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Original Article

Propofol alleviates hepatic ischemia/reperfusion injury via the activation of the Sirt1 pathway

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Abstract: Background and objectives: Propofol exerts protective effects on multiple organs, including the liver. The aim of the present study was to investigate whether the protective effects of propofol on the liver are related to Sirt1, an NAD^+-dependent deacetylase with anti-inflammatory and antioxidant properties. Methods: After treatment with propofol, hepatic I/R injury was induced in mice. Liver injury, oxidative stress, antioxidant capacity, cytokine production, and apoptotic markers were investigated to assess the effects of propofol pretreatment on hepatic I/R injury. The expression of Sirt1 was assessed by immunohistochemical and western blot analyses, and the expression levels of NF-κB/p65, IκBα, Bcl-2 and Bax were analyzed by western blot. Results: After 70% hepatic I/R injury, the mice that were pretreated with propofol showed considerably less liver injury, enhanced anti-inflammatory and antioxidant capacity, and less apoptosis. Additional studies revealed that propofol pretreatment prior to I/R injury results in reduced NF-κB activation and apoptosis through Sirt1 activation. Conclusions: The present study is the first to reveal that propofol can significantly reduce hepatic I/R injury by regulating the expression of Sirt1, and these effects may be related to anti-inflammatory and antioxidant effects. Our results suggest that propofol may be an effective therapeutic strategy for the treatment of hepatic I/R injury in hepatobiliary surgery.

Keywords: Ischemia/reperfusion injury, Sirt1, propofol, oxidative stress, inflammation

Introduction

Ischemia/reperfusion (I/R) injury is a major risk in liver transplantation, liver resection, and trauma. Various mechanisms interact to achieve complex pathophysiological changes [1]. Previous studies have shown that inflammation and oxidative stress play critical roles in the early stages of I/R injury [2]. Therefore, it is important to identify effective strategies to mitigate inflammatory and oxidative stress to protect the liver from I/R injury.

Sirt1 is an NAD^+-dependent class III histone deacetylase that removes acetyl (Ac) groups from non-histone targets, causing changes in cell metabolism, survival, and senescence [3]. A number of recent studies have indicated that Sirt1 can alleviate I/R injury in multiple organs, such as the heart, liver, kidney, and intestines [4-7]. The application of SRT1720 (a Sirt1 agonist) attenuates ischemic liver injury and enhances mitochondrial recovery and autophagy [6]. In Sirt1-deficient mice, the levels of inflammation, oxidative stress, and apoptosis increase in response to I/R injury [7]. Thus, Sirt1 is regarded as a promising target for the treatment of hepatic I/R injury.

Propofol (2,6-diisopropylphenol), a widely used injectable anesthetic, is primarily administered for the sedation of surgical or critically ill patients. The chemical properties of propofol are similar to alpha tocopherol, which is regarded as a free radical scavenger that protects organs through anti-inflammatory and antioxidant effects under diverse pathophysiological conditions, including focal cerebral ischemia, drug-induced neurotoxicity, and intestinal I/R injury [8-11]. However, whether the anti-inflammatory and antioxidant effects of propofol are associated with Sirt1 and the effects on hepatic I/R injury remains unknown.

To investigate the protective effects and intrinsic mechanisms of propofol on hepatic I/R injury, we established a 70% hepatic I/R injury mouse model, focusing on the dynamic changes in Sirt1 expression during hepatic I/R injury and the role of Sirt1 in injury. Our results show that propofol protects the liver by reducing inflammation and oxidative stress in response to hepatic I/R injury, which is associated with propofol-induced overexpression of Sirt1.

Materials and methods

Animals and ethics statement

Male C57BL/6 mice (6-8 weeks, weighing 20-25 g) were purchased from Hunan SJA Laboratory Animal Company Limited (Changsha, China). The mice were housed individually in wire-bottomed cages and were maintained under pathogen-free conditions for one week prior to the experiments. The experimental protocol and design was approved by the Nanchang University Animal Experimentation Committee, and experiments were performed according to the Nanchang University Guidelines for Animal Experimentation.

Administration of drugs

Propofol (commercial product Diprivan, AstraZeneca Plc., London, UK) is available as an intralipid solution. The drug was intraperitoneally administered daily at a dose of 0.025 mg/g (no sedative effect), 0.05 mg/g (sedative effect), or 0.1 mg/g (anesthetic effect) for 3 days prior to surgery. The dose of propofol was based on previous studies; specifically, the sedative effect on mice was characterized by a lack of response to pain stimuli while demonstrating responsiveness to acupuncture stimulation. At a dose of 0.1 mg/g, the mice achieved satisfactory anesthetic effects, which manifested as limb relaxation, loss of eyelash reflex, loss of consciousness without significant myocardial depression, and hypotension [12, 13]. As a control for propofol treatment, intralipid (20%, Sigma, St. Louis, MO, USA), the propofol vehicle solution, was intraperitoneally injected at the same volume of propofol for 3 days prior to surgery.

