabbit model of myocardial ischemia-reperfusion and studied the effects of pulmonary remote postconditioning in this model.

Methods: Serum levels of creatine kinase (CK), superoxide dismutase (SOD), and malondialdehyde (MDA), protein expression of endothelial nitric oxide synthase (eNOS), Rho kinase (ROCK-2), and protein kinase B (Akt) in myocardial cells and the apoptosis index of myocardial cells were examined.

Results: Pulmonary remote postconditioning decreased CK, significantly decreased MDA, and increased SOD. Postconditioning significantly increased eNOS protein expression. Administration of eNOS inhibitor, L-NAME, dramatically suppressed the postconditioning-induced eNOS protein expression and serum SOD level, but significantly increased MDA level. The two longer sessions of postconditioning increased Akt, although this increase was not accompanied by changes in levels of the Akt inhibitor, ROCK-2. Blocking eNOS activity with L-NAME had no visible effect on either Akt or ROCK-2.

Conclusion: Our results suggest a role for Akt in remote postconditioning-induced myocardial protection, but do not support an involvement of eNOS in Akt-mediated action.

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When the heart has undergone a period of ischemia, either during cardiac bypass surgery or a myocardial infarction, reperfusion to the ischemic areas allows the anoxic tissue to recover but the re-oxygenation also causes injury through an increase in reactive oxygen species (ROS), as well as calcium overload, mitochondrial dysfunction and intracellular proteolysis in the ischemic area and by uncoordinated excess contractile activity [1]. This phenomenon is called ischemia-reperfusion injury [2]. One method to lessen this damage is postconditioning; a series of short ischemia-reperfusion sequences given after the ischemic episode and before continuous reperfusion is begun [3,4]. The postconditioning procedure has been performed on the heart muscle itself and remote postconditioning has been performed on other tissues or organs with similarly protective results [5,6].

Previous studies suggest that postconditioning activates endothelial nitric oxide synthase (e-NOS), and that this activation sets into effect downstream actions, via the phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt) pathway, that result in reduction of mitochondrial permeability transition pore (MPTP) opening, loss of the membrane potential, loss of ATP synthesis and, finally, apoptosis [3,7]. Clinical application of postconditioning has potential in interventional thrombolytic therapy; however, if the action site for postconditioning is the coronary artery, the potential risk is of ischemia-reperfusion injury great.

In 2005, Kerendi *et al.* reduced myocardial infarct size by inducing transient ischemic-reperfusion of the renal artery before myocardial reperfusion in rats [8]. They called this protective effect remote postconditioning [8]. This study confirmed that remote postconditioning is adenosine-dependent, and that its underlying mechanism is similar to that of myocardial pre- and postconditioning. Organs and tissues used in remote postconditioning studies have included kidney, lungs, distal trachea and limbs. Remote postconditioning uses safer sites than the coronary artery, and is therefore more promising for clinical application. The most convenient remote postconditioning sites are the limbs, and remote postconditioning using these sites has been reported to effectively reduce infarct size in pigs [9]. Using limbs as the action site has shortcomings, because the repeated expansion and deflation of the occlusive device may also cause complex effects on bones, muscles and nerves, and these effects may interfere with accurate interpretation when investigating the mechanism of the remote postconditioning.

The current study established a rabbit model of myocardial ischemia-reperfusion using Japanese large-ear rabbits and studied the effects of pulmonary remote postconditioning in this

model. Serum levels of creatine kinase (CK), superoxide dismutase (SOD), and malondialdehyde (MDA), expression of eNOS, Rho kinase (ROCK-2), and Akt in myocardial cells, and the apoptosis index of myocardial cells were examined. In this way, the role of PI3K-Akt/eNOS pathway and ROCK-2 in pulmonary remote postconditioning and myocardial protection was explored.

Methods

Experimental grouping

Healthy male Japanese large-ear rabbits ($n=70$), weighing $2.1 \text{ kg} \pm 0.2 \text{ kg}$, were purchased from Nanchang Longping Rabbit Co., Ltd. (Jiangxi, China). The study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Second Affiliated Hospital of Nanchang University. The general protocol followed was ligation of the descending coronary artery, varying periods of occlusion of the left pulmonary artery, injection of nitric oxide synthase inhibitor (L-NAME; Sigma, St. Louis, MO, USA) in some groups and 180 min coronary artery reperfusion. Animals were randomly divided into seven groups with 10 animals in each group: control group, ligation and reperfusion only; RIP1 group, ligation, 3 min occlusion, reperfusion; RIP2 group, ligation, 5 min occlusion, reperfusion; RIP3 group, ligation, 10 min occlusion, reperfusion; L1, ligation, 3 min occlusion, L-NAME given 3 min before reperfusion, reperfusion; L2 group, ligation, 5 min occlusion, L-NAME given 5 min before reperfusion, reperfusion; L3 group, ligation, 10 min occlusion, L-NAME given 10 min before reperfusion, reperfusion. Details of the time sequence for each group are shown in Figure 1.

