

Original Article

Myocardial ischemic post-conditioning attenuates ischemia reperfusion injury via PTEN/Akt signal pathway

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Abstract: Objectives: To investigate whether myocardial ischemic post-conditioning attenuates ischemia reperfusion injury via PTEN/Akt signal pathway. Design: Forty-five male Sprague-Dawley rats were randomly divided into three groups: Sham, Ischemia reperfusion (I/R) and Ischemic post-conditioning (IPost) group. After the experiment finished, myocardial infarction area was examined. Serum creatine phosphokinase and lactate dehydrogenase activity were detected at baseline and the end of reperfusion. The protein levels of PTEN, Akt, p-Akt, Bax and Bcl-2 were measured by Western blot method. Results: Myocardial infarct size was significantly reduced in IPost as compared to I/R. Results were confirmed by serum creatine phosphokinase and lactate dehydrogenase activity. In addition, PTEN and Bax protein expression were inhibited and the p-Akt and bcl-2 protein expression were enhanced in IPost compared with I/R ($P < 0.05$). At the same time, the ratio of Bax and Bcl-2 was decreased in IPost ($P < 0.05$). However, ischemic post conditioning did not affect the total Akt level ($P > 0.05$). Conclusions: We confirmed that ischemic post-conditioning protects the heart against reperfusion injury. It is important that we demonstrated that the cardioprotective effect of ischemic post-conditioning was involved in the inhibition of PTEN, activation of the PI3K/Akt pathway and reduction of the cardiomyocyte apoptosis.

Keywords: PTEN, post-conditioning, ischemia reperfusion injury, Akt, apoptosis

Introduction

In recent years, the morbidity and mortality of coronary artery disease gradually grow [1]. Coronary artery disease is becoming a common disease with many patients dying each year due to myocardial infarction [2]. It can be estimated that coronary artery disease will probably become an important cause of death among global diseases in 2020 [3]. Prolonged ischemia and reperfusion result in cardiac cell death and ventricular dysfunction. Protecting the heart against ischemia reperfusion injury may reduce disease mortality [4, 5]. Ischemic preconditioning was first reported that 4 cycles of 5 minutes of ischemia alternating with 5 minutes of reperfusion limited infarct size by 75% in dog hearts [6]. It has been demonstrated to reduce the infarct size, prevent appearance of reperfusion arrhythmias, diminish apoptosis and enhance the recovery of cardiac function

[7, 8]. Ischemic post-conditioning was reported as another powerful endogenous protective way to against the ischemia reperfusion injury by Zhao in 2003 in which a short series of repetitive cycles of brief reperfusion and re-occlusion of the coronary artery was applied immediately at the onset of reperfusion [9]. Because of the unpredictability of the ischemia, the application of ischemic preconditioning was limited in clinical.

Phosphatase and tensin homologue deleted chromosome 10 (PTEN) is a tumor suppressor gene, which was discovered in 1997 by Li et al [10]. It is a regulator in cell growth, apoptosis, migration, signal transmission, etc. In the past years, the studies mainly concerned about PTEN in tumors [11]. In recent years, studies have found that PTEN/Akt signal pathway plays an important role in myocardial remodeling, cardiac hypertrophy, myocardial fibrosis and myocar-

dial ischemia reperfusion injury [12-14]. There has been reported that application of specific drugs to inhibit the activity of PTEN can reduce the myocardial infarction size and improve heart function [15]. PTEN activity is reduced after preconditioning with cycles of brief ischemia reperfusion and restored when the protective effect of preconditioning decays [16]. In the present study, we tested the hypothesis that myocardial ischemia post-conditioning may attenuate ischemia reperfusion injury through PTEN/Akt signal pathway.

Material and methods

Animal preparation

Forty-five male Sprague-Dawley rats weighing 300 ± 20 g were purchased from Shandong University of Traditional Chinese Medicine Animal Center (Jinan, China) (production license: SCXK (lu), 20110003). The study has been approved by Ethics Committee of Shandong University.

