Am J Transl Res 2015;7(12):2603-2611
www.ajtr.org /ISSN:1943-8141/AJTR0018920

Original Article

Later phase cardioprotection of ischemic post-conditioning against ischemia/reperfusion injury depends on iNOS and PI3K-Akt pathway

Gongming Wang[1*], Xin Li[1*], Hong Wang[2], Yan Wang[1], Ligong Zhang[1], Le Zhang[1], Bei Liu[1], Mengyuan Zhang[1]

[1]Department of Anesthesiology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, China; [2]Department of Nephrology, Taian Central Hospital, Taian 271000, China. [*]Equal contributors.

Received November 1, 2015; Accepted December 7, 2015; Epub December 15, 2015; Published December 30, 2015

Abstract: Background: The cardioprotection of ischemic post-conditioning (IPO) has been well demonstrated after a short period of reperfusion. However, little is known about the long-term effects of IPO. This study aimed to investigate the long term cardioprotection of IPO in a rat myocardial ischemia/reperfusion model and to explore the potential mechanism. Methods and results: Rats were either sham-operated (Sham group) or underwent 30-min left anterior descending coronary artery ischemia followed by immediate reperfusion (I/R group) or post-conditioning with 5 cycles of 10-s ischemia and 10-s reperfusion (IPO group). At 24 h after reperfusion, infarct size reduced from 34.7±1.1% in I/R group to 24.9±1.3% in IPO group ($P<0.05$) and the iNOS expression in IPO group was 4.7-fold higher than in I/R group. iNOS inhibitor 1400 W (1 mg/kg, 5 min before postconditioning or reperfusion) prevented the increase in iNOS expression and abolished IPO-induced protection (34.4±1.0%, $P>0.05$ vs. I/R group). When rats were treated with PI3K inhibitor LY294002 5 min before reperfusion (0.3 mg/kg), p-Akt expression at R 3 h and iNOS expression at R 24 h were significantly inhibited. Moreover, the delayed infarct-sparing effect of IPO was absent in the presence of LY294002. Conclusion: IPO has prolonged cardioprotective effects and iNOS as an important downstream effector of PI3K-Akt pathway contributes to the delayed phase cardioprotection of IPO.

Keywords: Delayed cardioprotection, ischemic post-conditioning, ischemia-reperfusion injury, phosphatidylinositol 3-kinase, Akt, inducible nitric oxide synthase

Introduction

The most effective treatment for patients with acute myocardial infarction (AMI) is to restore the blood supply to the ischemic myocardium-timely [1]. However, sudden reperfusion after a relatively long ischemia would result in ischemia-reperfusion (I/R) injury [2]. In order to alleviate this injury, numerous studies have focused on the interventions that can protect the heart against I/Rinjury [3]. In 1986, Murry et al. [4] first demonstrated that several brief episodes of I/R stimulus performed before prolonged ischemia could reduce infarct size: they named this phenomenon "ischemic preconditioning (IPC)". Therapeutic exploitation of this powerful protective effect would be appealing but is hindered by the unpredictability of ischemic events [5]. In this regard, ischemic post-conditioning (IPO) defined as a serious of repetitive cycles of I/R applied at the beginning of reperfusion after prolonged ischemia has attracted increasing attention for its applicability and availability [5, 6]. Lots of studies have confirmed the protective effect of postconditioning in all species tested so far [7], and the protection is related to the reduction in infarct size, improvement of endothelial dysfunction and attenuation of neutrophil accumulation and so on. In addition, clinical trails have shown that postconditioning applied during percutaneous coronary invention (PCI) may reduce infarct size and improves functional class in patients with ST-segment-elevation myocardial infarction (MI) [8].

