Ischemic preconditioning increases GSK-3β/β-catenin levels and ameliorates liver ischemia/reperfusion injury in rats

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Abstract. Ischemic preconditioning (IPC) ameliorates ischemia/reperfusion (I/R) injury in a number of organs, and the glycogen synthase kinase-3β (GSK-3β)/β-catenin signaling pathway regulates I/R-induced proliferation and apoptosis in the central nervous system and heart. However, the function of this signaling pathway in IPC during liver I/R remains unclear. Thus, in this study, we aimed to investigte the role of the GSK-3β/β-catenin pathway during I/R and following ischemic preconditioning. For this purpose, 30 Sprague-Dawley rats were randomly divided into the sham-operated, the I/R and the IPC groups (n=10). Following reperfusion, liver pathology, as well as alanine aminotransferase (ALT), aspartate aminotransferase (AST), maleic dialdehyde (MDA) and superoxide dismutase (SOD) levels were assessed. Western blot analysis was performed to quantify the GSK-3β, Ser9-phospho-GSK-3β (p-GSK-3β), cytosolic and nuclear β-catenin, vascular endothelial growth factor (VEGF), Bcl-2 and survivin levels. In addition, the Bcl-2 and survivin mRNA levels were assessed by RT-qPCR. Compared with the sham-operated group, I/R increased serum ALT, AST and MDA activity and decreased SOD levels, while IPC significantly decreased serum ALT, AST and MDA activity and increased SOD levels, compared with the I/R group. Simultaneously, I/R increased p-GSK-3β protein expression, and decreased Bcl-2 and survivin protein and mRNA levels. IPC further increased the protein expression of p-GSK-3β, and also increased cytosolic and nuclear β-catenin and VEGF expression compared with the I/R group; the expression of Bcl-2 and survivin was also increased by IPC, both at the mRNA and protein level. The total GSK-3β expression remained unaltered in all the groups. In conclusion, our data demonstrate that IPC exerts protective effects against liver injury induced by I/R and activates the GSK-3β/β-catenin signaling pathway.

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Introduction

Liver ischemia/reperfusion (I/R) is a common physiopathological phenomenon that occurs during surgery. The causes of liver I/R mainly include infection, shock, cardiopulmonary dysfunction, repair of liver trauma, tumor resection and transplantation. Liver I/R injury is of great clinical significance as it not only affects the graft or remaining liver following major hepatectomy, but also remote organs, such as the intestines, kidneys, lungs and brain; it can even result in multiple organ dysfunction syndrome (MODS) (1). In addition, approximately 10% of transplant patients present with liver failure in the early post-operative stage due to I/R. Indeed, I/R injury also participates in acute and chronic reactions (2). Therefore, in recent years, increasing attention has been paid to finding methods of alleviating the injury caused by I/R in surgery as much as possible.

Murry *et al* (3) reported that brief intermittent periods of ischemia and reperfusion of the coronary artery did not lead to irreversible damage of myocardial cells as did longer periods of I/R; on the contrary, it surprisingly protected the cardiomyocytes from the damage caused by subsequent long periods of ischemia. They termed this phenomenon ischemic preconditioning (IPC). Of note, brief periods of focal ischemia have also been shown to induce ischemic tolerance in rat brains, and IPC has been shown to exert profound protective effects on the liver and intestines, and to enhance donor lung preservation (4-7).

The molecular mechanisms underlying IPC are not yet fully understood. Indeed, the involvement of several signaling pathways and molecules has been suggested, including protein kinase C, nitric oxide (NO), cGMP-dependent protein kinase, Akt (protein kinase B), extracellular signal-regulated kinase

(ERK) and p38 MAP kinases, AMP-dependent protein kinase, and the mitochondrial ATP-sensitive potassium channel (8-10).

Compared to other organs, liver IPC has additional features as it reduces inflammation and promotes hepatic regeneration (11). Recently, it was demonstrated that the inhibition of glycogen synthase kinase-3β (GSK-3β) prior to hemorrhagic shock regulates the inflammatory response, and improves hepatic microcirculation and hepatocellular function (12). GSK-3β belongs to a family of conserved serine/threonine kinases present in eukaryotic groups and its activity is regulated by various pathways in addition to the phosphoinositide 3-kinase (PI3K)-PKB/Akt-dependent pathway, including Wnt signaling. The phosphorylation of GSK-3β results in increased β-catenin levels and its translocation to the nucleus (13). β-catenin has been shown to be a critical mediator during development and angiogenesis (14); it is phosphorylated in a cytosolic multiprotein complex containing the adenomatous polyposis coli (APC) protein, axin and GSK-3β (15-17). When the phosphorylation of β-catenin is blocked, the protein accumulates and translocates to the nucleus, where it forms a complex with T-cell transcription factor/lymphoid-enhancer binding factor (TCF/LEF) and activates or represses several important target genes, including c-Myc, cyclin D1, fibronectin, vascular endothelial growth factor (VEGF), Bcl-2 and survivin (18-20). However, whether IPC similarly protects the liver against I/R injury by involving the GSK-3β/β-catenin signaling pathway remains unknown.