Preparation of animal models of myocardial ischemia-reperfusion

Animals were anesthetized with an intravenous injection of 3 mg/kg sodium pentobarbitone (Sigma) through the marginal ear vein and fixed in a supine position on the operating table. A midline incision was made in the neck, and the trachea was dissociated, opened, cannulated, and connected to the animal ventilator (Medical instrument factory of Zhejiang University), which was set in the volume control mode at a tidal volume 21 ml, respiratory rate 60 times/min, inspiratory/expiratory ratio: 1:3. The left jugular vein was dissociated, and needle electrodes were placed into the muscles of the limbs for the ECG limb leads. Baseline ECG was then recorded (ECG machine purchased from Nihon Kohden Corporation, Japan). The skin was cut longitudinally on the left side of chest 1.0 cm away from the left parasternal line, and dissociated to expose the ribs. The internal mammary artery and vein were ligated in the second

FIGURE 1. Time lines for the seven protocols for pulmonary remote postconditioning.

RIP1, 3 min pulmonary occlusion; RIP2, 5 min pulmonary occlusion; RIP3, 10 min pulmonary occlusion; L1, 3 min pulmonary occlusion and L-NAME injection; L2, 5 min pulmonary occlusion and L-NAME injection; L3, 10 min pulmonary occlusion and L-NAME injection.

intercostal space using a No.1 mouse suture. The third and fourth ribs were removed, and the pericardium was lifted up using tweezers, cut open longitudinally using ophthalmic scissors and fixed onto the chest wall using a small curved forceps. The heart was exposed, and the left anterior descending coronary artery located on the left ventricular surface. A 6-0 pro-

lene suture was passed through the superficial myocardium under the left anterior descending coronary artery, 1.5 to 2.0 cm inferior to the lower edge of the left atrial appendage, and the left anterior descending coronary artery was clipped shut for 30 min using a rubber tube locking clip, followed by 180 min reperfusion. The ischemia-reperfusion model was exam-

ined using ECG, and successful model construction was indicated by ST segment elevation.

Preparation of the animal model of adaptation after pulmonary ischemia

After successful establishment of the reperfusion model, the small curved forceps were released. The pericardium and the heart were pushed to the right side to expose the left hilum. The left pulmonary artery was located inferior to the left superior pulmonary vein. Ophthalmic scissors were used to dissociate the pulmonary artery from the vein up to the left main bronchus. A non-invasive arterial clamp was used to occlude the left pulmonary artery close to the left main bronchus.

Determination of serum markers

Blood samples (2–4 mL) were collected from the left jugular vein at the following three times: after successful tracheal intubation, before reperfusion and after the 180 min reperfusion. The blood samples were immediately centrifuged at 4200 rpm for 5 min, and the serum collected and placed in an ice bag at -15 °C. The samples were then stored in a refrigerator at -20 °C until assayed.

Serum CK levels were measured using a creatine kinase (CK) test kit (Olympus Diagnostic Products Co., Ltd., Tokyo, Japan) and an Olympus AU2700 automatic analyzer (Olympus Corporation). Serum MDA levels were measured using a MDA test kit (Jiancheng Bioengineering Institute, Nanjing, China). Serum SOD activity was measured using a SOD test kit (Jiancheng Bioengineering Institute).

HE staining of the heart and lung tissues

At the end of the experiment, the animals were killed and bilateral lung tissues were collected. Specimens were cut at the levels inferior and superior to the hilum and rinsed with 0.9% sodium chloride solution before being fixed in neutral formalin. Procedures for embedding and slicing were same as those used for myocardial tissue (see subsequent paragraph). HE staining was then performed.

Immunohistochemical staining of the myocardial specimens

At the end of the experiment, the animals were killed and the heart was excised and cut open using a cross-shaped incision at the ligation place of the coronary artery. Heart tissue samples were rinsed with 0.9% sodium chloride solution and fixed in neutral formalin. Tissue samples were embedded in paraffin and serial sections with a thickness of 4 µm were cut 0.3 cm inferior to the cross-shaped incision of the coronary artery in

the coronary plane. The sections were then placed on slides covered with poly-L-lysine and heated at 58 °C for 1 h.