Accumulating evidence suggests that I/R-elicited injury is an on-going process which occurs not only at the acute phase of reperfusion, but extends to prolonged reperfusion [9]. However,

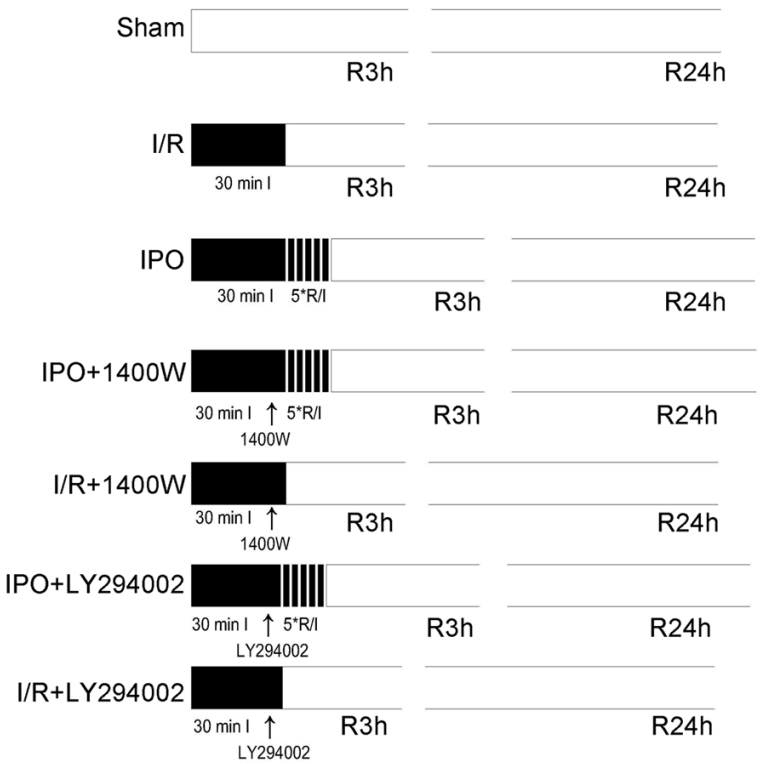

Figure 1. Experimental design. 5*R/I: 5 cycles reperfusion/ischemia (10 s/10 s); 1400 W: iNOS inhibitor, i.v. infusion (1 mg/kg) 5 min prior to reperfusion; LY294002: PI3K inhibitor, i.v. infusion (0.3 mg/kg) 5 min prior to reperfusion; I: ischemia; R: reperfusion.

most studies focus on IPO performed after a short period of reperfusion (less than 6 h) [7, 10, 11]. Little information is about the long-term effect of IPO. A persistent reduction of I/R injury rather than a simple delay in myocardial injury would make IPO more clinically appealing. Therefore, this study aimed to investigate whether IPO has delayed cardioprotective effects against I/R injury and further explore the mechanism underlying this protection. In a rat myocardial I/R injury model, our results demonstrated that IPO could reduce infarct size after a prolonged reperfusion, which was related to the activation PI3K-Akt signaling pathway and increased iNOS expression.

Materials and methods

Animals and materials

All the experimental protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Provincial Hospital affiliated to Shandong University, and the procedures were conducted according to the Guide for the Care and Use of Laboratory Animals published by the Ministry of Health, PRC and the National Research Council (Washington, D.C., National Academy Press, 1996).

Healthy male Wistar rats weighting 220-250 g were purchased from the Experimental Animal Center of Shandong University. Antibodies against iNOS (Santa Cruz Biotechnology, Inc, CA, USA), Akt and phosphor-Akt (p-Akt) (Cell Signaling Technology, Inc., Boston, MA, USA), triphenyltetrazolium chloride (TTC), 1400 W and LY294002 (Sigma. St. Louis, MO, USA), and creatine kinase (CK) assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used in the present study.

Experimental groups

A total of 126 rats were divided into following seven groups and 18 rats in each group received 3-h or 24-h reperfusion (n=9 pertime point). I/R group: 30-min left anterior descending coronary artery (LAD) ischemia, followed by 3 h or 24 h of reperfusion; IPO group: 30 min of LAD ischemia, followed by 5 cycles of I/R (10 s/10 s) and subsequent reperfusion; IPO+1400 W group: animals received an i.v. infusion of 1400 W (1 mg/kg, caudal vein) 5 min before postconditioning; I/R+1400 W group: animals received 1400 W 5 min before reperfusion; IPO+LY294002 group: animals received an i.v. infusion of LY294002 (0.3 mg/kg, caudal vein) 5 min before postconditioning; I/R+LY294002 group: animals received LY2940025 min before reperfusion; Sham: LAD was exposed without ligation and other interventions were the same to I/R group (**Figure 1**).

