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Simvastatin ameliorates total liver ischemia/reperfusion injury via KLF2-mediated mechanism in rats

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KEYWORDS
Hepatic;
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Summary
Objective: The total hepatic ischemia/reperfusion injury (IRI) involves the fact that both liver and gut are subjected to warm ischemia, which is a complex unavoidable process encountered during liver transplantation and a serious threat to graft outcome. The ways to improve hepatic IRI are currently limited. The aim of the present study was to explore the protective effect of simvastatin on total hepatic IRI and examine the underlying mechanisms.
Methods: Male Sprague Dawley rats were subjected to total (100%) hepatic warm ischemia to induce hepatic IRI. Thirty-six male rats (250—300 g) were randomly divided into three groups: sham, IRI control and simvastatin (1 mg/kg) pretreatment 0.5 h before surgery. Serum samples and liver tissues were collected after reperfusion at 6 and 24 h for further studies.
Results: Simvastatin pretreatment significantly decreased the values of the transaminases alanine aminotransferase and aspartate aminotransferase and improved histological alterations according to improved Suzuki's Score ($P < 0.05$). Moreover, simvastatin upregulated the expression of Kruppel-like factor 2 (KLF2), phosphorylated endothelial nitric oxide synthase and thrombomodulin ($P < 0.05$). Furthermore, simvastatin pretreatment affected superoxide

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dismutase and malondialdehyde activities ($P < 0.05$) to reduce oxidative stress, and inhibited levels of high-mobility group box-1, CD68, toll-like receptor 4, tumor necrosis factor α, interleukin-1β and interleukin-6 ($P < 0.05$) to suppress inflammatory response.
Conclusion: Simvastatin pretreatment ameliorates total hepatic IRI via a KLF2-mediated protective mechanism. Simvastatin may be used as a potential prophylactic treatment strategy for clinical trials against hepatic IRI.

Introduction

Hepatic ischemia/reperfusion injury (IRI) is an unavoidable process during liver transplantation and clamping of liver vessels during resection, and may lead to severe liver injury and dysfunction of the liver [1,2]. Although the initial organ damage due to warm ischemia results from oxygen and nutrient deprivation, the more extensive injury occurs during the period of reperfusion, and is driven by pro-inflammatory cytokines and oxidative stress [3]. Eventually, it may lead to liver cell necrosis and severe tissues damaged. Therefore, IRI represents a significant therapeutic problem which urgently needs a solution.

Kruppel-like factor 2 (KLF2) is a vasoprotective transcription factor, which is induced by a physiological blood laminar flow and primarily expressed by the endothelial cells, and playing an important role in maintaining the endothelial cell function [4—6]. More importantly, it induces protective factors like expression of vasodilator, anti-thrombotic and anti-oxidant genes. It inhibits expression of inflammatory genes [e.g. endothelial nitric oxide synthase (eNOS), thrombomodulin (TM) and heme oxygenase-1 (HO-1)] and suppresses the expression of adhesion molecules (vascular cell adhesion molecule 1 (VCAM-1) and E-selectin) [7,8]. In addition, recent studies by our group showed that KLF2 attenuated IRI in livers [9] and kidneys [10] from donors after circulatory death when it was induced by biomechanical stimuli like shear stress during machine perfusion and by drug induction (statins).

Clinically, statins, which are HMG-CoA reductase inhibitors, possess anti-thrombotic and anti-inflammatory effects. In addition, statins have shown other vasoprotective properties via inducing KLF2 expression [11,12]. The effects of statins obviously were similar to those seen from of KLF2. Recently, Tuuminen et al. reported that statins could ameliorate kidney IRI in rats by means of preventing renal microvascular dysfunction [13]. Another group have demonstrated that simvastatin could significantly protect the liver against IRI through upregulating the KLF2 expression [14—17]. However, the mechanisms of statins-mediated protection effects on hepatic IRI have not been fully defined.

The current study was undertaken to investigate the effects of a prophylactic simvastatin pretreatment on total hepatic IRI and to examine the underlying mechanisms. We hypothesized that simvastatin could ameliorate total hepatic IRI by reducing oxidative stress and apoptosis and inhibiting the levels of pro-inflammatory cytokines, which may be associated with the upregulation of the KLF2 pathway.