Ex527 (Selleck Chemicals, Houston, USA) is a potent, selective inhibitor of Sirt1 that effectively inhibits Sirt1 deacetylase activity but does not affect the gene and protein expression of Sirt1. Ex527 was dissolved in dimethyl sulfoxide (DMSO) (Sigma), and DMSO was diluted to a final concentration of 2% with normal saline for each medium to minimize any nonspecific or toxic effects. The mice were pretreated for 7 days (0.02 mg/g/day i.p.) with Ex527 prior to surgery, and the dose of Ex527 was based on previous studies [14].

Model of partial hepatic I/R injury and experimental groups

The mice were anesthetized with pentobarbital (50 mg/kg, i.p.) after drugs administration (**Figure 1A**). Based on previous studies, a model of 70% hepatic ischemia-reperfusion was established [15]. Briefly, we made a midline abdominal incision to expose the porta hepatis, after which we clamped the portal structure to the left and median lobes using an atraumatic vascular clip. The surgical incision was covered with wet gauze. After 45 min of 70% hepatic ischemia, the clip was removed, and the abdominal cavity was sutured using 4-0 silk sutures. After reperfusion for 4 hours (IR4h) or 24 hours (IR24h), the animals were sacrificed by exsanguination under anesthesia, and the blood samples and left lateral and median lobes of the liver were collected for further analysis. In the sham operation group, a laparotomy was performed in mice without vascular occlusion.

Initially, the mice were randomly divided into three groups: (1) wild-type group (WT), in which the mice were not subjected to any treatments, (2) 0.1 mg/g propofol pretreatment group (Pro0.1 group), and (3) intralipid pretreatment group (intralipid group). Each group comprised 18 mice divided into three subgroups: Sham group, IR4h group and IR24h group, with n=6 mice per group. The mice were subsequently divided into the following groups according to the dose of drugs administered and the surgery received, with n=6 mice per group: (1) 0.025 mg/g propofol pretreatment group, followed by IR4h (IR4h+Pro0.025 group), (2) 0.05 mg/g propofol pretreatment group, followed by IR4h (IR4h+Pro0.05 group), (3) 0.1 mg/g propofol pretreatment, followed by IR4h (IR4h+Pro0.1 group), (4) Ex527 pretreatment, followed by IR4h (IR4h+Ex527 group), (5) 0.1 mg/g propofol and Ex527 pretreatment, followed by IR4h (IR4h+Pro0.1+Ex527 group), and (6) intralipid pretreatment, followed by IR4h (IR4h+intralipid).

Hematoxylin & eosin and immunohistochemical (IHC) staining

The liver specimens were fixed in 10% formalin, embedded in paraffin, and sectioned. For histological analysis, the liver tissue sections were deparaffinized and stained with hematoxylin & eosin (H&E) to detect morphological changes. The Suzuki method was used to calculate the extent of liver damage.

For IHC experiments, deparaffinized sections were soaked in sodium citrate buffer (10 mmol/l, pH 6.0) and subjected to microwave antigen retrieval for 15 min. Subsequently, the sections were incubated in 0.3% H_2O_2 for 20 min and blocked with 10% serum for 15 min, followed by rabbit anti-mouse monoclonal Sirt1 antibody (Abcam Trading Shanghai Company, Shanghai, China) incubation overnight at 4°C. The next day, the sections were incubated with secondary antibodies, followed by horseradish peroxidase (HRP)-labeled streptavidin stock solution (Boster, Wuhan, China). Finally, the sections were stained using DAB developer kits (Boster) and counterstained with hematoxylin.

Quantification of liver injury

The blood samples were centrifuged at 4,000 r/min for 10 min to obtain serum for analysis. Serum ALT and AST levels were assessed using an automatic chemical analyzer (Olympus Company, Tokyo, Japan).

TdT-mediated dUTP-biotin nick-end labeling (TUNEL)

Paraffin-embedded liver tissues were cut into 4-μm-thick sections, after which they were stained with a TUNEL-based cell death detection kit (Roche-Boehringer, Mannheim, Germany) performed according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

The serum TNF-α and IL-6 levels were detected according to the manufacturer's instructions using ELISA kits (Boster).

Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity

Liver tissues were homogenized into 10% solutions in normal saline, after which the homogenates were subjected to three freeze-thaw cycles and then centrifuged at 4,000 r/min for 15 min to obtain supernatants. The MDA content and SOD activity in the liver tissues were determined according to the manufacturer's instructions (Jiancheng Bioengineering Ltd, Nanjing, China).

Western blot analysis

Total proteins and nuclear proteins were extracted using total protein and nuclear protein extraction kits according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). The protein concentrations were measured using the bicinchoninic acid method (Applygen Technologies, Beijing, China).