Surgery procedures

The surgical procedures were performed as previously described [12]. Rats were anesthetized with chloral hydrate (10%, 0.3 ml/100 g). Before thoracotomy, rats received mechanical

Table 1. Rats finally included in this study and analyzed at each time points

Group	R 3 h	R 24 h	Total
Sham	8	8	16
I/R	7	7	14
IPO	7	7	14
IPO+1400 W	8	7	15
I/R+1400 W	8	8	16
IPO+LY294002	6	7	13
I/R+LY294002	7	6	13
Total	51	50	101

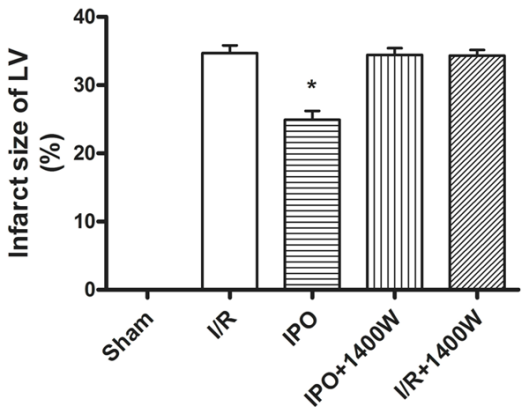

Figure 2. Infarct size of LV at R 24 h. *P<0.05 *vs.* I/R group. LV: left ventricular; R: reperfusion.

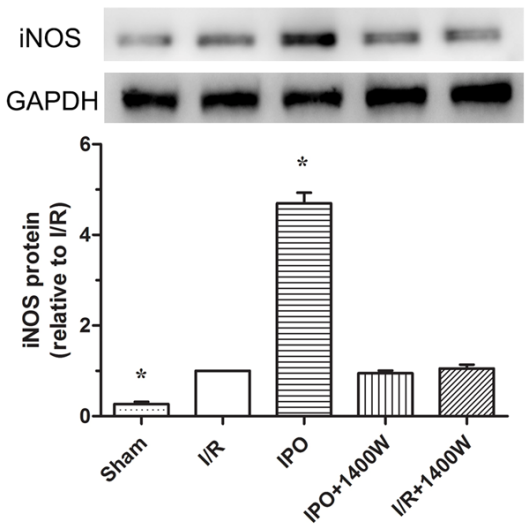

Figure 3. iNOS expression (normalized to I/R group) at R 24 h. *P<0.05 *vs.* I/R group. R: reperfusion.

ventilation (60 breaths per min, tidal volume of 3 ml/100 g). A left thoracotomy was carried out between the third and fourth intercostal space. The pericardium was opened gently, and the cardiac venous network and the LAD were clearly observed on the heart surface. LAD was ligated with a 6/0 silk suture (1-3 mm from tip of left auricle). The left ventricular apex and anterior wall became pale immediately once the right vessel was occluded.

Before closing the wounds, a small tube was left in the chest for the air evacuation from the chest. Mechanical ventilation was stopped until spontaneous breathing was present. Prophylactic therapy was administered after surgery. Ischemic myocardium was allowed to be reperfused for 3 or 24 h. At R 3 h and R 24 h, 3 rats were randomly selected from each group for measuring infarct size. Myocardial tissues were collected from remaining animals for immunohistochemistry and Western blot assay.

Infarct size measurements

The hearts were harvested after reperfusion 3 or 24 h. After excision of atria and right ventricular, the left ventricular was frozen at -20°C for 20 min and then transversely cut into 5 slices. All slices were incubated in 1% TTC at 37°C for 20 min until the necrotic area (pale) could be differentiated from the non-necrotic (red) area. Then, the slices were immersed in 4% formalin to enhance contrast. The stained slices were photographed, and analyzed with Image-Pro Plus 6.0.

Serum CK activity

Blood samples were collected from the tail vein at baseline and before killing. After centrifugation at 3000 rpm for 10 min, the serum was collected and processed for the detection of CK activity which was expressed as U/L.