The present study aimed to clarify the role of the GSK-3β/β-catenin signaling pathway in the protective effects induced by IPC agaisnt liver I/R injury. Liver morphology, and serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, as well as liver maleic dialdehyde (MDA) and superoxide dismutase (SOD) activity were assessed. In addition, the levels of GSK-3β, phosphorylated (Ser9) GSK-3β (p-GSK-3β), cytosolic and nuclear β-catenin, as well as those of VEGF were quantified. Finally, the expression levels of the anti-apoptotic markers, Bcl-2 and survivin, were also evaluated.

Materials and methods

Animals. Male Sprague-Dawley, rats weighing 180-220 g, were obtained from the Animal Center of Dalian Medical University (Dalian, China) (Institutional protocol no. SCXK 2008-0002), and maintained under standard laboratory conditions with free access to food and water. The rats were housed in a barrier system kept at 25˚C with 12/12 h light-dark cycles. They were allowed to acclimatize for 1 week prior to the commencement of the experiments. All procedures were conducted according to our institutional animal care guidelines and approved by the Institutional Ethics Committee (Peking University People's Hospital).

Surgical procedures and experimental groups. A total of 30 rats were randomly divided into 3 groups, including the sham-operated, the liver I/R and the IPC groups. The rats in the sham-operated group underwent surgery, with the portal vein and artery isolated without occlusion. In the I/R group, the animals were subjected to 70% liver ischemia for 45 min, followed by 3 h of reperfusion, as previously described (21). In the IPC group, the rats were subjected to 10 min of ischemia and 10 min of reperfusion prior to the sustained ischemia, as previously described (22). At the end of reperfusion, blood and liver samples were collected and preserved for the subsequent procedures.

The animal experiments were approved by the ethics committee of our institution. The animals were kept under pathogen-free conditions under a 12-h light/dark cycle (4-6 animals per cage) and allowed free access to food and water. Care was taken to minimize the suffering of the animals as much as possible. The outcomes of the preliminary experiment were taken into consideration when designing the sample size and operation standard. A daily observation was performed to examine the physiological and mental state of the animals, to make sure the animals were kept at a normal state. At the endpoint of the experiment, the mice were quickly sacrificed by an intraperitoneal antesthetic injection. After the mice were sacrificed, blood and liver samples were collected.

Liver morphological assessment. The liver tissues were harvested and fixed in 10% formalin. Consecutive 5-μm-thick sections from paraffin-embedded liver tissues were prepared for hematoxylin and eosin staining and subsequently evaluated as previously described (23). Briefly, the liver specimens were evaluated at x200 magnification by a point-counting method for the severity of liver injury with an ordinal scale as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, loss of intercellular borders and mild to moderate neutrophil infiltration; and grade 3, severe injury with disintegration of hepatic cords, hemorrhaging and severe polymorphonuclear (PMN) cell infiltration. An average of 100 adjacent points on a 1-mm^2 grid was graded for each specimen.

Serum ALT and AST levels. Blood samples were drawn from the abdominal aorta and centrifuged at 3,000 rpm for 15 min to yield serum. Subsequently, serum ALT and AST levels, which are generally considered the most sensitive indexes of acute liver injury, were measured using an Olympus AU1000 automatic analyzer (Olympus Optical, Tokyo, Japan) according to the manufacturer's instructions (Nanjing Jiancheng, Nanjing, China).

Liver MDA and SOD activity assay. Liver tissues were harvested and homogenized immediately on ice in 5 volumes of normal saline. The homogenates were centrifuged at 3,000 rpm for 5 min. MDA and SOD activity in the supernatants was determined using specific assay kits (Nanjing Jiancheng), according to the manufacturer's recommendations. MDA and SOD activity was expressed in nmol/mg protein and U/mg protein, respectively.