Materials and methods

Animals and treatment

Male inbred Sprague Dawley rats from the Animal Experiment Center of Wuhan University weighting 250—300 g were used. All rats were maintained on standard animal care conditions and had free access to food and water. All experimental designs were approved by the Experimental Animal Regulations of the People's Republic of China and the Guide for the Care and Use of Laboratory Animals of the USA. To investigate whether simvastatin could ameliorate total liver IRI, rats were pretreated with either simvastatin (1 mg/kg, MedChem Express, NJ 08852, USA [IP]) [17] or vehicle (dimethyl sulfoxide, 0.1% DMSO, Barcelona, Spain [IP]) 0.5 h prior to warm ischemia. Rats were sacrificed after reperfusion at 6 and 24 h time point. Liver injury was analyzed.

Model establishment and experimental design

A rat model of total hepatic warm ischemia was induced as described previously [18]. Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg, IP). After an abdominal midline laparotomy, the hepatic hilum was clamped for 30 min with a microvascular clip for the IRI groups. Then, the microvascular clip was loosened, and the liver was reperfused for 6 h or 24 h. The total hepatic ischemia was observed by color changes in the total liver and severe mesenteric congestion. No rats died within 24 h. After reperfusion, the abdominal incision was closed with surgical sutures and the rats were kept in a warm environment. A total of thirty-six rats were randomly divided into three groups as follows:

- group 1, sham group: rats received by only laparotomy and mobilization of the hepatic hilum without IRI;
- group 2, IRI-Control group: rats were intra-peritoneally injected with vehicle (dimethyl sulfoxide, 0.1% DMSO, Sigma-Aldrich) 0.5 h before laparotomy with total hepatic IRI;
- group 3, IRI-Simvastatin group: rats were intra-peritoneally injected with simvastatin (1 mg/kg, MedChem Express, NJ 08852, USA) 0.5 h before laparotomy with total hepatic IRI.

All groups of rats were humanely sacrificed at 6 and 24 h after total hepatic reperfusion ($n = 6$ each). All blood samples and liver tissue samples were collected and stored for further experiments.

Analysis of liver enzymes. Measurements of transaminases of alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

All blood samples were centrifuged at 3,000 × g for 15 min. Hepatocyte injury was determined by the levels of the transaminases alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum (the clinic laboratory of the Zhongnan Hospital of Wuhan University) following the manufacturer's protocols. The levels of serum tumor necrosis factor α (TNF-α), interleukin-6 (IL-6) and 8-hydroxy-desoxyguanosine (8-OHdG) were measured with ELISA (rat enzyme-linked immunosorbent assay) kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) following the manufacturer's protocols.

Oxidative stress analysis

For the evaluation of oxidative stress, frozen liver tissue samples were determined by using a rat colorimetric assay kit specific for malondialdehyde (MDA) and superoxide dismutase (SOD) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following manufacturer's instructions. The results were given as nmol/mg protein.

Western blotting

Western blotting was performed as previously described [10]. We used the following primary antibodies: rabbit anti-KLF2 (1:400, Biosynthesis Biotechnology, Beijing, China), rabbit anti-phosphorylated eNOS (p-eNOS) at Ser1177 (1:1000, Cell Signaling, Danvers, MA), rabbit anti-total eNOS (1:1000, Cell Signaling, Danvers, MA) and rabbit anti-β-actin (1:1000, Proteintech, Manchester, UK)

The bands were developed by chemiluminescent ECL reagent and protein expression was analyzed by densitometry analysis using the Image J Station (NIH, Bethesda, USA). All Bands of each protein was normalized to the ratio of β-actin.

Quantitative real-time PCR analysis

Total RNA was extracted from frozen rat liver tissues using the Trizol reagent (Invitrogen Inc, Grand Island, NY, USA), and subsequent RNA was reversely transcribed to complementary (cDNA) (Thermo Scientific Revert Aid, USA) according to the manufacturer's instructions. SYBR green (Shanghai yeasen biotechnology co. LTD, Shanghai, China) quantitative RT-PCR was used to assay the expression of target genes. The results were normalized to β-actin mRNA. All primers are as previously described [9].