Western blot assay

Total protein was extracted from myocardial tissues using RIPA lysis buffer. Samples containing equal amounts of protein (60 μg) were separated on SDS-PAGE and transferred onto PVDF membrane which was then blocked with 5% nonfat milk for 1 h and incubated with corresponding antibodies overnight at 4°C. After washing with Tris-buffered saline containing 0.1% Tween 20 (TBST) thrice, the membrane was incubated with horseradish peroxidase-

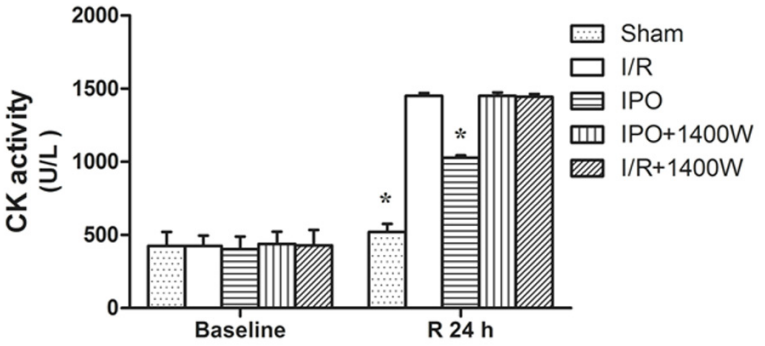

Figure 4. CK activity at different time points. *P<0.05 *vs.* I/R group at the same time points. R: reperfusion.

conjugated secondary antibody for 1 h. Following washing thrice in TBST again, the membrane was visualized with enhanced chemiluminescence-plus reagents. GAPDH was used as an internal control. The bands of target proteins in different groups were normalized to those of I/R group.

Statistical analysis

SPSS 16.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. All the data are expressed as mean ± standard deviation (SD). Independent samples t-test and one-way ANOVA followed by Bonferroni post hoc test were used. A value of P<0.05 was considered statistically significant.

Results

General data

A total of 126 rats were used for the experiments and 25 rats were excluded due to severe bleeding during operation (n=9), ventricular fibrillation during ischemia (n=4) and death after surgery (n=12). Thus, 101 animals were included for the final analysis (**Table 1**).

iNOS expression increased after 24-h reperfusion in IPO group and mediated the delayed cardioprotection of IPO in vivo

At R 24 h, IPO resulted in significant decrease in infarct size of LV from 34.7±1.1% in I/R group to 24.9±1.3% in IPO group rats (P<0.05), suggesting the delayed cardioprotection of IPO (**Figure 2**). At the same time point, iNOS expression increased significantly in IPO group (4.7-fold higher than in I/R group) (**Figure 3**). To determine whether iNOS plays an essential role in the delayed phase of IPO, rats were treated with 1400 W (a selective iNOS inhibitor) 5min before reperfusion. There were no significant differences between I/R group and IPO+1400 W group in the infarct size and iNOS expression (**Figures 2** and **3**).

CK activity, an indicator of myocyte injury, was comparable in all groups at baseline, but increased at the end of reperfusion, suggesting the myocyte injury. Notably, the increased serum CK activity following I/R injury significantly reduced by IPO. Similarly, the cardioprotection of IPO was abolished by 1400 W as indicated by increased CK activity at R 24 h. Findings in CK activity corresponded to those observed on infarct size (**Figure 4**).

These findings demonstrated that IPO has delayed beneficial effect on myocardia which is related to iNOS.

PI3K-Akt signaling pathway mediates iNOS expression

Given that the delayed phase is the extension of acute phase, this study focused on the protective mechanism in acute phase to explore the signaling pathway by which iNOS increased as a response to the delayed cardioprotection of IPO. At R 3 h, a significant increase was observed in p-Akt in IPO group (6-fold higher than in I/R group) (**Figures 5** and **6**) and this difference between two groups vanished during the delayed phase (R 24 h). The iNOS expression was comparable between two groups in the acute phase (R 3 h) whereas elevated at R 24 h in IPO group as compared to I/R group (**Figure 5**). On basis of these results, we hypothesized that the increased iNOS expression following IPO in the delayed phase depended on the activation of PI3K-Akt pathway in the acute phase. To address the relationship between PI3K-Akt and iNOS, rats were treated with selective PI3K inhibitor LY294002 5 min before reperfusion. Results showed that the p-Akt activation was abolished at R 3 h and the increased iNOS expression was prevented at R