The protein expression levels of Sirt1, NF-κB/p65, IκBα, Bcl-2, Bax, TBP, and β-actin were determined by western blot analysis. Primary antibodies against the following proteins were used: Sirt1, NF-κB/p65, IκBα (Abcam Trading Shanghai Company), Bcl-2, Bax, TBP (Proteintech, Wuhan, China), and β-actin (Beijing Golden Bridge Biotech, Beijing, China). The proteins were loaded onto 10% or 12% SDS-PAGE gels for electrophoresis and then transferred to PVDF membranes. The PVDF membranes were blocked with 5% non-fat dry milk/TBST, after which they were incubated with primary antibodies overnight at 4°C. The next day, after washing with TBST, the membranes were incubated with HRP-conjugated antibodies for 1 h at room temperature. The bands were detected using a ChemiDoc™ MP System (Bio-Rad Laboratories, Hercules, CA, USA) and were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The data are expressed as the means ± SD. Statistical analyses were performed using GraphPad PRISM software (GraphPad Software, CA, USA) and the SPSS 22.0 software package (SPSS, Chicago, USA). Student's t-tests were used to analyze differences between two groups, while one-way ANOVA was used to analyze differences between three or more groups. The statistical significance level was set at $P<0.05$.

Results

Propofol reduces hepatic I/R injury

To evaluate the effects of propofol on hepatic I/R injury, we analyzed serum AST and ALT lev-

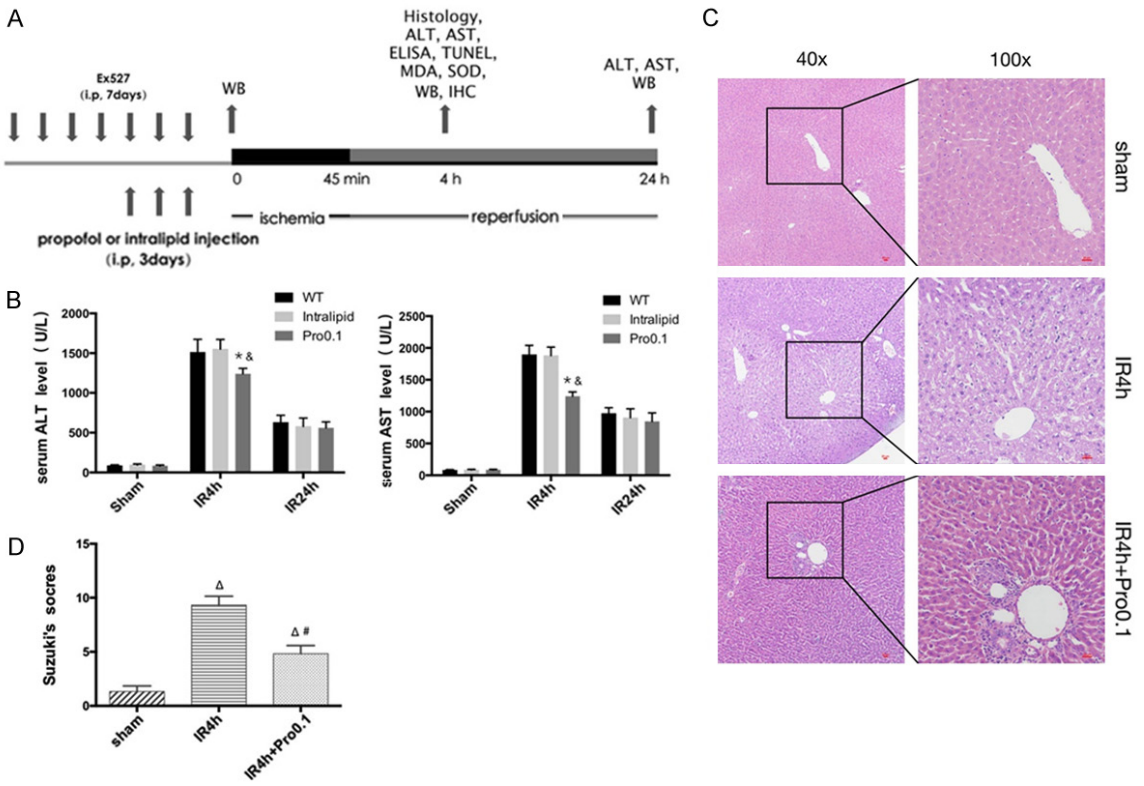

Figure 1. Propofol reduces hepatic I/R injury. A. Flowchart of the experimental protocol. B. Serum AST and ALT levels in the sham and the experimental groups after partial ischemia and 4 h or 24 h reperfusion. C. Partial ischemia and 4 h reperfusion induced dynamic pathologic changes in the sham and experimental groups as shown by H&E staining (×40, ×100). D. Suzuki classification. Mean ± SD, *P<0.05 versus the WT group, $^{\&}$P<0.05 versus the intralipid group, $^{\Delta}$P<0.05 versus the sham group; $^{\#}$P<0.05 versus the IR4h group, n=6 mice per group.