Histopathology and TUNEL staining

Fresh liver tissues were fixed in 4% buffered paraformaldehyde and subsequently embedded in paraffin. The liver sections (5 μm) were stained with hematoxylin-eosin (H&E) and assessed for IRI by Suzuki's criteria, the Score was graded from 0 to 4 dependent on the degree of neutrophil infiltration, congestion and necrosis [19].

Apoptosis was detected by using the TUNEL staining (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. The number of total hepatocytes and TUNEL-positive cells were calculated by 3 randomly chosen views (× 100 magnification) for each liver sections. The percentage of apoptosis (number of TUNEL-positive cells/total number of hepatocytes × 100 %) in each views was counted by Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Statistical methods

All statistical analyses were performed using SPSS 16.0 statistical software for Windows (SPSS Inc, Chicago, IL, USA). All values are presented as the mean ± standard deviation. The analysis of variance (ANOVA) was evaluated to compare for significant differences between groups. P-values < 0.05 were considered significant.

Results

Simvastatin pretreatment ameliorates total hepatic IRI in rats

To evaluate the effects of simvastatin pretreatment as compared to total hepatic IRI in rats, hepatic function was analyzed by measuring the levels of alanine ALT and AST.

As shown in Fig. 1A and Fig. 1B, after total hepatic warm ischemia for 30 min, the serum levels of ALT and AST were remarkably increased at 6 and 24 h compared with those in the sham group, with the highest values at 6 h after total hepatic IRI. However, treatment with simvastatin prior to total hepatic IRI significantly decreased the serum levels of ALT and AST by 52% and 55%, respectively, at 6 h compared with the IRI-Control group ($P < 0.05$). Fig. 1C and Fig. 1D show the same trends in the histological alterations in the liver tissues with H&E staining. Total hepatic warm ischemia caused a significant increase of necrotic areas, congestion and neutrophil infiltration compared to sham group at 6 and 24 h after reperfusion, while simvastatin pretreatment were dramatically reduced compared to the IRI-Control group by Suzuki's Score of IRI ($P < 0.05$). The results indicated that simvastatin pretreatment significantly ameliorated total hepatic IRI in rats.

Simvastatin pretreatment maintains the expression of KLF2 and its protective target genes (eNOS and TM)

In kidney, statins exert protective effects against IRI by activating KLF2 and eNOS [13]. In the present experiments, with total hepatic warm ischemia for 30 min, the rat livers exhibited a reduction in KLF2 protein and mRNA expression after both time points (6 and 24 h) after reperfusion compared with the sham group (Fig. 2A, Fig. 2B and Fig. 2D). The IRI-Control group showed the same trend in the expression of p-eNOS (protein and mRNA levels, Fig. 2C and Fig. 2E) and TM (Fig. 2F). However, simvastatin pretreatment significantly