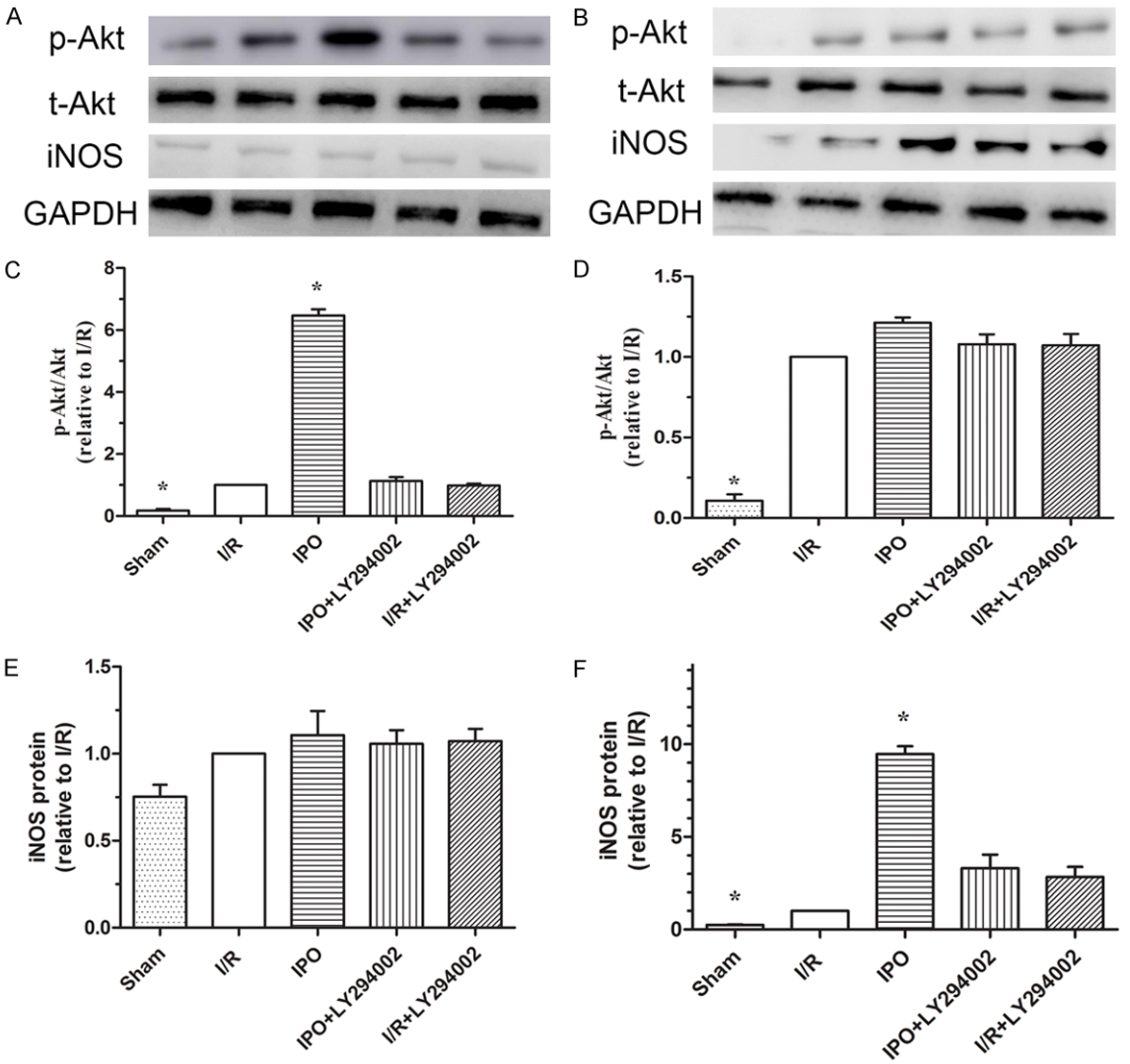

Figure 5. Effects of various treatments on protein expression. A, C, E. Western blot assay; B, D, F. Quantitative analysis of p-Akt/t-Akt and iNOS expressions (normalized to I/R group) at R 3 h and R 24 h.*P<0.05 vs. I/R group at the same time point; R: reperfusion.

24 h, as well. Furthermore, in the presence of LY294002, the ability of IPO to protect myocardia against I/R injury was attenuated at R 24 h, confirming a requirement for PI3K-Akt activation in the long-term cardioprotective effect of IPO (**Figures 5** and **7**).