els after reperfusion. As shown in **Figure 1B**, after reperfusion for 4 h, the AST and ALT levels significantly increased in the WT, intralipid, and Pro0.1 groups and then gradually decreased after reperfusion for 24 h (P<0.05). We observed no detectable difference between the intralipid and the WT groups after reperfusion for 4 and 24 h, whereas the AST and ALT levels in the Pro0.1 group were significantly less than those in the other two groups after reperfusion for 4 h (P<0.05). However, this result did not reach statistical significance when the Pro0.1 group was compared with the other two groups after reperfusion for 24 h. The histological changes in the livers of the IR4h group (**Figure 1C**) showed serious hepatic tissue injury, including sinusoid damage, congestion, hepatocyte swelling, inflammatory cell infiltration, necrosis and cavitation. In contrast to the large area of necrosis in the IR4h group, mice in the IR4h+Pro0.1 group showed less severe liver damage, manifesting as minor or non-existent necrotic areas. The IR4h+Pro0.1 group also demonstrated lower Suzuki scores (P<0.05) (**Figure 1D**), suggesting that propofol pretreatment markedly reduces hepatic I/R injury.

Propofol attenuates oxidative stress, inflammation, and apoptosis induced by hepatic I/R injury

Inflammation and oxidative stress are the main mechanisms in the early stages of I/R injury [16]. To assess the effects of propofol pretreatment on hepatic inflammation and oxidative stress, blood and hepatic samples from each group were collected after reperfusion for 4 h. Subsequently, the activity of the antioxidant enzyme SOD and the levels of the membrane lipid peroxidation indicator MDA were examined. As shown in **Figure 2A**, compared with the MDA content in the IR4h group, the MDA content (P<0.05) in the IR4h+Pro0.1 group significantly decreased, whereas the SOD activity (P<0.05) increased, suggesting that propofol pretreatment reduces the level of oxidative