For immunohistochemical staining, the avidin-biotin peroxidase complex SABC method was used. Briefly, the primary antibody (anti-eNOS, anti-ROCK-2, or anti-Akt, SABC kit, Boster Biological Engineering Co., Ltd., Wuhan, China) was added and the sample was incubated at 37 °C for 1.5 hours. All primary antibodies were diluted 1:100. Biotinylated goat anti-mouse IgG was added and the sample was placed at room temperature for 20 min and rinsed with PBS. SABC was added and the sample placed at room temperature for 20 min. Colour was developed using a DAB chromogenic kit: DAB was mixed well, added onto the slides and the color was developed at room temperature for 18 min, after which the samples were slightly stained with hematoxylin.

Ten visual fields (x400) were randomly selected from each slice under light microscopy (Olympus). A digital camera was fixed in front of the microscope using a tripod. The aperture and shutter values were fixed. The white balance was adjusted manually, and pictures were taken with the 4x optical zoom. Positive expression was indicated by dark brown or yellow brownish staining of the cytoplasm. Image-Pro Plus 6.0 image analysis measurement software was used for image analysis, and the positive area and the average optical density of each photograph were calculated.

Detection of apoptosis in myocardial specimens

Myocardial tissue was conventionally embedded and sliced and a terminal deoxynucleotidyl transferase (TUNEL) assay (Promega, Fitchburg, WI, USA) was performed according to the manufacturer's instructions.

Statistical analysis

Continuous variables were compared using a one-way analysis of variance (ANOVA). When a significance between groups was apparent, multiple comparisons of means were performed using the Bonferroni procedure with type-I error adjustment. Data are presented as means ± SD. All statistical assessments were two-sided and evaluated at the 0.05 level of significant difference. Statistical analyses were performed using SPSS 15.0 statistics software (SPSS Inc, Chicago, IL, USA).

Results

Lung tissue histology.

Figure 2 shows representative photographs of heart and right (ischemic) and left (non-ischemic) lung tissues after transient

FIGURE 2. Representative HE staining pictures of (A) heart and (B,C) lung tissues after transient ischemia-reperfusion cycles. (A) No obvious change in heart tissue. (B) Ischemic lung shows alveolar collapse and RBC infiltrate (arrows). (C) Non-ischemic lung shows no obvious changes. Magnification: heart, 200x; lung, 400x.

ischemia-perfusion cycles. Pathological examination of lung tissue showed that the transient ischemia-reperfusion cycles caused severe alveolar collapse and RBC infiltration in the ischemic lung (Figure 2B). The histology of the contralateral non-ischemic lung tissue showed no obvious damage (Figure 2C). Therefore, the 3 to 10 minute ischemia-reperfusion cycles were considered safe.

CK, MDA, and SOD levels.

There were no significant differences among the seven groups in serum concentrations of CK and MDA and activity of serum SOD before occlusion or before reperfusion ($P > 0.05$, Figure 3). In the control group, the 180 minute reperfusion after ischemia increased the levels of CK and MDA, the respective biomarkers for myocardial damage and oxidative stress [10], and decreased levels of SOD, the biomarker for antioxidant ability [11] (Figure 3). The 3 min, 5 min and 10 min remote postconditioning protocols all partly reversed the reperfusion-induced increase in CK and MDA. Inhibiting eNOS with L-NAME prevented the postconditioning effects on CK and MDA from taking place. Only the changes in MDA induced by postconditioning and their reversal by L-NAME reached statistical significance (Figure 3B). The other changes induced by postconditioning on CK and their reversal by L-NAME, although consistent with the supposition that nitric oxide synthesis is responsible for the postconditioning effect on CK, were not statistically significant (Figure 3A).

Although at 180 min reperfusion, all three remote postconditioning protocols showed increased levels of SOD, the biomarker for antioxidant activity, compared with control levels at that time, this increase reached statistical significance only for the 5 minute postconditioning protocol (Figure 3C). Inhibiting eNOS with L-NAME decreased the increase in SOD activity, and this decrease was statistically significant compared with the corresponding postconditioning increase for the 5 and 10 min postconditioning time periods.