Experiment groups

A total of 45 rats were randomly divided into three groups ($n = 15$). 1) Sham group (Sham): Rats were subjected to the surgical procedures without the left anterior descending artery (LAD) occlusion; 2) Ischemia reperfusion group (I/R): All rats were subjected to 30 min LAD occlusion, followed by 3 h of reperfusion; 3) Ischemic post-conditioning group (IPost): After 30 min of LAD occlusion, reperfusion was initiated for 30 s followed by 30 s reocclusion, repeated for three cycles, then followed by 3 h reperfusion.

Surgical procedure

All animals were anesthetized with 10% of chloral hydrate (3 ml/100 g) through the intraperitoneal injection. During the experiment, additional anesthetic was administered as needed. After anesthesia, the animals were placed in a supine position and the electrodes of electrocardiogram (ECG) machine (XDH-3, Shanghai, China) were placed subcutaneous of limb to record the ECG. The rats were intubated and mechanically ventilated using an animal ventilator (CWE-830, Beijing, China). A left lateral thoracotomy (3 cm incision between the third and fourth ribs) was performed to expose the heart. After pericardiotomy, a 6-0 silk suture

was placed around the LAD at 2 to 3 mm from the tip of the left auricle. The end of the suture was threaded through a piece of tubing, forming a snare for reversible LAD occlusion. The presence of myocardial ischemia was confirmed by significant ST segment elevation indicated by ECG. Successful reperfusion was defined as complete ST segment resolution.

Analysis of myocardium at risk of infarction and infarct area

At the end of experiment, the coronary artery was reoccluded in the original position (except for Sham group), and 3 ml of 1% Evans blue dye was injected from the apex cordis. The non-ischemic zone (NIZ) was stained blue, thereby the area at risk (AAR) outlined. The heart was quickly excised after the dye was uniformly distributed. The left ventricular (LV) tissues were dissected and kept 30 min at -20°C . The frozen tissues were sliced into five sections each one of which was about 2 mm thick. And the AAR (uncolored by blue dye) was separated from the NIZ (colored by blue dye). Then the non-stained AAR was incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 for 15 min to differentiate the unstained gray area of necrosis (AN) from the stained AAR (brick red). The AAR was expressed as a percentage of the LV mass (AAR/LV), and the AN was expressed as a percentage of the AAR (AN/AAR). The mass of each area was calculated by tissue weight.

Measurement of serum creatine kinase (CK) and lactate dehydrogenase (LDH)

Serum CK and LDH concentration were determined as other indicators of myocardial damage induced by ischemia and reperfusion. Before ischemia and at the end of reperfusion, blood samples were collected and centrifuged at 3500 rpm for 10 min. The levels of serum CK and LDH were measured by a colorimetric method using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocols. The results were expressed as U/L.

Western blot analysis

At the end of experiment, protein was extracted and determined using a BCA Protein Assay Kit. Equivalent amounts of protein for each sample were separated by 10% SDS-PAGE. The samples were subsequently transferred to polyvi-

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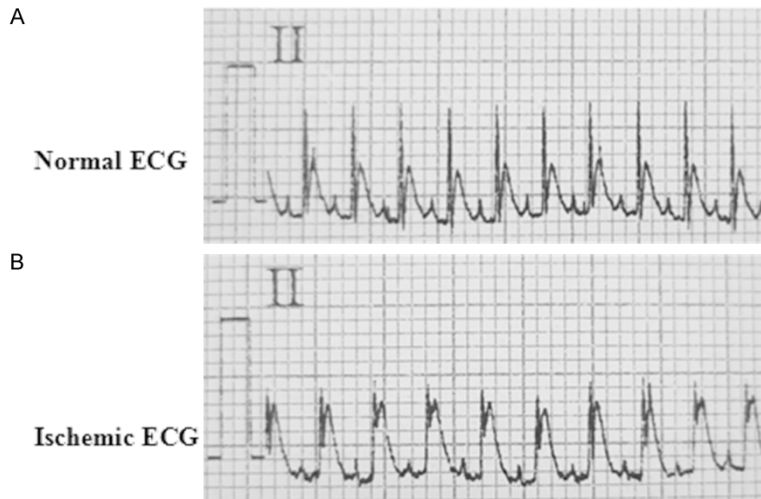


Figure 1. Normal and ischemic ECG. A. Normal ECG in lead II. B. Ischemic ECG in lead II.