Quantification of liver GSK-3β, p-GSK-3β, cytosolic and nuclear β-catenin, VEGF, Bcl-2 and survivin protein levels by western blot analysis. Cytosolic, nuclear and total protein samples were obtained from snap-frozen tissues using a protein extraction kit (Beyotime Institute of Biotechnology, Nantong, China). The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with appropriate gel concentrations (10% for β-catenin, GSK-3β, p-GSK-3β and VEGF; 15% for Bcl-2) and then electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA,

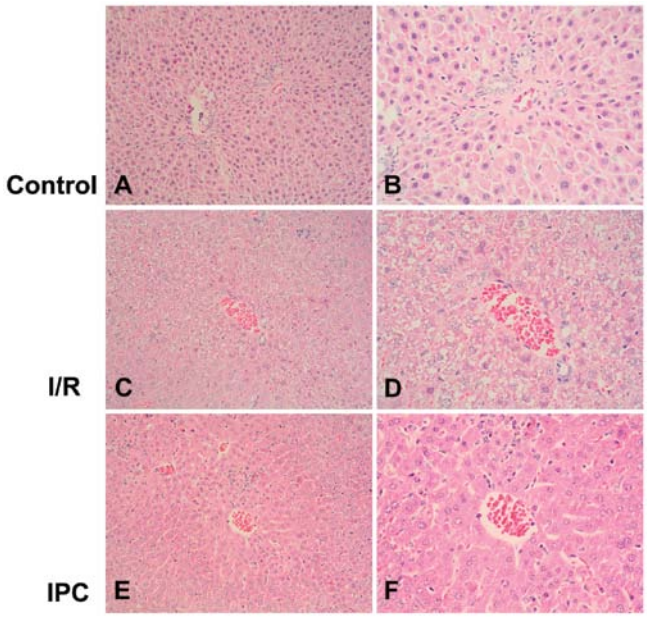

Figure 1. Histopathology of the liver by H&E staining revealed normal organ appearance in the sham-operated (control) group, hemorrhaging and hepatocyte necrosis in the ischemia/reperfusion (I/R) group, and significantly alleviated hepatocyte injury in the ischemic preconditioning (IPC) group. (A, C and E) x200 magnificationl; (B, D and F) x400 magnification.

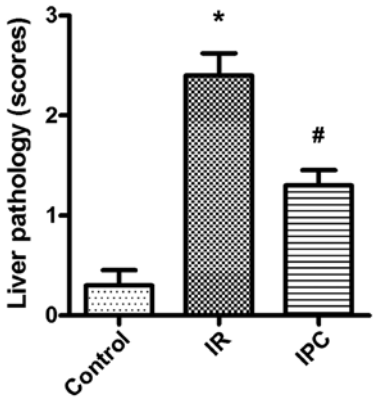

Figure 2. Scores of liver pathology. *P<0.01 vs. sham-operated (control) group; #P<0.01 vs. ischemia/reperfusion (I/R) group; n=10 per group. IPC, ischemic preconditioning.

USA) for 2 h. The membranes were then incubated overnight at 4˚C with antibodies raised against β-actin (sc-47778; 1:1,000), GSK-3β (sc-9166; 1:800), p-GSK-3β (sc-11757; 1:600), β-catenin (sc-7963; 1:500), VEGF (sc-7269; 1:600), Bcl-2 (sc-7382; 1:600) and survivin (sc-17779; 1:500; all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Secondary antibodies (ZDR-5308, ZDR-5306 and ZDR-5307) conjugated to horseradish peroxidase (HRP; 1:2,000) were from Beijing Zhongshan Golden Bridge Biological Technology (Beijing, China). The signals were visualized using a chemiluminescent substrate kit (Thermo Fisher Scientific, Rockford, IL, USA) and analyzed using a gel imaging system (Kodak System EDAS120; Kodak, Tokyo, Japan). Gray values were normalized to those of β-actin.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was quantified using the Quant-iT™ RiboGreen® RNA Assay kit (Invitrogen, Eugene, OR, USA). Equal amounts of mRNA were reverse transcribed into single-stranded cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Framingham, MA, USA). The expression of the target genes was measured by quantitative PCR using Power SYBR-Green PCR Master mix, on an ABI PRISM 7300 sequence detection system (both from Applied Biosystems). The following primer sets were used in quantitative PCR: Bcl-2 forward, 5'-AGCCCTG TGCCACCTGTGGT-3' and reverse, 5'-ACTGGACATCTCT GCAAAGTCGCG-3'; survivin forward, 5'-AGGACCACC GGATCTACACCTTCA-3' and reverse, 5'-CTCGGTAGGGC AGTGGATGAAGC-3'. All results were normalized to β-actin (forward, 5'-CCCATCTATGAGGGTTACGC-3' and reverse, 5'-TTTAATGTCACGCACGATTTC-3') and the values were calculated using the $2^{-\Delta Ct}$ method.

Statistical analysis. All data are presented as the means ± SD. One-way analysis of variance (ANOVA) followed by LSD was used to compare the differences between the 3 groups. A P-value <0.05 was considered to indicate a statistically significant difference. All statistical analyses were carried out using the Statistical Product and Service Solutions (SPSS 16.0) statistical software package (SPSS Inc., Chicago, IL, USA).