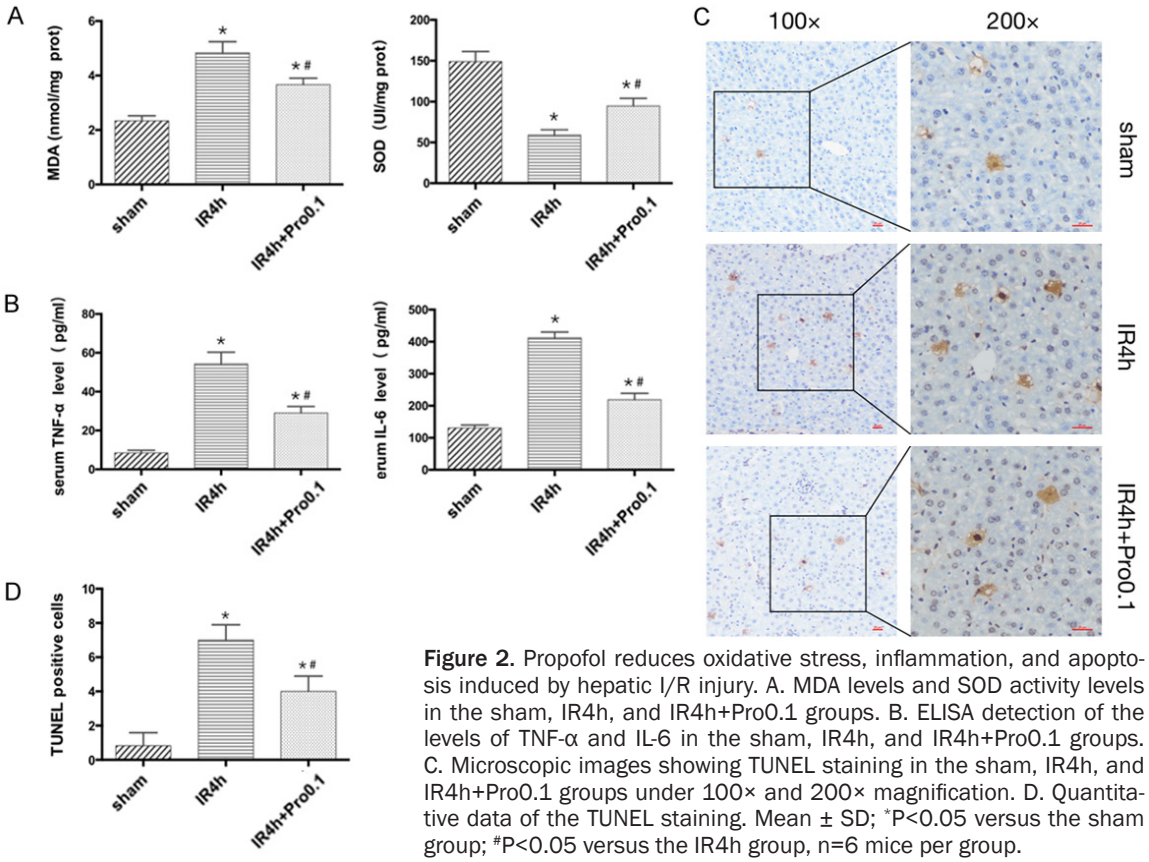

Figure 2. Propofol reduces oxidative stress, inflammation, and apoptosis induced by hepatic I/R injury. A. MDA levels and SOD activity levels in the sham, IR4h, and IR4h+Pro0.1 groups. B. ELISA detection of the levels of TNF-α and IL-6 in the sham, IR4h, and IR4h+Pro0.1 groups. C. Microscopic images showing TUNEL staining in the sham, IR4h, and IR4h+Pro0.1 groups under 100× and 200× magnification. D. Quantitative data of the TUNEL staining. Mean ± SD; *P<0.05 versus the sham group; #P<0.05 versus the IR4h group, n=6 mice per group.

stress caused by hepatic I/R injury. We also measured the levels of the cytokines TNF-α and IL-6 by ELISA and found that the levels of serum TNF-α ($P<0.05$) and IL-6 ($P<0.05$) in the IR4h+Pro0.1 group were lower than those in the IR4h group, indicating that propofol pretreatment also decreases the levels of inflammatory cytokines (**Figure 2B**). In addition, the level of hepatocyte apoptosis was detected by TUNEL. There was a remarkable reduction in TUNEL-positive cells in the IR4h+Pro0.1 group compared with that in the IR4h group, indicating that propofol pretreatment could stop the apoptosis of hepatocytes in mice (**Figure 2C** and **2D**).

Propofol increases the expression of Sirt1 after hepatic I/R injury

The expression of Sirt1 at 4 and 24 h after reperfusion in mice was investigated by IHC and western blotting to determine the dynamic changes in Sirt1 after hepatic I/R injury. IHC results showed that the expression of Sirt1 in hepatic tissue sharply decreased 4 h after reperfusion and recovered after 24 h (**Figure 3A**). The western blot results were consistent with the IHC results, showing that the Sirt1 levels decreased to 50.7% of the sham treatment group 4 h after reperfusion and subsequently increased to 72.8% of the sham group 24 h after reperfusion (**Figure 3B**). Thus, Sirt1 expression decreases during the early stages of hepatic I/R injury and then gradually recovers.

Next, we sought to determine how propofol pretreatment could affect Sirt1 levels after hepatic I/R injury. After the administration of different pretreatment doses of propofol (0.025, 0.05, and 0.1 mg/g/d for 3 days), western blot analysis of Sirt1 expression levels (**Figure 3C**) revealed that with increasing doses of propofol, the Sirt1 levels in liver tissue gradually increased. Moreover, in contrast with propofol-induced Sirt1 overexpression, we observed no statistically significant difference in the Sirt1 expression between the IR4h+intralipid and IR4h groups by comparing Sirt1 expression (**Figure 3D**). These results suggest that propofol increases the expression of Sirt1 after