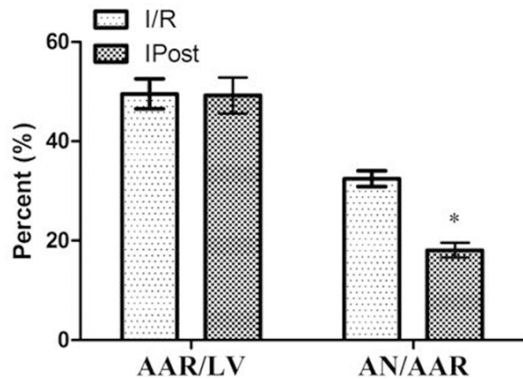


Figure 2. Bar graph showing area at risk (AAR) expressed as a percentage of the left ventricle (LV) and area of necrosis (AN) expressed as a percentage of the AAR. I/R = ischemia reperfusion; Ipost = ischemic post-conditioning. As shown in this figure, the AAR/LV (%) was similar between the I/R and IPost. However the tissue necrosis (AN/AAR) was significantly decreased in IPost. *P < 0.05 IPost vs. I/R. Values are means \pm SD.

nylidene difluoride membranes (PVDF) and blocked with 5% nonfat dry milk. The membranes were then incubated over night at 4°C with antibodies against PTEN, Akt, phospho-Akt (Ser473), Bcl-2, Bax (Cell Signaling Technology) and β -actin (Beijing Zhongshan Golden Bridge Biological Technology CO, LTD, China). After three washes in TBST, the membranes were incubated with a secondary antibody (Beijing Zhongshan Golden Bridge Biological Technology CO, LTD, China) for 1 h at room temperature. Signals were detected with an enhanced chemiluminescence (ECL) kit (millipore).

Statistical analysis

All data were expressed as means \pm standard deviation (SD). Differences between groups were analyzed by one-way analysis of variance (ANOVA). A P-value less than 0.05 was considered to be statistically significant. Data analyses were performed with the SPSS 17.0 software.

Results

ECG

The change of ST segment is the main indicator to define the success of ischemia and reperfusion model. The presence of myocardial ischemia was confirmed by significant ST segment elevation indicated by ECG in lead II (Figure 1).

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Area at risk and infarct size

Rats in the Sham were not performed the LAD occlusion, so there was no obvious ischemia and infarct. As shown in Figure 2, the area placed at risk by LAD occlusion was comparable between I/R (49.55 \pm 3.01%) and IPost (49.21 \pm 3.60%). However, infarct size in IPost was significantly decreased compared with that in I/R (18.08 \pm 1.51% vs. 32.46 \pm 1.57%).

Serum CK and LDH activity

The CK and LDH activity were used to confirm myocardial infarct size quantified by TTC staining. As shown in Table 1, both CK and LDH activity were no statistical differences among the three groups at baseline (P > 0.05). During ischemia and reperfusion, they were significantly increased in I/R and IPost. At the end of reperfusion, the activity of CK and LDH in IPost were significantly less than that in I/R (P < 0.05). Meanwhile, the differences in I/R and IPost were consistent with those observed about infarct size.

The expression of PTEN, Akt, p-Akt, Bcl-2 and Bax protein

As western blot analysis shown in Figure 3, the expression of PTEN and Bax protein were inhibited and the expression of p-Akt and Bcl-2 pro-

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Table 1. The activity of CK (U/L) and LDH (U/L)

Group	Base		R3h	
	CK	LDH	CK	LDH
Sham	528.4±88.7	989.1±115.5	537.2±109.9	964.9±141.5
I/R	545.9±115.6	955.7±123.4	1219.2±157.5*	2247.5±189.6*
IPost	541.2±83.4	934.3±145.8	810.4±117.2*#	1451.7±159.9*#

Base = baseline; R3h = end of reperfusion 3h; CK = creatine kinase; LDH = lactate dehydrogenase; I/R = ischemia reperfusion; IPost = ischemic post-conditioning. *P < 0.05 I/R, IPost vs Sham at 3 h of reperfusion. #P < 0.05, IPost vs. I/R at 3 h of reperfusion. Values are means ± SD.

tein were enhanced in IPost relative to I/R (P < 0.05). Thus, the ratio of Bax and Bcl-2 (Bax/Bcl2) was decreased in IPost (P < 0.05). However, compared with I/R group, ischemic postconditioning did not affect the total Akt level (P > 0.05).