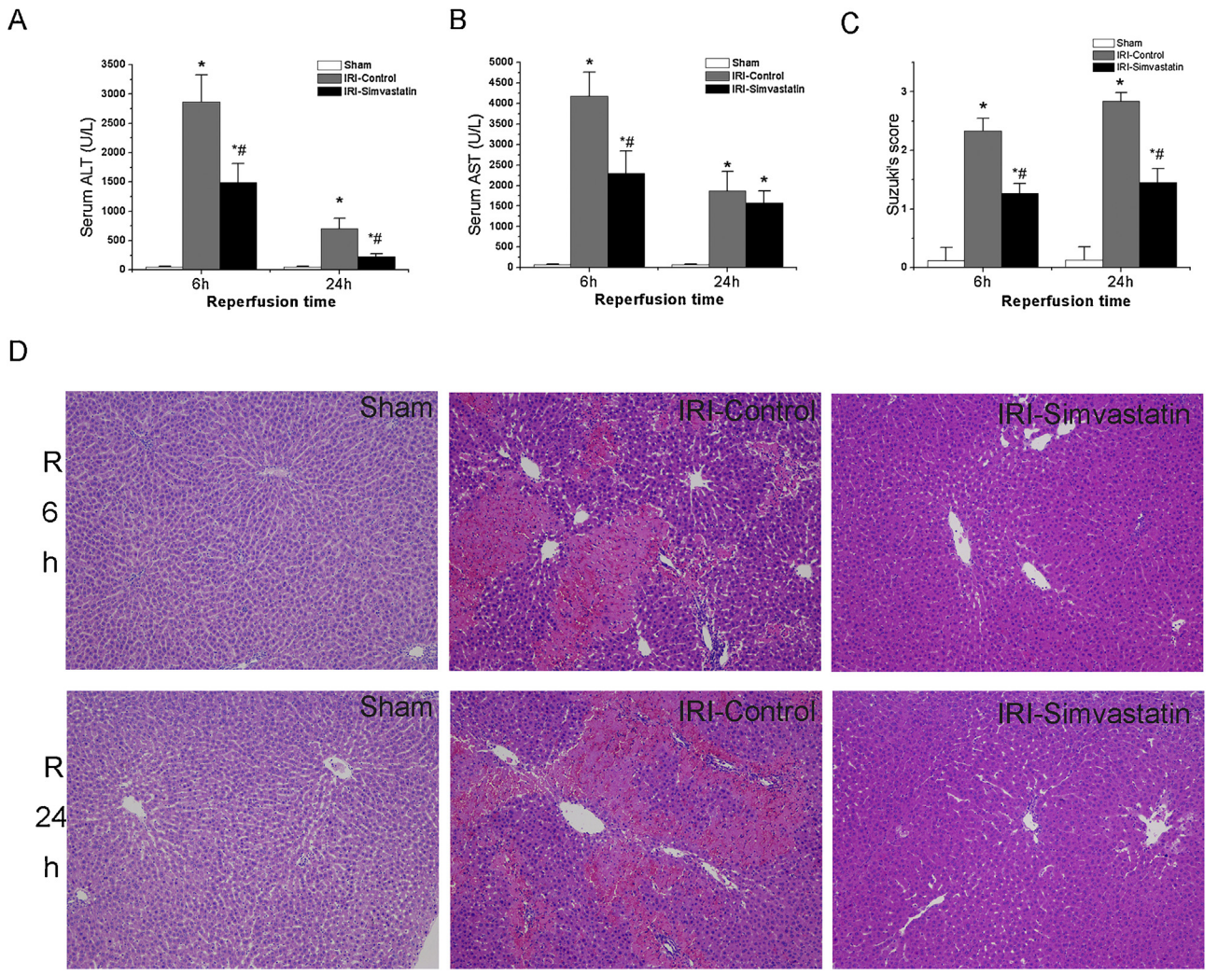

Figure 1 Simvastatin pretreatment ameliorates total hepatic IRI. Hepatic injury evaluated as release of transaminases (ALT and AST) in liver serum at 6 and 24 h reperfusion. (A) Serum ALT and (B) AST levels were expressed as the mean ± SD ($n = 6$; $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. IRI-Control group). (C) The liver tissue sections stained with HE were assessed for IRI with Suzuki's histological Score. (D) Representative HE stained of liver tissue sections from total hepatic IRI in rats. Original magnification, × 100. R 6 h, reperfusion 6 h; R 24 h, reperfusion 24 h. ($n = 6$; $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. IRI-Control group).

and effectively increased expression of KLF2, eNOS and TM (Fig.2A-2 F, $P < 0.05$). These results provided strong evidence that simvastatin pretreatment significantly and effectively maintained the expression of KLF2, eNOS and TM in total hepatic IRI in rats.

Simvastatin pretreatment reduces oxidative stress and attenuates hepatocyte apoptosis in total hepatic IRI

As shown in Fig.3A, hepatic tissue SOD levels in the IRI-Control group were significantly lower than in the sham group after reperfusion, while simvastatin pretreatment increased SOD activity compared with the IRI-Control group ($P < 0.05$). Consistently, simvastatin pretreatment significantly reduced hepatic tissue MDA levels after 6 and 24 h of reperfusion in comparison with the IRI-Control group (Fig.3B, $P < 0.05$). To investigate further, TUNEL staining was used to determine the percentage of hepatocyte apoptosis in the three groups (Fig. 3D). Furthermore, as shown in Fig.3D, the results of TUNEL staining demonstrated that a vast amount of apoptotic cells were seen in the IRI-Control, while less apoptotic cells were seen in the IRI-Simvastatin group. These differences were significantly different (Fig. 3C, $P < 0.05$). The results demonstrated that simvastatin pretreatment significantly reduced oxidative stress and attenuated hepatocyte apoptosis in total hepatic IRI in rats.