Results

IPC decreases the severity of morphological and pathological changes induced by I/R injury. Morphological observations indicated that the liver tissues from the rats in the sham-operated group were normal. On the contrary, the liver tissues from the rats in the I/R group appeared swollen and were dark red with mass effusion in the abdominal cavity. In the rats from the IPC group, the liver tissues presented only a mild increase in volume and slight effusion was observed. In agreement with these results, light microscopy revealed the presence of disorganized liver tissues in the I/R group, with the disintegration of hepatic cords, blood stasis in the central vein and small vessels within the portal area, edema and hemorrhaging, as well as neutrophil infiltration. Compared with the I/R group, IPC significantly decreased the severity of liver injury; the organs showed more regularly arranged hepatocytes, and less edema, hemorrhaging, blood stasis and neutrophil infiltration (P<0.01) (Figs. 1 and 2).

Effects of IPC on I/R-induced acute liver injury. As general markers of acute liver injury, the serum levels of ALT and AST were determined. As expected, there was a significant increase in the serum ALT and AST levels in the I/R group compared with sham-operated animals, indicating severe liver damage caused by I/R. Of note, IPC exerted protective effects against I/R-induced liver injury, which resulted in decreased ALT and AST levels. The ALT levels were 42.80±5.51, 625.30±76.04 and 410.20±61.10 U/l in the sham-operated group (controls), the I/R group and IPC group, respectively; the AST levels were 25.20±7.19, 60.20±7.48

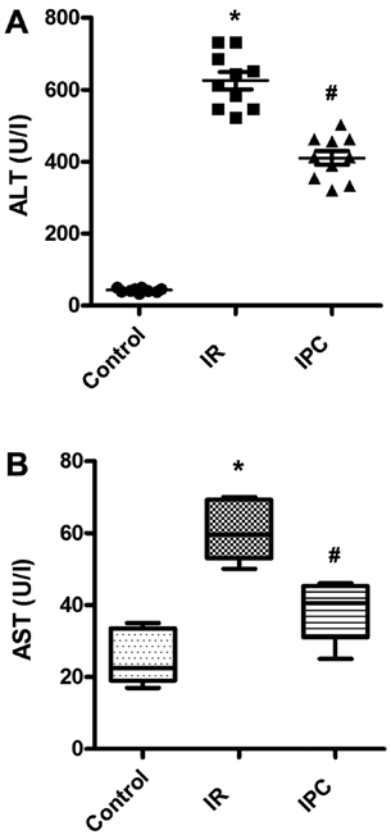

Figure 3. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in the different groups. *P<0.01 vs. sham-operated (control) group; #P<0.01 vs. ischemia/reperfusion (I/R) group; n=10 per group. IPC, ischemic preconditioning.

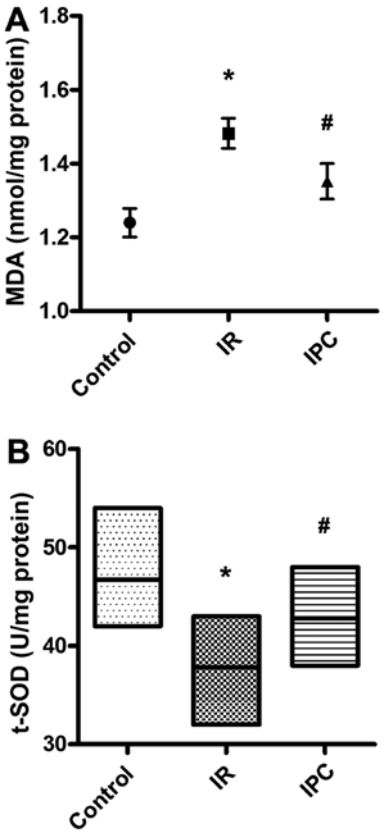

Figure 4. Liver tissue maleic dialdehyde (MDA) and superoxide dismutase (SOD) activity in the different groups. *P<0.01 vs. sham-operated (control) group; #P<0.01 vs. ischemia/reperfusion (I/R) group; n=10 per group. IPC, ischemic preconditioning.

and 38.20±7.97 U/l in the sham-operated group (controls), the I/R group and IPC group, respectively (all P<0.01) (Fig. 3).

Effects of IPC on liver MDA and SOD activity. MDA is the degradation product of lipid peroxidation caused by oxygen free radicals attacking polyunsaturated fatty acids in biological membranes, which results in cross-linking and the polymerization of macromolecules, such as proteins and nucleic acids, leading to cell cytotoxicity (24). Generally, MDA levels indirectly reflect the degree of cell membrane damage (24). Compared with the sham-operated group, liver tissue MDA levels in the I/R group were significantly increased (1.24±0.12 vs. 1.48±0.13 nmol/mg protein, P<0.01). However, the MDA levels were markedly decreased in the IPC group compared with the I/R group (1.48±0.13 vs. 1.35±0.15 nmol/mg protein, P<0.01) (Fig. 4A). On the contrary, SOD is an active substance that removes harmful metabolic products and plays an important role in the balance between oxidation and anti-oxidation (25). Liver tissue SOD levels in the I/R group decreased significantly (46.70±3.83 vs. 37.80±3.52 U/mg protein, P<0.01) compared with the sham-operated animals. Of note, the SOD levels were increased in the IPC group compared with the values obtained for the I/R group (37.80±3.52 vs. 42.80±3.46 U/mg protein, P<0.01) (Fig. 4B).