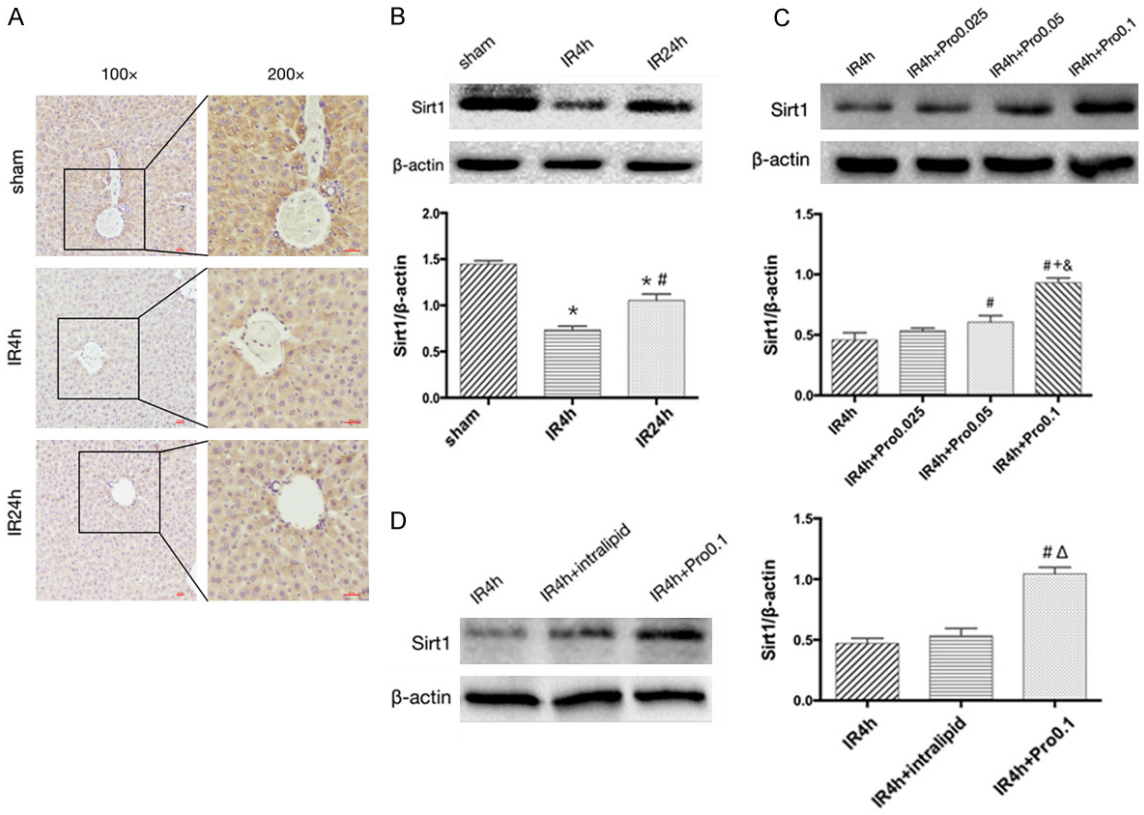

Figure 3. Propofol increases the expression of Sirt1 after hepatic I/R injury. A. IHC staining of Sirt1 in the sham, IR4h, and IR24h groups (×100, ×200). B. The Sirt1 and β-actin protein expression levels were analyzed by western blot in the sham, IR4h, and IR24h groups, and the relative quantities of Sirt1 to β-actin were assessed. C. The Sirt1 and β-actin protein expression levels were analyzed by western blot in the IR4h, IR4h+Pro0.025, IR4h+Pro0.05, and IR4h+Pro0.1 groups, and the relative quantities of Sirt1 to β-actin were assessed. D. The Sirt1 and β-actin protein expression levels were analyzed by western blot analysis in the IR4h, IR4h+intralipid, and IR4h+Pro0.1 groups, and the relative quantities of Sirt1 to β-actin were assessed. Mean ± SD; *P<0.5 versus the sham group; $^{\#}$P<0.05 versus the IR4h group; $^{\Delta}$P<0.05 versus the IR4h+intralipid; $^{+}$P<0.05 versus the IR4h+Pro0.025; $^{\&}$P<0.05 versus the IR4h+Pro0.05, n=6 mice per group.

hepatic I/R injury in a dose-dependent manner.

Propofol regulates NF-κB and apoptosis through Sirt1

NF-κB is a Sirt1 deacetylation target protein that has critical functions in inflammation and oxidative stress in response to hepatic I/R injury. NF-κB/p65 is a subunit of NF-κB, whose post-translational modification fine-tunes the transcriptional activation of the NF-κB complex [17] Therefore, we sought to determine the effects of propofol-induced Sirt1 overexpression on NF-κB/p65 and its inhibitor IκBα. To this end, we treated mice with the Sirt1 inhibitor Ex527. Western blot results showed that Ex527 did not inhibit the expression of Sirt1 protein during hepatic I/R injury but remarkably increased the level of NF-κB/p65, corresponding to reduced expression of IκBα (**Figure 4A**). Compared with the level of NF-κB/p65 in the IR4h group, the level of NF-κB/p65 in the IR-4h+Pro0.1 group was significantly decreased, and the level of IκBα showed a corresponding increased. However, Ex527 treatment reduced Sirt1 deacetylase activity and blocked the effects of propofol pretreatment on the expression of NF-κB/p65 and IκBα in the IR4h+Pro0.1+Ex527 group (**Figure 4A**). These results suggest that the regulation of NF-κB by propofol after hepatic I/R injury depends at least in part on Sirt1.

The ratio of Bcl-2 to Bax is a determinant "molecular switch" of apoptosis [18]. The Bcl-2/Bax ratio was lower in the IR4h group than in the sham group, and propofol pretreatment dr-