Simvastatin pretreatment inhibits the levels of inflammatory cytokines in total hepatic IRI

HMGB1 and TLR4 are activated by oxidative stress and inflammatory cytokines, they are also key mediators of hepatic IRI [20]. As shown in Fig.4A-4C, the serum levels of TNF-α, IL-6 and 8-OHdG were increased in the IRI-Control

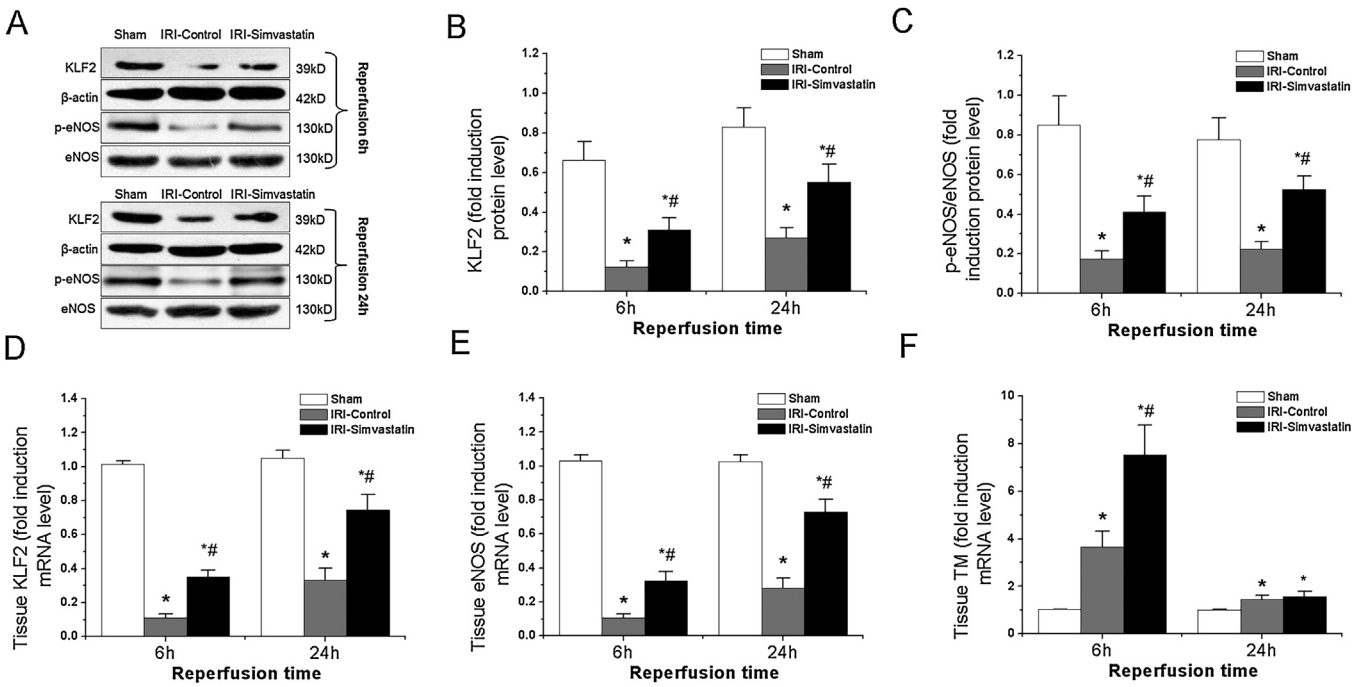

Figure 2 Simvastatin pretreatment maintains the expression of KLF2 and its protective target genes (eNOS and TM). (A) Western blot analysis of KLF2, phosphorylation eNOS and total eNOS in liver tissue. β-actin was normalized as the loading control. Densitometric analysis of the protein expression of KLF2 (B) and p-eNOS/eNOS (C) ($n = 6$; $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. IRI-Control group). The mRNA expression of KLF2 (D), eNOS (E) and TM (F) were assessed by RT-PCR. The experiments were repeated by three times and all results are shown as the mean ± SD ($n = 6$; $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. IRI-Control group).