Infract size

No myocardial infarction was found in Sham group. There was a significant increase in infarct size at R 24 h as compared to that at R 3 h (25.0±1.2 at 3 h vs. 34.7±1.1% at 24 h, P<0.05). At R 3 h, infarct size reduced by 28.4% in IPO group as compared to I/R group (17.9± 1.1 vs. 25.0±1.2%, P<0.05), and at R 24 h, 28.2% in IPO group as compared to I/R group (24.9±1.3 vs. 34.7±1.1%, P<0.05). Although IPO decreased infarct size, there was an increasein infarct size at R 24 h as compared to that at R 3 h, suggesting that IPO alone could not completely prevent the dynamic progression of infarction during the reperfusion, and thus combined therapy may provide better protection during the prolonged reperfusion (**Figure 8**).

Discussion

Previous studies have well demonstrated that IPO is able to alleviate myocardial I/R injury after a short period time of restoring blood sup-

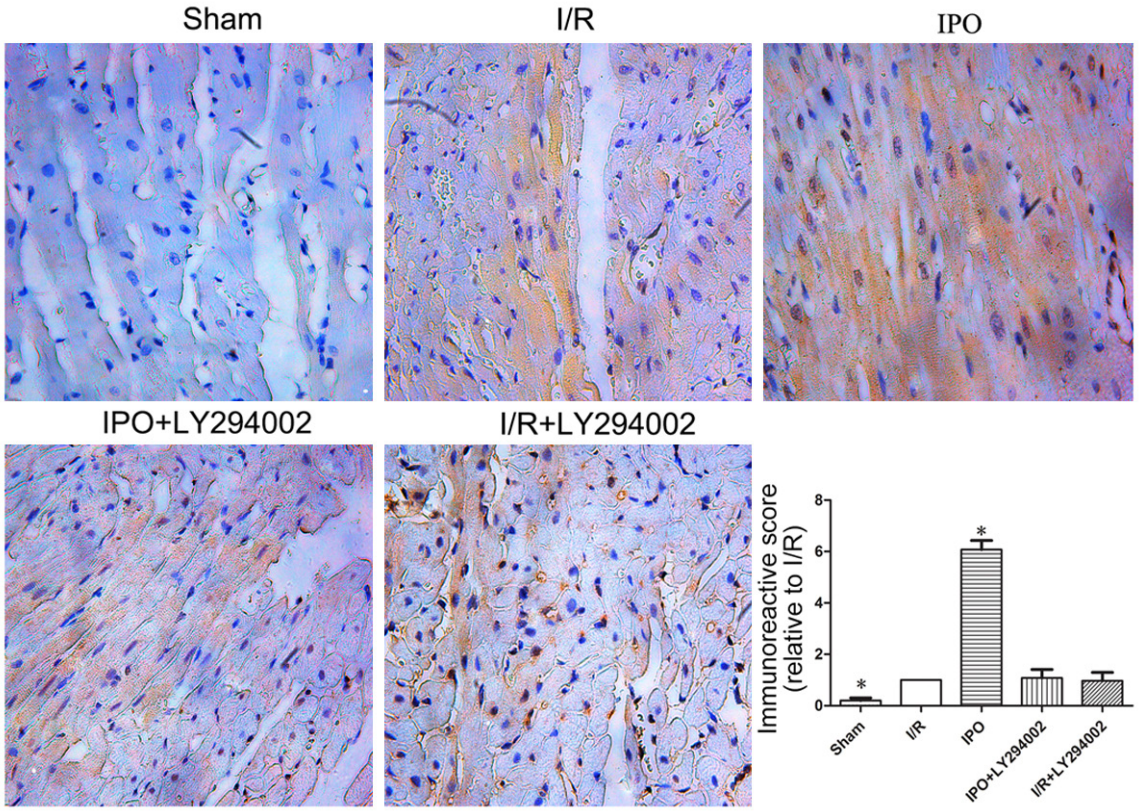

Figure 6. Detection of p-Akt expression by immunohistochemistry at R 3 h (400×). *P<0.05 vs. I/R group; R: reperfusion.

