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Ischemic Postconditioning Attenuates Ischemia/Reperfusion-induced Injury Through Activating Inflammatory Signaling Pathways

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Abstract: The role of post-treatment in protecting organ ischemia and re-perfusion damage is increasingly recognized, however, its mechanism of the action is not very clear, all in all, it still needs further research. The purpose of this experiment is to investigate whether IPO can reduce I/R-induced liver damage through inhibiting inflammatory signaling pathways in rats. Rats were randomly divided into sham, I/R, IPO and LY294002+IPO groups. The levels of AST and ALT were assessed. The expression levels of IL-1, Akt, NF-κB-P65 and TNF-α were analyzed using western blot analysis. The expression levels of ALT, AST, IL -1, TNF-α and NF-κB-P65 were significant reduction in the IPO group compared with those in the I/R group. Furthermore, the protein expression level of phosphorylated Akt was observed to be significant increase in the livers of the rats in the IPO group compared with those in the I/R group. Moreover, LY294002 was found to offset the advantages of IPO. To the best of our knowledge, this study provided the clear evidence to show that IPO significantly reduced the injury caused by I/R, and it might protect the liver from hepatic injury through activating the phosphoinositide 3-kinase pathway, which increased the expression of Akt, and inhibited the protein expression of IL-1, NF-κB-P65 and TNF-α.

Keywords: Ischemic Postconditioning, Inflammatory, Signaling Pathways

1. Introduction

It is well established that ischemia followed by reperfusion leads to liver injury. Ischemia-reperfusion (I/R) injury occurs during liver transplantation and trauma, as well as during liver resection. It has been proposed that inflammatory signaling cascades may be activated during the process of I/R. Inflammatory signaling cascades have been found to induce the activation of macrophages, as well as increase infiltration by neutrophils and lymphocytes [1]. These cells produce several mediators, including reactive oxygen species (ROS), proteases and cytokines, which cause cell damage. Kupffer cells are activated following reperfusion [2, 3] and have been reported to release inflammatory cytokines and free radicals [4-6]. It has previously been reported that tumor necrosis factor (TNF)-α [7], interleukin (IL) 1 [8], ROS [7, 9] and leukotrienes [10] recruit neutrophils to the liver [11], which may have an important role in acute liver injury and lead to cell death, as well as activate internal signaling pathways, for example the cytochrome *c*-caspase signaling pathway. Therefore, attenuation of I/R injury is of critical importance in surgery. Several protective and treatment strategies have been reported, including ischemic pre- and postconditioning [12-14]. Such interventions may be performed at different time-points. Liver pre- and postconditioning may be used during liver transplantation and resection. Ischemic post conditioning (IPO) may be an important strategy to protect the liver from (I/R) injury. Zhao *et al* [15] were the first to discover that in the heart, postconditioning using the repetitive application of ischemia during early reperfusion had a cardio-protective effect through attenuating reperfusion injury. At the start of reperfusion, three cycles of 30 sec reperfusion and 30 sec left anterior descending artery reocclusion preceded the 3 h of reperfusion [15]. Infarct size was found to be significantly reduced in the pre- and postconditioning groups compared with the controls.

Postconditioning was observed to be as effective as preconditioning in reducing infarct size and preserving endo thelial function. Therefore, postconditioning may be clinically applicable for coronary intervention, coronary artery bypass surgery, organ transplantation and peripheral revascularization where reperfusion injury is a problem [15]. However, there have been few reports on IPO in the liver and whether IPO in the liver is capable of activating inflammatory signaling pathways has yet to be elucidated. Therefore, the present study investigated the mechanisms underlying postconditioning in the liver.

2. Material and Methods

2.1. LY294002 and Experimental Animals

The phosphoinositol-3-kinase inhibitor 2-(4-morpholinyl)-8-phenyl-4 H-1-benzopyran-4-one (LY294002) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Male Sprague-Dawley rats (weight, 200-250 g; n=28) were obtained from China. All procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with recommendations published in the Guide for Care and Use of Laboratory Animals by the National Research Council.