IPC activates GSK-3β/β-catenin signaling during liver I/R. GSK-3β is an enzyme that is specifically inactivated after phosphorylation. To further determine the effects of IPC on I/R injury, we assessed the levels of GSK-3β phosphorylated at Ser9 (p-GSK-3β) by western blot analasis In the sham-operated group, p-GSK-3β was barely detectable, while the expression of total GSK-3β was relatively high. An increase in p-GSK-3β expression was observed following I/R (Fig. 5). Of note, IPC further increased the expression of p-GSK-3β compared with the I/R group, while total GSK-3β expression remained unaltered. The p-GSK-3β levels were 0.09±0.00, 0.31±0.01 and 0.56±0.03 in the sham-operated group, the I/R group and IPC group, respectively, (all P<0.05); the total GSK-3β levels were 0.59±0.03, 0.56±0.01 and 0.57±0.01 in the sham-operated group, the I/R group and IPC group, respectively (all P>0.05) (Fig. 5). As regards β-catenin, its expression was detected at significantly greater levels in the cytosolic and nuclear fractions in the IPC group in comparison with the I/R group (cytosolic fraction, 0.17±0.01 vs. 0.40±0.01, P<0.01; nuclear fraction, 0.36 ±0.01 vs. 0.57±0.03, P<0.01) (Fig. 6A and B).

VEGF is a downstream effector of the GSK-3β/β-catenin signaling pathway. When β-catenin accumulates in the cytosol and translocates to the nucleus, it binds to TCF/LEF, which results in the activation of VEGF and an increase in its expression (37). To further determine whether this pathway is activated, we measured the expression of VEGF. Compared with the I/R group, VEGF expression in the IPC group increased significantly (0.19±0.01 vs. 0.41±0.01, P<0.01) (Fig. 6C), similar to the expression of p-GSK-3β and β-catenin.

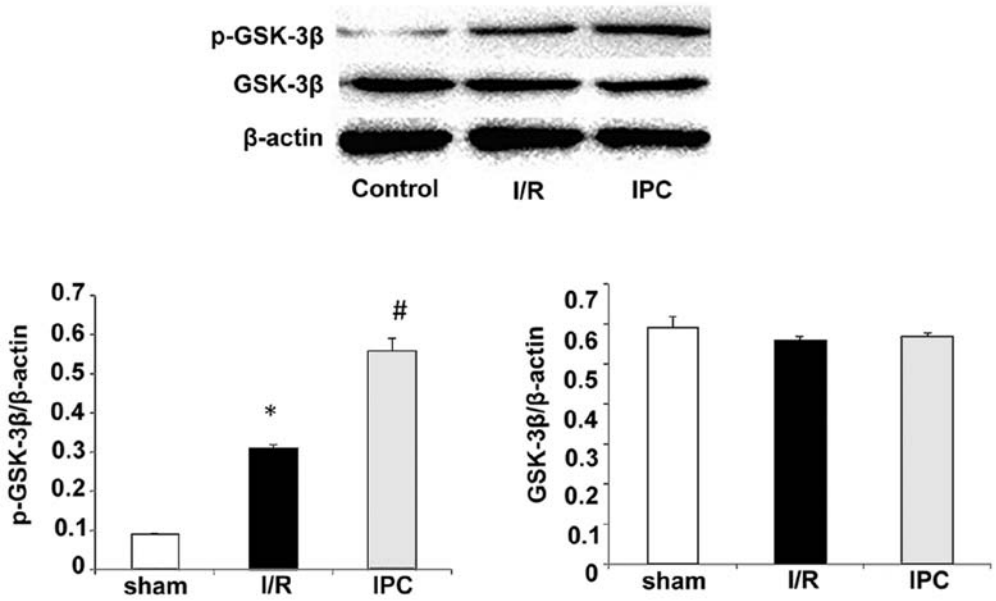

Figure 5. Protein expression of phosphorylated-glycogen synthase kinase-3β (p-GSK-3β) and GSK-3β in liver tissues (western blot analysis). Gray values were normalized to those of β-actin. The results revealed an increased protein expression of p-GSK-3β in the ischemic preconditioning (IPC) group in comparison with the ischemia/reperfusion (I/R) group; GSK-3β was maintained at comparable levels in all groups. *P<0.05 vs. sham-operated group; #P<0.05 vs. I/R group, values are the means ± SD, n=10 per group.