Addition of the NOS inhibitor L-NAME, as expected, blocked the significant increase in eNOS caused by postconditioning (Figure 4B). Neither postconditioning nor eNOS inhibition affected the expression of ROCK2 (Figure 4C), a negative regulator of the P13/Akt pathway and inhibitor of apoptosis. The 5 and 10 min postconditioning protocols did, however, cause an increase in the Akt/P13 activity, as shown by the increased number of Akt positive cells (Figure 4A). This increase was unaffected by eNOS inhibition. Figure 5 shows representative photographs of Akt, eNOS, and ROCK-2 staining myocardial tissues in control, RIP, and L groups.

The representative TUNEL staining pictures of myocardial tissues in control, RIP and L groups are shown in Figure 6A-G. All postconditioning protocols showed trends to decreased apoptosis, which were blocked by eNOS inhibition (Figure 6H); however, these results were only trends and did not reach statistical significance for any protocol.

FIGURE 3. Serum levels of CK and MDA and activity of serum SOD among the groups.

*Indicates a significant difference between the given group and control group.

§Indicates a significant difference between the RIP1 and L1 groups.

||Indicates a significant difference between the RIP2 and L2 groups.

¶Indicates a significant difference between the RIP3 and L3 groups.

FIGURE 4. Numbers of Akt, eNOS and ROCK-2-positive cells in myocardial tissue among the groups at the end of the 180 min reperfusion period.

*Indicates a significant difference between the given group and control group.

†Indicates a significant difference between the given RIP group and RIP1 group.

‡Indicates a significant difference between the given L group and L1 group.

§Indicates a significant difference between the RIP1 and L1 groups.

||Indicates a significant difference between the RIP2 and L2 groups.

¶Indicates a significant difference between the RIP3 and L3 groups.

FIGURE 5. Representative immunohistochemistry photographs of Akt-, eNOS-, and ROCK-2-positive cells in myocardial tissues after reperfusion. Left column: Akt; middle column, eNOS; right column, ROCK-2. Row 1, Control; rows 2-4, L-NAME injected groups; rows 5-7, pulmonary ischemia groups. The corresponding positive cells in each photograph are brown. Magnification: Akt and eNOS (200X); ROCK-2 (400X).

FIGURE 6. Apoptosis in myocardial tissue among the groups revealed by TUNEL assay. (A-G) The representative TUNEL staining pictures of myocardial tissue. A, Control; B, RIP1; C, RIP2; D, RIP3; E, L1; F, L2, G, L3. (H) The histogram of TUNEL results for all groups.

Discussion

Our results showed pulmonary remote postconditioning produced a slight but not significant decrease in CK, a significant decrease in oxidative stress (MDA), and an increase SOD that reached significance only for the 5 min postconditioning protocol. Postconditioning significantly increased eNOS; an effect blocked by the eNOS inhibitor, L-NAME. Inhibiting the postconditioning induced an increase in eNOS, a slight but not significant decrease in the postconditioning effect on CK, and a significant decrease in the postconditioning effect on MDA. The two longer sessions of postconditioning increased Akt although this increase was not accompanied by changes in levels of the Akt inhibitor, ROCK-2. Blocking eNOS activity with L-NAME had no visible effect on either Akt or ROCK-2.

One effect of postconditioning is to trigger the myocardial reperfusion injury salvage kinase (RISK) pathway, a pathway that achieves myocardial protective effects through activating the phosphoinositide kinase - protein kinase B (PI3K-Akt) pathway and extracellular signal-regulated kinase (ERK1/2) [12]. Cultured endothelial cells transfected with IGF-1 and VEGF can induce synthesis of eNOS through the PI3K pathway [13]. Akt and eNOS can be co-localized at the cell membrane, causing Akt to be phosphorylated, and PI3K inhibitors can reduce Akt activity and phosphorylation levels. Activated Akt phosphorylates Bcl-2 antagonist of cell death (BAD), thus inhibiting expression of apoptosis genes and exerting an anti-apoptotic role [14]. Our results support a role for Akt in remote postconditioning-induced myocardial protection, but do not support an involvement of eNOS in this pathway.

ROCK2 plays a negative regulatory role in the PI3K/Akt system [15], by phosphorylating and activating phosphatase and tensin homolog (PTEN), an upstream negative regulator of this system. Inhibiting ROCK2 increases Akt signal expression, reduces PTEN phosphorylation and leads to release of NO [16]. ROCK can increase apoptosis by inducing Bax and caspase-3 expression and subsequent action on the mitochondria. Administration of a ROCK inhibitor has been shown to significantly reduce cardiomyocyte apoptosis, showing myocardial protective effects [17]. Our results do not support a role for ROCK2 in remote postconditioning.