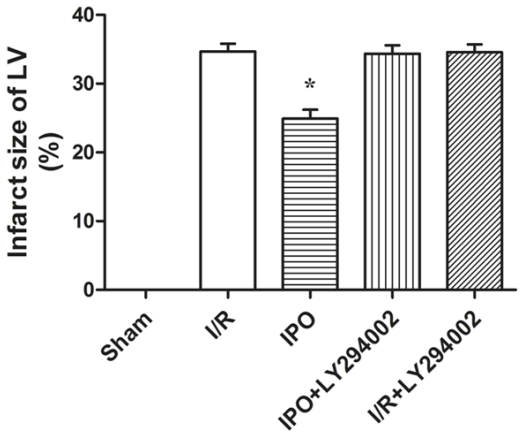

Figure 7. Infarct size of LV at R 24 h. *P<0.05 *vs.* I/R group. LV: left ventricular; R: reperfusion.

ply. Results from the present study showed that the reduction in infarct size of LV was still evident with IPO after 24-h reperfusion, which was accompanied by a comparable reduction in serum CK activity. Furthermore, this delayed protection was found to be likely related to the PI3K-Akt dependent up-regulation of iNOS expression, because either 1400 W or LY294002 was able to attenuate this cardioprotection. Although PI3K-Akt signaling pathway has been shown to be activated by postconditioning during acute phase of reperfusion, to our knowledge, the role of this pathway in the long-term cardioprotection of IPO against I/R injury has not yet been well defined.

Even though IPO could decrease infarct size, the infarct size increased progressively after reperfusion, but the specific mechanisms remain elusive. The results of Zhao *et al.* [9, 13, 14] were partially similar toours. They found a linear relationship between polymorphonuclear (PMN) accumulation and delayed development of myocardial infarction, which suggests that PMN may, in part, play a role in the increase in infarct size following reperfusion [9]. Excessive release of reactive oxygen species (ROS) from activated PMN may up-regulate the expression of adhesion molecules, which in turn results in the migration and accumulation of PMN [9, 15,

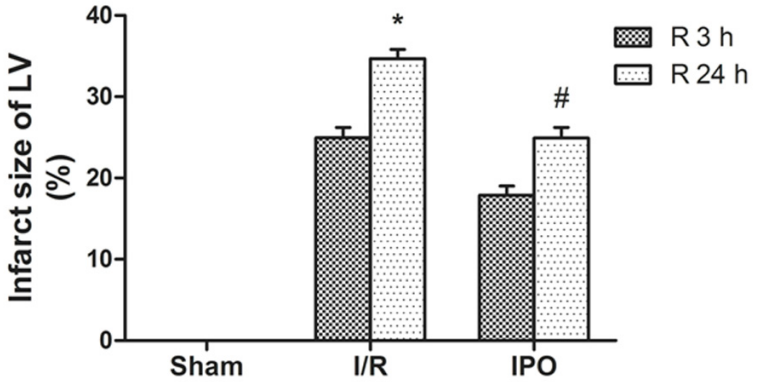

Figure 8. Changes in infarct size at R 3 h and R 24 h. *P<0.05 R 24 h *vs.* R 3 h in I/R group; #P<0.05 R 24 h *vs.* R 3 h in IPO group. LV: left ventricular; R: reperfusion.

16]. The potential injury caused by PMN has been explained by the oxidative burst and robust generation of oxygen radicals as well as release of proteolytic enzymes [17]. Furthermore, in a canine model, Zhao and his colleagues demonstrated that postconditioning could reduce PMN accumulation after both 3-h and 24-h reperfusion, thus alleviating reperfusion injury [18]. Interestingly, Argaud et al. reported that, in a rabbit model, protection of postconditioning could be detectable after 72-h reperfusion. Therefore, further study is needed to elaborate the difference between species in the pathological changes during reperfusion, and elucidate the mechanisms (more than 6 h of reperfusion) of cardioprotective effects of IPO during the later phase.