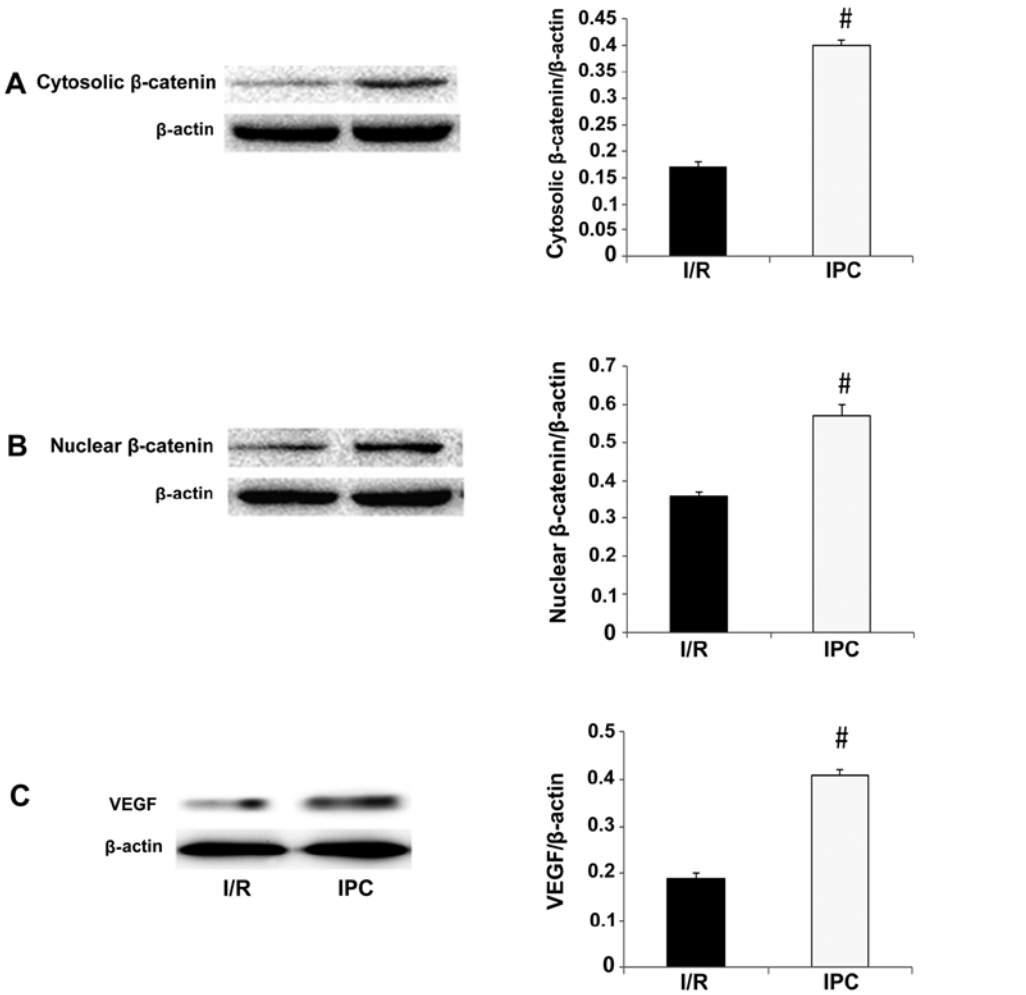

Figure 6. Expression of (A) cytosolic β-catenin, (B) nuclear β-catenin and (C) VEGF in liver tissues (western blot analysis). All protein levels were increased in the ischemic preconditioning (IPC) group compared with the ischemia/reperfusion (I/R) group. #P<0.01 vs. I/R group, values are the mean ± SD, n=10 per group.