2.2. Experimental Groups and Protocols

Adult male Sprague-Dawley rats were used as the experimental animals and were maintained in a temperature-controlled environment (25-30°C) on a standard diet and water *ad libitum*. The rats were randomly divided into four groups (n=7/group): Sham, I/R, ischemia postconditioning (IPO) and LY294002+IPO groups. The rats in the I/R group underwent occlusion of the porta hepatis for 60 min, followed by persistent reperfusion for 2 h. In the rats in the IPO group, following the induction of ischemia, the livers were postconditioned using five cycles of reperfusion for 10 sec followed by ischemia for 10 sec, performed three times. The livers were then reperfused for 2 h. In the rats in the LY294002+IPO group, ischemia was induced in LY294002 (15 μmol/l)-treated livers, followed by IPO as described for the IPO group, subsequent to liver reperfusion for 2 h. Following a midline laparatomy incision, an atraumatic vascular clip was placed on the vessels blocking the portal venous and hepatic arterial blood supply to the median and left lateral lobes of the liver, which resulted in ~70% rat liver I/R injury. The rats in the sham group underwent the same surgical procedure as the rats in the other groups, except vessels clips were not applied. Blood samples and liver tissues were taken from the rats in each group for analysis following reperfusion for 2 h.

2.3. Serum Liver Function Assay

Blood samples were obtained from the rats following reperfusion for 2 h. Serum alanine amino- transferase (ALT) and aspartate transaminase (AST) levels were measured using a standard clinical automated analyzer (ILab 600; Shimadzu Corporation, Kyoto, Japan).

2.4. Phosphorylated (p)-Akt, IL-1, TNF-α and Nuclear Factorκ-light-chain-enhancer of Activated B Cells (NF-κB)-P65 Protein Expression

Total protein was extracted from the hepatic tissue samples and quantified using the Bradford assay. Equal quantities of protein (50 μg) were separated using SDS-PAGE. Proteins were then transferred onto poly- vinylidene difluoride (PVDF) membranes. Membranes were incubated overnight at 4°C with anti-Akt, - IL-1, -TNF-α and NF-κB-P65 rabbit polyclonal antibodies, at a 1:500 dilution. Membranes were then incubated with horseradish perox idase-conjugated secondary antibodies (dilution, 1:2,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Membranes were re-incubated with anti-β -actin antibodies at a 1:5,000 dilution (Santa Cruz Biotechnology, Inc.) to control for protein loading. Immunoreactive bands were visualized using the enhanced chemiluminescence Advance Western Blotting Detection Kit (GE Healthcare, Little Chalfont, UK) in a GeneGnome system (Synoptics Ltd, Cambridge, UK). Band intensity was measured using Quantity One® software (Bio- Rad, Hercules, CA, USA).

2.5. Statistical Analysis

Data are presented as the mean ± standard deviation. Multiple comparisons were performed using analysis of variance. Comparisons between two groups were performed using Student's t-test. All analyses were conducted using SPSS software (version 18.0; SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

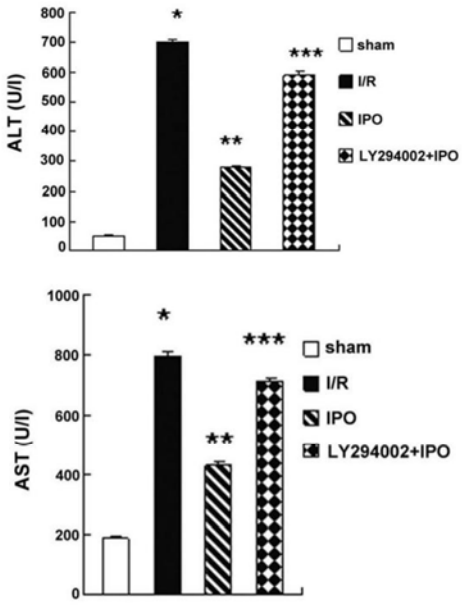

Figure 1. *Serum levels of (A) ALT and (B) AST in rats. ALT and AST serum levels were assessed at the end of the treatment conditions. The levels of ALT and AST were significantly decreased in the IPO- treated rat liver tissues. LY294002 attenuated the IPO-induced decreases in ALT and AST. Data are presented as the mean ± standard deviation. *P<0.05 vs. sham; **P<0.05 vs. I/R; ***P<0.05 vs. IPO (n=7/group). ALT, alanine aminotransferase; AST, aspartate transaminase; I/R, ischemia-reperfusion; IPO, ischemic postconditioning.*

3. Results

3.1. Physiological Function of IPO in Hepatic injury Caused by I/R (HIRI)

We observed that the obvious differences of Serum levels of ALT and AST measured after 2 h of reperfusion following 1h of ischemia occurred in these groups. The value of ALT and AST in the I/R group was observed to be significantly increased compared with those in the rats in the sham group. IPO treatment was found to significantly reduce ALT and AST serum levels compared with those in the I/R group (Figure 1A and B). Furthermore, LY294002 was not observed to inhibit the protective effect of IPO.