group compared with those in the sham group at both measured time points, with a peak at 6 h after IRI. Simvastatin pretreatment significantly inhibited the levels of inflammatory cytokines ($P < 0.05$). In addition, quantitative real-time PCR was used to detect the mRNA expression of these inflammatory factors. As shown in Fig. 4D-4I, compared with the IRI-Control group, simvastatin pretreatment dramatically reduced the mRNA expression of the high mobility group box-1(HMGB-1), CD68, toll-like receptor 4 (TLR4), IL-6, IL-1β and intercellular adhesion molecule 1 (ICAM-1) at each time point of reperfusion. In conclusion, these results provided strong evidence demonstrated that simvastatin pretreatment could significantly inhibit the release of HMGB1, TLR4, CD68, TNF-α, IL-1β, IL-6, and ICAM-1 to suppress inflammatory response.

Discussion

The total hepatic IRI involves the fact that both liver and gut are subjected to warm ischemia in vivo, which is a complex but unavoidable process encountered during liver transplantation and conventional liver resection and a serious threat to the outcome of the procedure involved. Therefore, in this study, it was our aim to investigate whether simvastatin pretreatment would have a beneficial effect on total hepatic IRI and explored the underlying mechanisms. We could show that:

- simvastatin pretreatment ameliorates total hepatic IRI in rats;
- the protective effect of simvastatin pretreatment was mediated via upregulating the expression of KLF2 and its protective target genes;
- simvastatin pretreatment reduces oxidative stress and attenuates hepatocyte apoptosis in total hepatic IRI;
- simvastatin pretreatment inhibits the levels of inflammatory cytokines in total hepatic IRI.

In the present study, we found that simvastatin was able to ameliorate IRI in well-established model of total hepatic IRI, as evidenced by the reduction of serum levels of ALT and AST (Fig. 1A and Fig. 1B) as well as by attenuation of the pathological changes as described by Suzuki Score (Fig. 1C and Fig. 1D). Our results demonstrated that simvastatin pretreatment have a positive effect on total hepatic IRI in rats.

The serum levels of ALT and AST were used as biochemical injury markers of liver cell [21,22]. Our results showed that simvastatin pretreatment significantly decreased serum levels of ALT and AST compared with those in the IRI-Control group which is a potential benefit.

Our previous studies could show that hypothermic machine perfusion for organ preservation decreased rabbit warm ischemia injury in kidneys via upregulation of KLF2 and inhibition of the TGF-β signaling after transplantation [10]. In addition, the upregulation of KLF2 may have a protective effect on the functional recovery in livers and kidneys [13,17]. Interestingly, simvastatin could induce KLF2 expression. In our study showed that total hepatic warm ischemia injury resulted in down-regulating the expression of KLF2 and p-eNOS, while simvastatin pretreatment signifi-