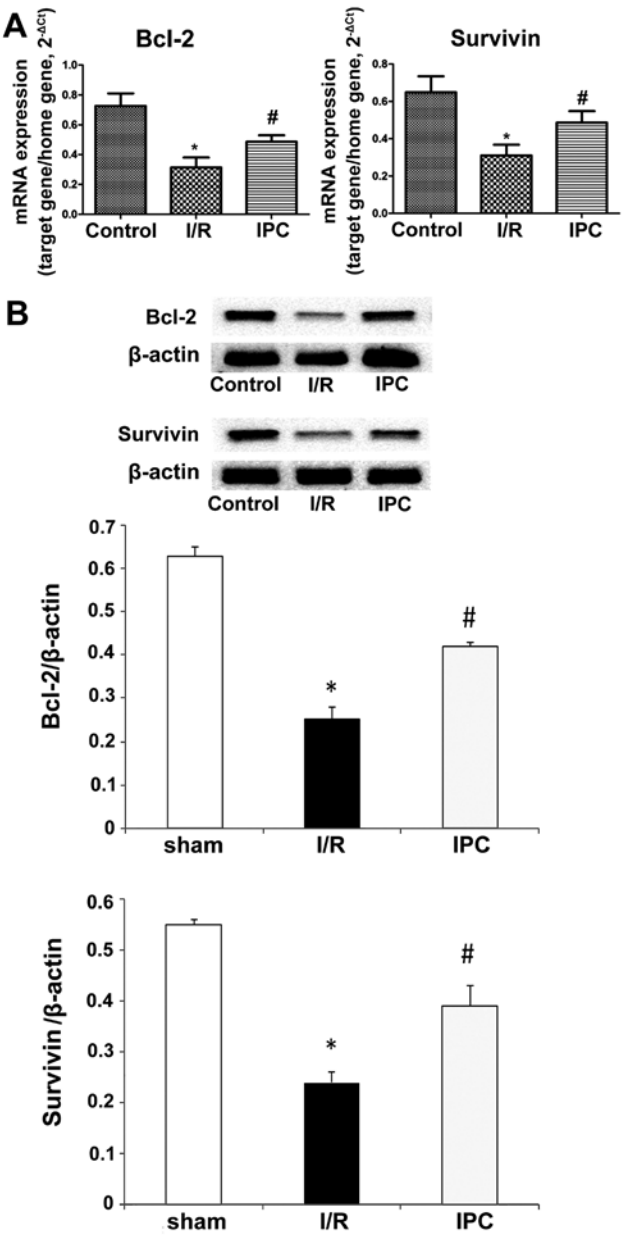

Figure 7. (A) mRNA and (B) protein expression levels of Bcl-2 and survivin. *P<0.01 vs. sham-operated group; #P<0.05 vs. ischemia/reperfusion (I/R) group, values are the mean ± SD; n=10 per group. IPC, ischemic preconditioning.

These results indicate that IPC activates the GSK-3β/β-catenin signaling pathway during liver I/R.

IPC upregulates Bcl-2 and survivin mRNA expression and attenuates apoptosis. Bcl-2 and survivin are anti-apoptotic proteins that inhibit the release of cytochrome *c* and the induction of subsequent apoptosis by various inducers (37). RT-qPCR revealed that both Bcl-2 and survivin were detected in the 3 groups. I/R conspicuously downregulated Bcl-2 and survivin expression compared with the sham-operated animals, while IPC significantly increased the mRNA expression of Bcl-2 and survivin in comparison with the I/R group. For Bcl-2, relative mRNA amounts of 0.73±0.08, 0.31±0.06 and 0.49±0.04 were obtained in the sham-operated group, I/R group and IPC group, respectively (all P<0.01); the relative mRNA levels for survivin were 0.65±0.09, 0.31±0.06 and 0.49±0.06 in the sham-operated group, I/R group and IPC group, respectively (all P<0.05) (Fig. 7A). The results from western blot analysis corroborated these findings. Indeed, the relative Bcl-2 protein levels were 0.63±0.02, 0.25±0.03 and 0.42±0.01 in the sham-operated group, I/R group and IPC group, respectively (all P<0.01); the survivin levels obtained in the sham-operated, I/R group and IPC group were 0.55±0.01, 0.24±0.02 and 0.39±0.04, respectively (all P<0.05) (Fig. 7B).

Discussion

In this study, we demonstrated that IPC ameliorates I/R-induced-liver injury at the morphological and pathological levels. This was confirmed by the decreased serum ALT and AST levels observed in the IPC group compared with the I/R group. In agreement with our findings, remote ischemic preconditioning (RIP) and N-acetylcysteine with RIP, as well as other ischemic preconditioning methods have been shown to exert protective effects against reperfusion injury in rats (26-28). Of note, in our study, IPC reversed the I/R-induced increase in MDA activity, as well as the I/R-induced decrease in SOD activity in the liver tissue, as mentioned above. These data indicate that IPC modulates the liver oxidant-antioxidant system during I/R injury in rats as has been reported for other ischemic preconditioning methods (28-30).

Liver I/R leads to severe injury and even liver failure, and IPC is a simple method for ameliorating liver I/R injury. The mechanisms underlying the protective effects of IPC against I/R injury remain controversial. Previous studies (31,32) have demonstrated that IPC significantly reduces inflammatory cell infiltration so as to improve hepatic microcirculation through the activation of transcription regulators, such as nuclear factor-κB (NF-κB) and hypoxia-inducible factor-α (HIF-α). Consequently, the transcription of inducible nitric oxide synthase (iNOS) is regulated and NO synthesis is increased, which results in the amelioration of hepatic microcirculation and decreased oxygen free radical damage. Moreover, the expression of tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β has been shown to be downregulated after liver I/R; this significantly reduces the inflammatory response and cell apoptosis (33). In this study, we demonstrated that IPC activated the GSK3β/β-catenin signaling pathway.