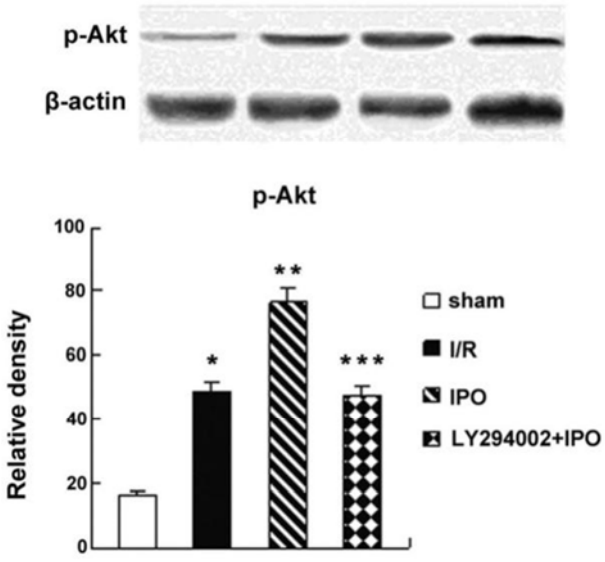

Figure 2. *P-Akt expression detected using western blot analysis. Rats were ran- domly divided into sham, I/R, IPO and IPO+LY294002 groups. The rats in the I/R group underwent occlusion of the porta hepatis for 60 min, followed by per sistent reperfusion for 2 h. In the rats in the IPO group, following the induction of ischemia, the livers were postconditioned using five cycles of reperfusion for10 sec followed by ischemia for 10 sec, performed three times. The livers were then reperfused for 2 h. In the rats in the LY294002+IPO group, ischemia was induced in LY294002 (15 μmol/l)-treated livers, followed by IPO treatment. as described for the IPO group, subsequent to liver reperfusion for 2 h. At the end of the treatment, liver samples were obtained from the rats and subjected to western blot analysis using anti-p-Akt antibodies. Densitometric scanning was performed to assess p-Akt protein expression which was normalized to that of β-actin. Data are presented as the mean ± standard deviation from four independent experiments (n=7/group). *P<0.05 vs. sham; **P<0.05 vs. I/R; ***P<0.05 vs. IPO. I /R, ischemia-reperfusion; IPO, ischemic postconditioning p-, phosphorylated; Akt, protein kinase B.*

3.2. p-Akt, IL- 1, TNF-α and NF-κB-P65 Protein Expression

To assess whether IPO protected the liver against I/R injury through activating the prosurvival kinases PI3K and Akt, thereby inhibiting the expression of IL-1, TNF-α and NF-κ B-P65, the protein expression of p-Akt, IL-1, TNF-α and NF-κB-P65 was analyzed using western blot analysis. p -Akt expression was found to be significantly increased in the liver tissue in the rats in the I/R group compared with that in the rats in the sham group. Furthermore, p-Akt expression was observed to be significantly higher in the liver tissues in the rats in the IPO group, compared with that in the I/R group. Moreover, LY294002 was found to significantly attenuate the IPO-induced increases in p-Akt expression (Figure 2). Inflammatory signaling pathways have been reported to be associated with the protective effects of preconditioning [16-18]. Therefore, the purpose of this experiment is to investigate whether IPO altered the activation of liver I/R-induced inflammatory signaling. The expression of IL -1, TNF-α and NF-κB-P65 was assessed. Compared with the rats in the sham group, IL-1, TNF-α and NF-κB-P65 protein expression were signifcantly increased in the rats in the I/R group. Furthermore, compared with the I /R group, IL -1, TNF-α and NF-κB-P65 protein expression was significantly decreased in the rats in the IPO group (Figures 3-5). LY294002 was observed to significantly attenuate the IPO-induced increases in p-Akt expression and decreases in IL-1, TNF- α NF-κB-P65 expression.