Discussion

PTEN is a potent negative regulator of PI3K activity in many cell types [17], and thus the inhibition of PTEN is a potential target for the induction of protection against ischemia reperfusion injury. In this study, we confirmed that ischemic post-conditioning could protect the heart against reperfusion injury. Ischemic post-conditioning induces downregulation of PTEN activity leading to Akt activation and cardiac protection. Ischemic post-conditioning reduced infarct size and decreased the activity of CK and LDH in rats. The results of LDH, CK levels and infarct size in this study were also consistent with our previous report [18].

It is well documented that cardiomyocyte apoptosis plays an important role in the pathogenesis of myocardial ischemic reperfusion injury [19]. Many studies have confirmed that suppressing of apoptosis could minimize cardiac injury and prevent myocardial damage induced by ischemic reperfusion. Bcl-2 family is the dominant regulators of apoptosis. It mainly consists of both antiapoptotic substrates such as Bcl-2 and Bcl-xl, and proapoptotic substrates including bad, Bax, etc. Korsmeyer [20] reported that Bcl-2/Bax is a rheostat that regulates an antioxidant pathway and cell death as early as 1993. The life or death depends on the balance of Bcl-2 and Bax in cell. Bax/Bcl-2 is always as an index of apoptotic activation. In this study, we showed that ischemic post-conditioning could increase expression of Bcl-2 and decrease the levels of Bax. Thus, the ratio of

Bax and Bcl-2 (Bax/Bcl-2) was decreased.

PI3K/Akt is an intracellular signaling pathway, which involve in the control of cell growth, proliferation, survival and migration [21]. It is well known that the activation of the PI3K/Akt pathway is essential for cardioprotection [22]. PI3K, when activated, has the ability to phospho-

phorylate PIP2 into the secondary messenger PIP3 and lead the activation of Akt. When Akt is activated, it may product its antiapoptotic effects via the phosphorylation of two categories of downstream substrates: (a) the antiapoptotic substrates, when phosphorylated, become active, such as: Bcl-2, eNOs, p70s6k and (b) the proapoptotic substrates which, when phosphorylated, become inactive, such as bad, Bax, caspase9, GSK-3β [23]. PTEN is a dual lipid and protein phosphatase, discovered by Li et al [10]. PTEN is the main negative regulator of PI3K/Akt pathway. PTEN can dephosphorylate PIP3 to PIP2, thereby blocking the PI3K signaling pathways. In the previous research, there existed the study about PTEN gene deletion in myocardial ischemia reperfusion model. Siddall [24] reported that PTEN haploinsufficiency alone does not induce cardioprotection in this model. However, it reduces the threshold of protection induced by ischemic post-conditioning. In the PTEN^{+/-} hearts in which protection against infarction could be achieved with less cycles than in the case of the littermate^{+/+} hearts. Rats treated with Bpv (a PTEN inhibitor) in ischemia reperfusion model limited the myocardial infarct size and improved the left ventricular function [15]. Similarly, Parajuli et al [12] use adenoviruses to transfect PTEN gene into the peri-infarct area of WT mice. They discovered that the activation of PTEN was involved in post-myocardial infarction remodeling through the Akt/interleukin-10 signaling pathway. In recently, a pharmacological mechanism study demonstrated that inhibition of PTEN and activation of Akt contributed to the anti-oxidant capacity and cardioprotection of ABPP [25]. In our study, we showed that ischemic post-conditioning treatment decreased PTEN level, increased p-Akt level, activated the antiapoptotic Bcl-2, and inhibited the activation of proapoptotic Bax. It indicates that ischemic