In general, iNOS is undetectable in normal tissues but highly induced by inflammatory cytokines, growth factors, tumor promoters, and other factors after stimulation or injury. Being one isoform of nitric oxide (NO) synthase, iNOS-may produce NO and decrease reperfusion-induced oxygen free radicals [19]. NO has been reported to open mitochondrial ATP-sentitive K^+ channels (mitoKATP) and/or inhibit the opening of mitochondrial permeability transition pore (mPTP) [20-23]. MPTP is voltage and Ca^{2+}-dependent, and opens at the onset of reperfusion due to Ca^{2+} overload and excessive production of ROS during the reperfusion. The opening of mPTP results in collapse of mitochondrial integrity and mitochondrial dysfunction, leading toabnormal coupling of the respiratory chain [24] and release of pro-apoptotic factors resulting in cellapoptosis or necrosis [25, 26]. Activation of mito-K_{ATP} during I/R protects the heart by attenuating mitochondrial anion channel opening and decreasing mitochondrial membrane potential and intramitochondrial Ca^{2+} influx, which influences the efflux of ROS to the cytoplasm and inhibits the opening of mPTP, protecting the cardiomyocytes [27]. In addition, the increase in iNOS expression may enhance the expression of antioxidant proteins [28]. However, few studies on postconditioning focus on iNOS. Given that iNOS plays an important role in the neovascularization during the growth and invasion of neoplasm [29], we hypothesized as well as demonstrated the involvement of iNOS in the IPO induced cardioprotection in delayed phase. When compared with I/R group, iNOS protein expression significantly increased at R 24 h in IPO animals, whereas no marked difference was observed between two groups at R 3 h. After 1400 W treatment, the iNOS expression reduced and the cardioprotection induced by IPO was also attenuated at R 24 h. However, different reports indicate that high NO produced from iNOS may induce apoptosis, while low NO produced from eNOS reduce apoptosis [30, 31]. This may be related to the different protocols for post-conditioning and the duration of prior ischemic insult. Therefore, the role of iNOS is still controversial and further study is needed to elucidate the role of iNOS in the setting of I/R injury.

Accumulating evidence has proven that PI3K-Akt is a key signaling pathway in mediating acute cardioprotection of IPO [32]. However, no studies have demonstrated the role of PI3K-Akt signaling pathway in the long-term protection of IPO *in vivo*. Western blot assay in the present study showed that, in IPO group, the expressions of p-Akt and iNOS increased at R 3 h and R 24 h, respectively. LY294002 prevented the up-regulation of iNOS expression and reduced the cardioprotection induced by IPO at both R 3 h and R 24 h. These demonstrated that the up-regulation of iNOS expression during the late phase of reperfusion is dependent on the activation of PI3K-Akt signaling pathway during the

acute phase of reperfusion. Activation PI3K-Akt signaling pathway may phosphorylated downstream effectors such as eNOS and Bcl-2 family, then transduces the IPO stimulus into the nucleus where the transcription of iNOS and other cardioprotective genes is up-regulated. The specific mechanisms underlying the protection of IPO against I/R injury have not been elucidated completely. In the setting of delayed IPC, it has been shown that iNOS is associated with and can modulate COX-2 [33]. It is now clear that the late IPO is a polygenic phenomenon that requires the simultaneous activation of multiple stress-responsive genes [34]. Therefore, the exact mechanisms underlying the delayed protective effect of IPO will require further investigations on the metabolism and genes.

In conclusion, in the rat myocardial I/R injury model, our results show IPO has long-term cardioprotection against I/R injury through up-regulating iNOS expression in a PI3K-Akt signaling pathway depedent manner. IPO may be clinically applicable in coronary angioplasty, organ transplantation, thrombolysis and other settings where I/R injury will occur because IPO offers sustained cardioprotective effect. In addition, for patients who cannot tolerate the coronary angioplasty, the identification of iNOS as a specific protein in charge of delayed IPO provides a new pharmacological or genetic target for the adjunctive therapy to thrombolysis, aiming at mimicking the long-term cardioprotective effects of IPO.

Conclusion

Our study demonstrates that IPO exerts persistent cardioprotective effects and iNOS as an important downstream effector of PI3K-Akt signaling pathway contributes significantly to the later phase cardioprotection of IPO.

Acknowledgements

This study was supported by the Natural Science Foundation of Shandong Province (No. ZR2012HM092) and the National Natural Science Foundation of China (No. 30872433).

Disclosure of conflict of interest

None.

Address correspondence to: Mengyuan Zhang, Department of Anesthesiology, Shandong Provincal Hospital affiliated to Shandong University, 324# Jingwu Road, Jinan, 250021, China; Tel: +8653186886471; Fax: +8653186886471; E-mail: zhangmy717@163.com

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