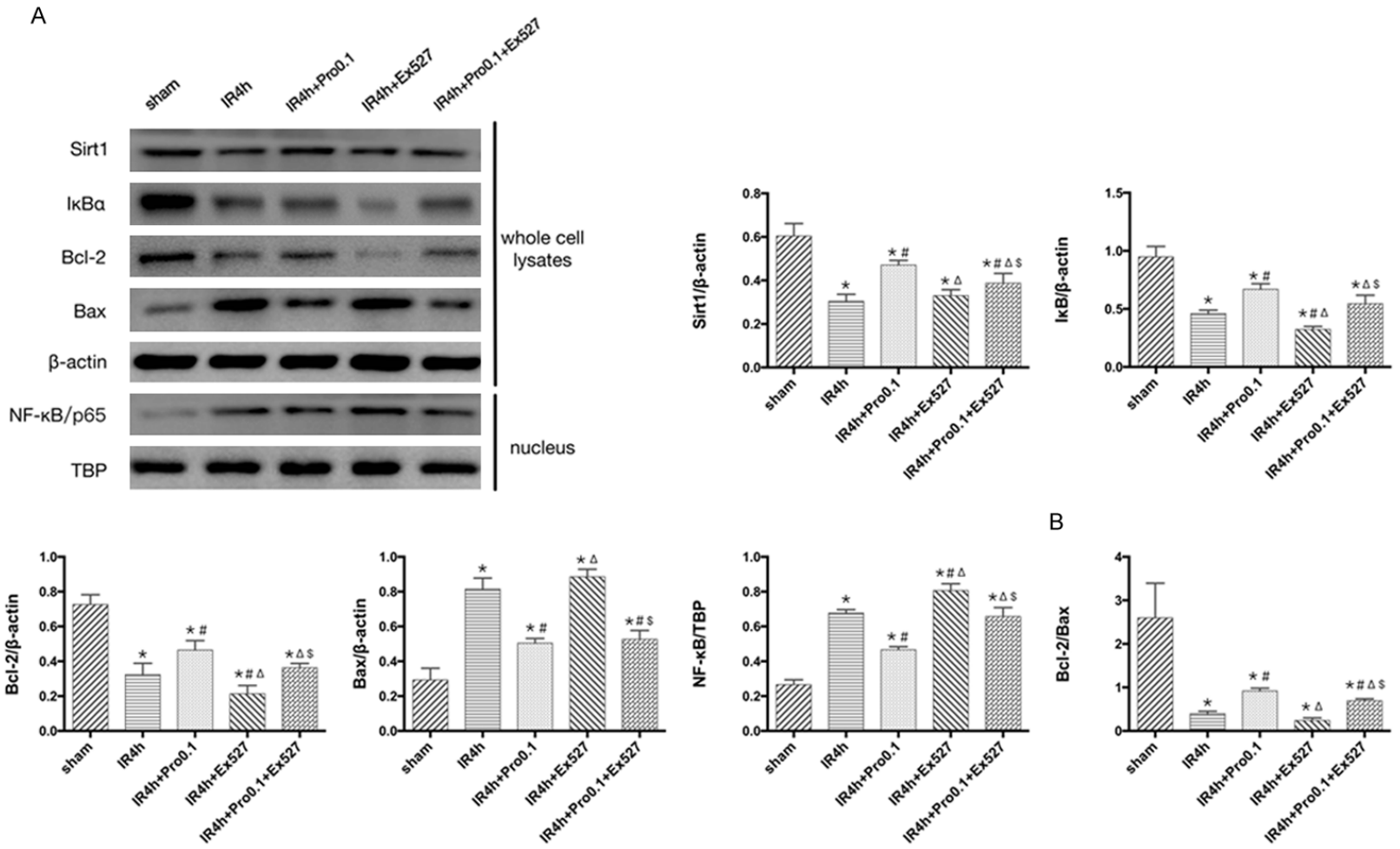

Figure 4. Propofol regulates NF-κB and apoptosis through Sirt1. A. The protein expression levels of Sirt1, NF-κB/p65, IκBα, Bcl-2, Bax, β-actin, and TBP were analyzed by western blot in the sham, IR4h, IR4h+Pro0.1, IR+Ex527, and IR4h+Pro0.1+Ex527 groups, and the relative quantities of Sirt1, IκBα, Bcl-2, and Bax to β-actin, as well as the relative quantities of NF-κB/p65 to TBP, were assessed. B. The anti-apoptotic Bcl-2/Bax ratio in each group. Mean ± SD, *P<0.05 versus the sham group; $^{\#}$P<0.05 versus the IR4h group; $^{\Delta}$P<0.05 versus the IR4h+Pro0.1 group; $^{\$}$P<0.05 versus the IR4h+Ex527 group, n=6 mice per group.

amatically increased the apoptotic Bcl-2/Bax ratio. These effects were diminished in the IR4h+Pro0.1+Ex527 group (**Figure 4A** and **4B**). These results suggest that propofol attenuates hepatocyte apoptosis induced by I/R injury through Sirt1.

Discussion

The main findings of the present study were that 1) propofol significantly alleviates the inflammation and oxidative stress induced by hepatic I/R injury and reduces hepatocyte apoptosis and 2) Sirt1 regulation plays a role in the propofol-mediated protection of hepatocytes. These results have not been reported in other studies.