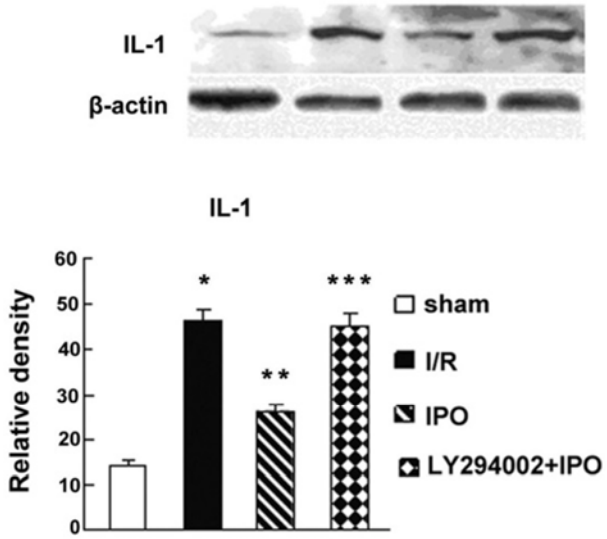

Figure 3. *IL -1 expression detected using western blot analysis. After 60 min of ischemia and 2 h of reperfusion, IL-1 protein expression in IPO-treated hepatic tissues was determined using western blot analysis. I/R treatment activated inflammatory signaling in the rat liver. IPO treatment inhibited IL-1 expression, while LY294002 significantly attenuated the IPO -induced inhibition of IL-1 expression. β-actin was used as a loading control. (A) Western blot of IL -1 expression. (B) Ratio of IL-1 to β-actin expression Data are presented as the mean ± standard (n=7/group). *P<0.05 vs. sham;. **P<0.05 vs. I/R; ***P<0.05 vs. IPO. I/R, ischemia-reperfusion; IPO, ischemic postconditioning; IL, interleukin.*

4. Discussion

I/R injury is associated with hepatic resection and liver transplantation and is a serious complication in clinical practice, despite several attempts to solve the problem. It is well established that the redox balance, which is essential for normal tissue function and tissue integrity, is dysregulated during I/R and causes an accumulation of ROS [19, 20]. The formation of ROS and oxidative stress are the most commonly reported mechanisms involved in HIRI [21, 19].

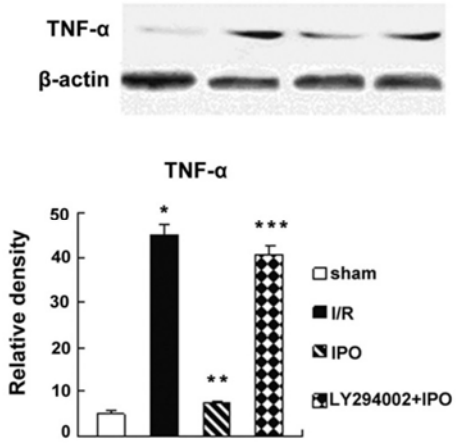

Figure 4. *TNF-α expression detected using western blot analysis. After 60 min of ischemia and 2 h of reperfusion, TNF-α protein expression was assessed in IPO-treated hepatic tissues using western blot analysis. I /R treatment activated inflammatory signaling in the rat liver which led to an increase in TNF-α protein expression. IPO treatment inhibited the IR-induced increases in TNF -α expression and LY294002 significantly attenuated the effect of IPO. β-actin was used as a loading control. (A) Western blot of TNF-α protein expression. (B) Ratio of TNF -α to β-actin expression. Data are presented as the mean ± standard deviation (n=7/group). *P<0.05 vs. sham; **P<0.05 vs. I/R; ***P<0.05 vs. IPO. I/R, ischemia-reperfusion; IPO, ischemic postconditioning; TNF, tumor necrosis factor.*

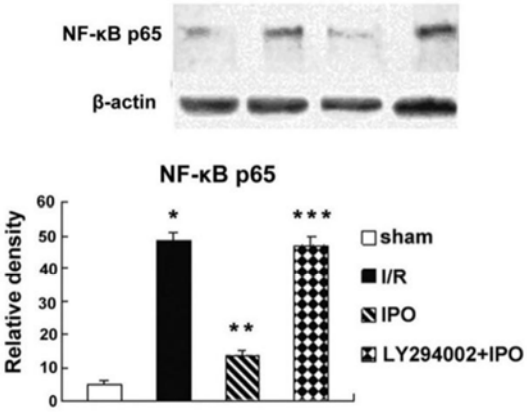

Figure 5. *NF-κB-P65 expression detected using western blot analysis. After60 min of ischemia and 2 h of reperfusion, NF-κ B-P65 protein expression was assessed in IPO-treated hepatic tissues using western blot analysis. I/R treatment activated inflammatory signaling in the rat liver which led to an increase in NF- κB-P65 protein expression. IPO inhibited the increase in NF- κB-P65 and LY294002 significantly attenuated the effect of IPO. β-actin was used as a loading control. (A) Western blot of NF-κB-P65 expression (B) Ratio of NF-κB-P65 to β-actin expression. Data are presented as the mean ± standard deviation (n=7/group). *P<0.05 vs. sham; **P<0.05 vs. I/R; ***P<0.05 vs. IPO. IPO. I/R, ischemia-reperfusion; IPO, ischemic postcondi- tioning; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells.*