GSK-3 is a widely expressed and multifunctional serine/threonine protein kinase, and includes the GSK-3α and GSK-3β subtypes. GSK-3β is constitutively active in its dephosphorylated form and has a pleiotropic function in the regulation of cell activation, differentiation and survival. It has previously (34) been suggested that GSK-3β regulates the expression of cAMP-response element binding protein (CREB), heat shock factor 1 (HSF1) and heat shock protein 70 (HSP70), as well as that of caspase and Bax, leading to cell apoptosis. Several signaling pathways participate in the regulation of GSK-3β activity, including the PI3K-PKB/Akt and Wnt pathways. The phosphorylation of GSK-3β leads to the release, stability and accumulation of β-catenin in the cytoplasm, followed by its translocation into the nucleus and combination with TCF/LEF, which activates or inhibits target genes, such as cMyc, cyclin D1, fibrin, VEGF, Bcl-2 and survivin (13,14,18-20,33). Tong *et al* (36) reported that IPC leads to the phosphorylation and inactivation of GSK-3β, exerting marked cardioprotective effects. The stability and

accumulation of β-catenin in the cytoplasm is the core event, while its translocation to the nucleus translates into the activation of the Wnt pathway. Kaga *et al* (37) found that IPC accelerates angiogenesis and anti-apoptosis by upregulating the expression of VEGF, Bcl-2 and survivin through GSK-3β/β-catenin signaling, significantly ameliorating myocardial I/R injury in rats. Therefore, in this study, we aimed to verify whether the same mechanism of GSK-3β/β-catenin signaling contributes to the protective effects of IPC against liver I/R injury.

Of note, p-GSK-3β was barely detectable in the sham-operated group; however, its expression increased following I/R and IPC, while the total GSK-3β levels were maintained at relatively high levels. These findings further confirmed the liver injury in the I/R and IPC groups. Importantly, it has been demonstrated that the inhibition of GSK-3β ameliorates hepatic I/R injury through the GSK-3β/β-catenin signaling pathway (38) and an IL-10-mediated immune regulatory mechanism (39). In this study, β-catenin significantly accumulated in the cytosol and nucleus along with the increased VEGF expression in the IPC group in comparison with the I/R group. These data suggest that IPC activates the GSK-3β/β-catenin signaling pathway, alleviating liver I/R injury.

The majority of neuronal and cardiac studies have suggested that the protective effects of GSK-3β inhibition occur through anti-apoptosis. Koh *et al* (40) used a transient middle cerebral artery occlusion model and verified that GSK-3β inhibition protected neuronal tissue from occlusion-induced damage through anti-apoptosis. Using adeno-shRNA, Thirunavukkarasu *et al* (41) found that the knockdown of β-catenin abolished the IPC-mediated cardioprotective effects by downregulating the target genes, Bcl-2 and survivin, in the ischemic rat myocardium. Kaga *et al* (37) clarified that SB216763 increased the accumulation of β-catenin in both the cytosol and nucleus, which activated GSK-3β/β-catenin and further enhanced anti-apoptotic signaling through the induction of Bcl-2 and survivin expression in the rat IPC myocardium.

Studies assessing different types of liver damage have also suggested that the protective effects of GSK-3β inhibition occur through anti-apoptosis (42-44). In this sutdy, we demonstrate that IPC leads to the phosphorylation and inactivation of GSK-3β, thus function as a GSK-3β inhibitor. Of note, the results from western blot analysis and RT-qPCR revealed higher levels of the anti-apoptotic factors, such as Bcl-2 and survivin in the IPC group compared with the I/R group, suggesting decreased apoptosis in the former group. The anti-apoptotic effects of GSK-3β inactivation in our model of liver I/R injury were consistent with those reported for other models (43,44).

In conclusion, in this study, to the best of our knowledge, we present the first evidence that the inactivation of GSK-3β by IPC in liver I/R induces β-catenin signaling and subsequently upregulates anti-apoptotic factors, such as Bcl-2 and survivin, leading to a significant amelioration of liver I/R injury. As reported in a previous study (45), liver I/R induces hepatocyte and non-parenchymal cell death through necrosis and apoptosis, as well as proliferation. However, it remains unclear whether the IPC-induced inactivation of GSK-3β also relieves liver proliferation and necrosis; further studies are required to clarify this. Furthermore, further studies are required to clarify whether inhibitors of GSK-3β and β-catenin affect other downstream target genes, such as HIF-α and NF-κB, which may enhance our understanding of these events. Overall, our data demonstrate partly how IPC ameliorates liver I/R injury and enhances anti-apoptosis through GSK-3β/β-catenin signaling.

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