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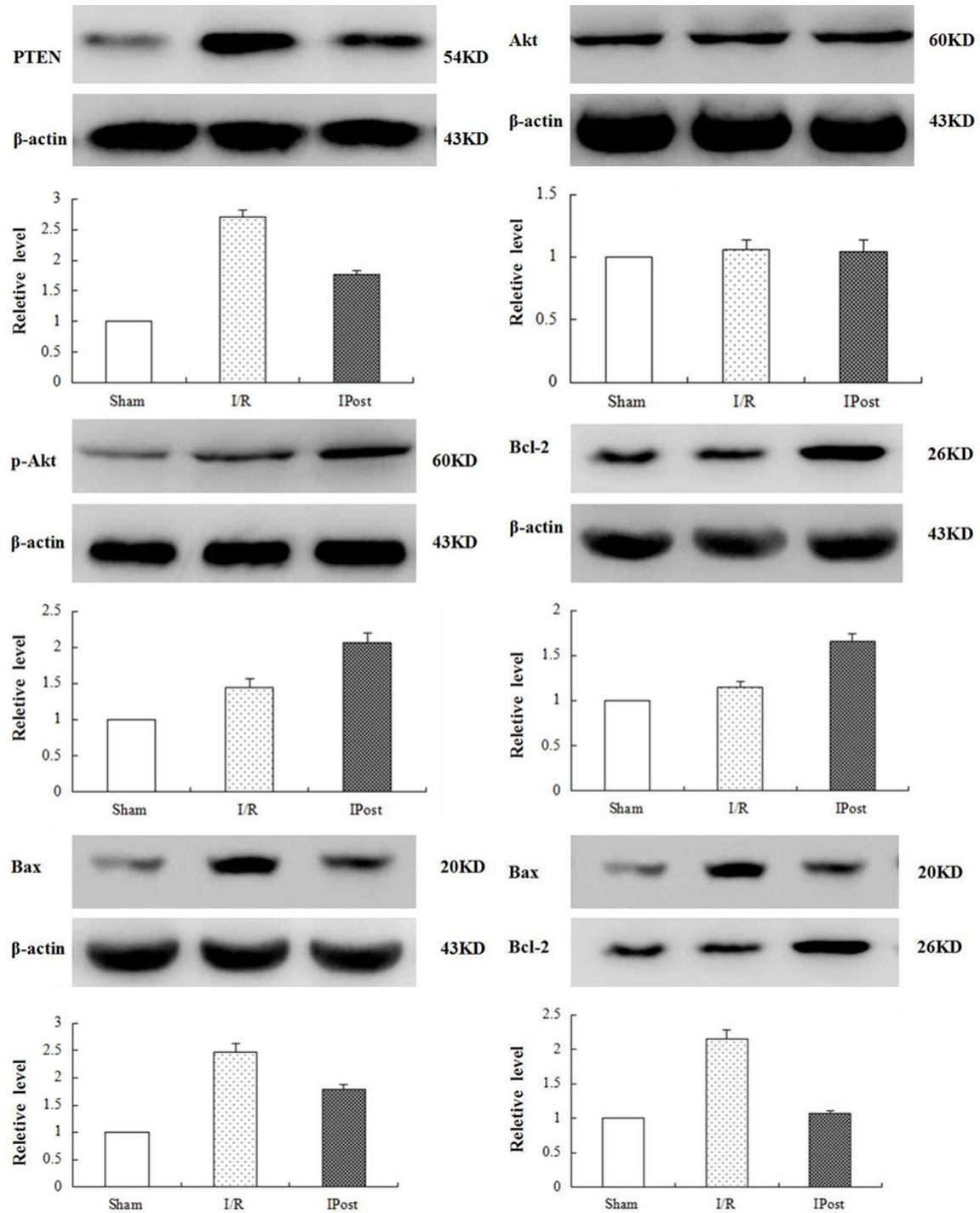


Figure 3. The expression of PTEN, Total Akt, p-Akt, Bcl-2 and Bax protein level.*P < 0.05 IPost vs. I/R. Values are means \pm SD.

post-conditioning blocks PTEN and activates PI3K/Akt prosurvival kinase pathway. Decreasing PTEN level is vital for post-conditioning to attenuate myocardial ischemia reperfusion injury.

Thus, it can be seen that ischemic post-conditioning protects the heart against reperfusion injury through the PTEN/Akt signal pathway. In addition, suppressing the expression of PTEN is an effective way to protect the myocardium

against reperfusion injury. It is a great opportunity to investigate cardioprotection of PTEN inhibition in ischemic heart disease. There needs further studies from basic research to clinical bedside.

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Disclosure of conflict of interest

None.

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