The present study aimed to investigate postconditioning strategies to overcome HIRI. Zhao *et al* [15] investigated whether postconditioning using the repetitive application of ischemia during early reperfusion may have a cardio-protective effect through attenuating reperfusion injury. They found that postconditioning was as effective as preconditioning in reducing infarct size and preserving endothelial function [15]. Therefore, postconditioning may be clinically applicable for coronary intervention, coronary artery bypass surgery, organ transplantation and peripheral revascularizа tion where reperfusion injury is a problem. Studies have shown that IPO may have two predominant effects on I/R injury. The first of which may be a more passive effect, in which the gradual reperfusion associated with IPO reduces the generation of ROS, decreases neutrophil aggregation [22] and inhibits mitochondrial calcium overloading [23]. The second is proposed to be a more active effect, in which IPO activates PI3K-Akt [24], ERK1/2 and the downstream endo- thelial nitric oxide synthase [25], as well as p70S6K [26], thereby inhibiting mitochondrial permeability transition pore opening [27], cell death and protein transcription, and ultimately protecting the ischemic organ. In the present study, p-Akt was found to be increased in the livers of rats in the IPO group compared with those in the rats in the I/R group. This IPO-induced increase in p-Akt levels was observed to be significantly attenuated by LY294002, suggesting that the activation of the PI3K pathway may be involved in IPO-induced liver protection. Furthermore, compared with the rats in the I/R group, the levels of the serum liver enzymes ALT and AST were found to be significantly decreased in the IPO group, suggesting that IPO may reduce HIRI, but that LY294002 may significantly inhibit the protective effect of IPO. Therefore, IPO may reduce HIRI through activating the PI3K pathway.

The release of cytokines caused by HIRI is an important factor. A variety of cytokines have been reported to be involved in the pathophysiology of HIRI [28], which act in autocrine and paracrine manners to exert their biological effects and are capable of causing hepatic injury independently or synergistically. These cytokines include IL-1, -6 and-10, as well as platelet-activating factor and TNF-α. TNF -αhas been reported to have a role in the initial development of HIRI and is highly correlated with HIRI [8]. TNF-α may not cause sinusoidal endothelial cell swelling directly, but may significantly increase the expression of adhesion molecules on the surface of sinusoidal endothelial cells, enhancing the interaction between polymorphonuclear neutrophils with sinusoidal endothelial cells, thus causing a microcirculation disorder [29]. Furthermore, TNF-α may activate the adhesion of polymorphonuclear neutrophils to the sinusoidal endothelial cells, resulting in the release of oxygen free radicals, causing liver damage [30]. IL -1 may not only induce Kupffer cells to produce TNF-α and increase the release of oxygen free radicals from polymorphonuclear neutrophils, but may cooperate with TNF-α on the endothelial cells, inducing the synthesis of thrombin and plasmin, thus destroying the endothelial cells [31] and increasing HIRI. The transcription factor NF-κB is a proinflammatory protein released by inflammatory mediators during HIRI. Activation of NF -κB has been reported to regulate the expression of a variety of molecules, including TNF-α, IL-6, IL -8, monocyte chemoattractant protein-1, inducible nitrogen oxide synthase, as well as adhesion molecules, including intercellular adhesion molecule 1, vascular cell adhesion molecule-1, E-selectin and P-selectin.

These molecules may directly or indirectly act on endothelial cells, leading to endothelial cell injury. Phosphorylation of inhibitor of NF-κB α causes NF-κB activation. Activated NF-κB then passes through the nuclear membrane receptors into the nucleus where it binds its target genes, rapidly induces target genes transcription and increases the expression of a variety of molecules, which promote the release of inflammatory mediators, resulting in tissue damage. The above-mentioned factors may be activated though the toll-like receptor (TLR) 4-NF-κB pathway, which may activate the PI3K pathway and downstream Akt and inhibit glycogen synthase kinase 3β activity, thereby hindering the activation of NF-κ B-P65 and the production of TNF- α and IL-1. The present study showed that the expression of NF-κB-P65, TNF- α and IL-1 were significantly increased in the I/R group and significantly reduced in the IPO group. However, LY294002 significantly inhibited the effect of IPO, with the expression of NF-κB-P65, TNF-α and IL-1 being significantly increased in the IPO+LY294002 group compared with those in the IPO group ($P<0.05$). It was reasoned that these factors may be primarily generated through this pathway, which reduced HIRI and protected livers.

5. Conclusions

All in all, we found that IPO had a protective effect on rat livers against I/R injury. IPO may activate the TLR4 receptor and in turn the PI3K pathway, promoting the phosphorylation and activation of Akt, thus inhibiting downstream NF-KB-P65, TNF -α and IL-1 expression. This process may ultimately reduce HIRI.

Acknowledgements

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