Remote postconditioning in the kidney has been reported to be adenosine-dependent [3]. Remote postconditioning in human limbs has been reported to provide myocardial protection from ischemia-reperfusion injury through protection of vascular endothelial cells [18]. Our study did not investigate whether adenosine or endothelial cells play a role in remote postconditioning, so can provide no further insight in this regard, but this could be tested using this experimental model.

Currently it is believed that only transient ischemia-reperfusion cycles performed at the very beginning of the long reperfusion can produce protective effects. A study in rat models suggested that the myocardial protective effects would disappear if the timing of postconditioning was postponed for even one min [19]; however, the time window for postconditioning varies in different animal species, and there is no consensus on the appropriate duration of the transient ischemia-reperfusion cycle or on the appropriate number of cycles. Zhang *et al.* [20] found that three cycles of 10 s ischemia-reperfusion achieved myocardial protection in mice. When the number of cycles was increased from three to six, no further protective effect on the myocardium was observed. In dogs, the duration of ischemia-reperfusion is usually 30-60 s. In human studies, the duration of ischemia-reperfusion is commonly 1 min. The length of the ischemia-reperfusion cycles used tends to increase with increase in body weight. This phenomenon may be related to the differences in the xanthine oxidase activity among different animal species [21]. Our study showed that increasing the duration of the ischemia-perfusion cycle repetitions beyond 3 minutes produced no additional protection.

The kidney was the first organ used to induce remote postconditioning; however, the kidney is sensitive to ischemia-reperfusion, and transient ischemia in this organ can lead to impaired kidney function [22]. Loukogeorgakis *et al.* [18] and Xu *et al.* [23] conducted studies on remote postconditioning using limbs as the postconditioning site. In remote limb postconditioning, a tourniquet is commonly used as a non-invasive means to induce ischemia-reperfusion cycles. The use of a tourniquet cannot control the duration of ischemia-reperfusion accurately or ensure the complete occlusion of the blood flow; therefore, it is difficult to use in small animal models. The lung is also a commonly used organ for inducing remote postconditioning. The lungs can better tolerate ischemia-reperfusion injury than the kidney, perhaps because they have a double blood supply (pulmonary artery and bronchial artery) and occluding the pulmonary artery does not completely prevent oxygenation. Malik *et al.*'s study suggested that 6 to 12 hour ischemia-reperfusion caused lung injury within an acceptable range [24]. Xu *et al.* [25] analyzed patients with lung cancer who underwent surgical treatment and found that occlusion of the pulmonary artery and pulmonary vein for 20-72 min separately caused no serious complications, suggesting that human lungs have relatively strong tolerance for ischemia-reperfusion injury. The present study adopted the lungs as the remote organ because of the aforementioned advantages. In

accordance with these results, our protocol produced only a slight change in lung histology.

A question that was not examined in the current study was whether the periods of left pulmonary artery occlusion that were part of the postconditioning procedure caused increased afterload in the right heart and therefore decreased preload and cardiac demand in the left heart. Because the entire pulmonary circulation was not blocked completely, the increase in right arterial blood flow should have compensated for the blockage of the left pulmonary artery.

Animal studies in dogs showed that the most common heart complications after pneumonectomy were fibrillation and sinus tachycardia, but that no short-term functional impairment was seen. [26] In our study on rabbits, the left pulmonary artery was blocked for only 3-10 min, which should not have an impact on left ventricular function. In humans, studies on patients with pneumonectomy showed that long-term blockage of unilateral pulmonary circulation can be tolerated [27,28] In addition, an animal study in rats reported that transient ischemia-reperfusion cycles in the lung protect the lung from injury caused by an attendant long ischemia-reperfusion period. [29] This result illustrates the safety of unilateral pulmonary artery blockage. Clinical application of our results should therefore be feasible and safe, since short-term pulmonary artery blockage should have a relatively small impact on circulation and cardiac function. This study has other limitations. The effect of L-NAME was determined only on preconditioning and not on ischemia-reperfusion itself. Cardiac functional data, such as dp/dt, left ventricular stroke work or ejection fraction, and cardiac output were not measured; thus, the effects of preconditioning on cardiac performance should be examined in future studies.

In conclusion, our results support a role of Akt in remote postconditioning-induced myocardial protection, but do not support an involvement of eNOS in this pathway. Nor do they support a role of ROCK2 in remote postconditioning.

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