Initially, we evaluated the changes of hepatic function during the early stages (IR4h) and late stages (IR24h) of hepatic I/R injury in mice, and we found that propofol protects the liver against hepatic I/R injury primarily during the early stages of hepatic I/R. In contrast, as a control for propofol, intralipid exerted no effect on liver function. Liver histological analysis also confirmed this conclusion. Therefore, in subsequent experiments, we selected the parameters of the IR4h group to further examine the protective mechanisms of propofol.

Inflammation and oxidative stress are the two most important mechanisms during the early stages of hepatic I/R injury when Kupffer cells produce large amounts of reactive oxygen species (ROS) and pro-inflammatory factors, such as TNF-α, IL-1, and IL-6 [2]. During later phases of I/R injury, these cytokines activate neutrophils, which further release cytokines, ROS, myeloperoxidase (MPO), and a variety of factors that exacerbate tissue damage [16]. Propofol is the most widely used clinical intravenous anesthetic and is listed on the WHO Model List of Essential Medicines as an indispensable drug in healthcare [19]. The chemical properties of propofol are similar to those of alpha tocopherol, and this compound may have antioxidant activity, as both molecules contain a phenolic hydroxyl group that inhibits lipid peroxidation and scavenges free radicals [8, 20]. Several studies have shown that propofol has anti-inflammatory and antioxidant effects in multiple organs [8-11]. Consistent with previous studies, in the present study, hepatic MDA levels decreased, whereas the activity of SOD increased after propofol pretreatment; these results are consistent with the antioxidant effects of propofol. The decreased levels of TNF-α and IL-6 suggest that propofol treatment reduces the inflammatory reaction in mice. In addition, TUNEL staining indicated that propofol pretreatment also prevents hepatocyte apoptosis; however, the intrinsic mechanism remains unclear.

Western blot analysis revealed that propofol pretreatment increases the expression levels of Sirt1, a member of the class III group of histone deacetylases, in a dose-dependent manner in response to I/R injury. Interestingly, many studies have shown that Sirt1 has hepatoprotective effects in hepatic I/R injury, and the main mechanism underlying Sirt1 function is anti-inflammatory and antioxidant effects [6, 21]. NF-κB is a Sirt1 substrate and is an important nuclear transcription factor in the context of hepatic I/R injury [1]. Typically, the NF-κB complex, which consists of a p50/p65 heterodimer, is retained in an inactive state in the cytoplasm by the inhibitory protein IκBα. When the liver is subjected to ischemia-reperfusion injury, IκBα becomes phosphorylated and recognized by a ubiquitin ligase, which results in IκBα ubiquitination and degradation. Thus, under I/R injury the inhibitory effect of IκBα on NF-κB is removed, which enables NF-κB to translocate to the nucleus and bind to the kappa B locus of the corresponding gene promoter and enhancer regions to regulate mRNA synthesis and the expression of inflammatory factors, chemotactic factors, and adhesion molecules [22]. After directly associating with the RelA/p65 subunit of NF-κB, Sirt1 deacetylates RelA/p65 at lysine 310, which further inhibits the transcriptional activity of NF-κB and reduces the inflammation and oxidative stress caused by NF-κB activation [17]. Consistent with previous studies, we induced Sirt1 expression in hepatic I/R injury by propofol treatment, which reduced NF-Kb/p65 levels in the nucleus and increased the expression levels of IκBα. Moreover, these effects were abolished by the concomitant inhibition of Sirt1 deacetylase activity. Therefore, the results of the present study demonstrate that propofol decreases the levels of inflammation and oxidative stress in the liver during I/R injury by regulating the Sirt1/NF-κB pathway, which increases the anti-apoptotic Bcl-2/Bax ratio and reduces hepatic apoptosis.

The present study has several limitations. For example, propofol is a short-acting alkylphenolic intravenous anesthetic, and the safety of this compound in humans and animals has been confirmed. Ex527, a specific deacetylase activity inhibitor of Sirt1, is an amide compound, and several studies have shown that propofol is safe for patients treated with amide drugs such as lidocaine (local amide-based anesthetics). However, it is not clear whether Ex527 in mice could affect the pharmacological properties of propofol, although neither drug is contraindicated. In addition, the experiments in the present study were conducted in vivo, and further in vitro studies are needed to investigate more in-depth mechanisms for propofol, Sirt1, and liver protection in hepatic I/R injury. Nevertheless, this study presents new insights into the protective effects of propofol on hepatocytes in hepatic I/R injury.

In summary, this study is the first to reveal that propofol can significantly reduce hepatic I/R injury through the regulation of Sirt1 expression, and these effects appear to be related to anti-inflammatory and antioxidant effects. These results suggest that propofol may be a promising therapeutic for the treatment of hepatic I/R injury in hepatobiliary surgery patients.

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Disclosure of conflict of interest

None.

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