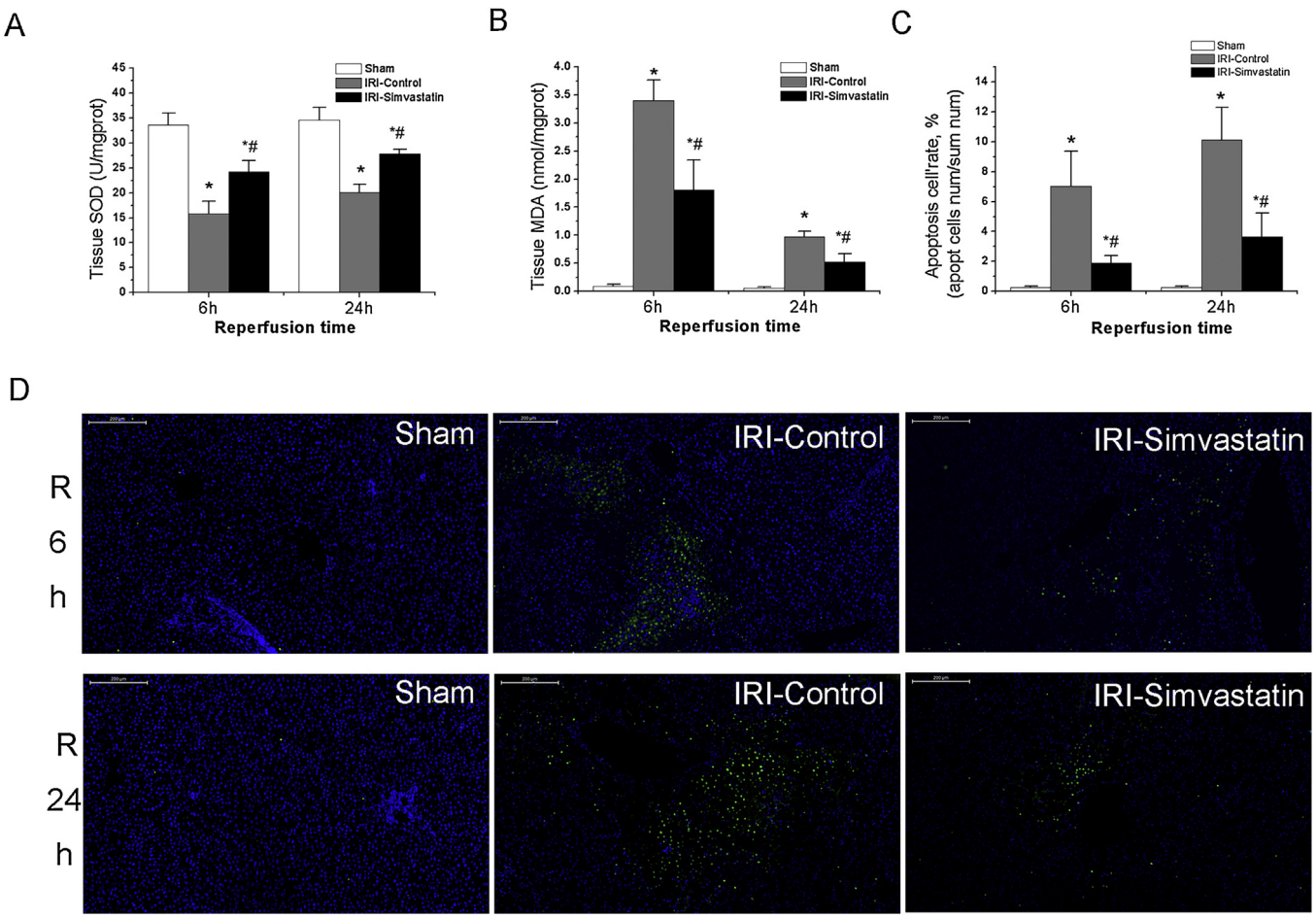

Figure 3 Simvastatin pretreatment reduces oxidative stress and attenuates hepatocyte apoptosis. (A) SOD level was measured in liver tissue. (B) MDA level was measured in liver tissue. All results are shown as the mean ± SD ($n = 6$; $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. IRI-Control group). (C) Quantitative analysis of liver apoptosis cells with TUNEL staining. (D) Representative images of TUNEL staining determined in liver tissue sections from total hepatic IRI in rats. Original magnification, × 100. R 6 h, reperfusion 6 h; R 24 h, reperfusion 24 h. ($n = 6$; $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. IRI-Control group).

cantly upregulated the protein of KLF2 and p-eNOS and the mRNA expression of KLF2, eNOS and TM (Fig. 2A—F). These observations demonstrated that the protective effects of simvastatin pretreatment on total hepatic IRI may be due to the upregulation of KLF2 expression.

Furthermore, we explored the association between simvastatin and oxidative stress. Hypoxia triggers the release of ROS which is a major injury marker of oxidative stress in hepatic IRI [20]. Therefore, it has widely been demonstrated that reduction of ROS could be important to ameliorate IRI. In the present study, we found that simvastatin pretreatment reduced oxidative stress in total hepatic IRI, which became manifest as increased total SOD activity and decreased lipid peroxidation, compared with IRI-Control group (Fig. 3A and Fig. 3B). These results were consistent with the degree of hepatocyte apoptosis. The TUNEL staining of liver cells showed that there were fewer liver cells undergoing apoptosis in the IRI-Simvastatin group (Fig. 3C and Fig. 3D).

The release of endogenous damage-associated molecular patterns (DAMPs) is a key contributor to the induced inflammatory response in IRI, which are produced from necrotic cells or damaged tissues [23,24]. The elevated endogenous DAMPs result in poor outcome during liver damage including the Toll-like receptors (TLRs) and HMGB-1 [25,26]. Similarly, HMGB-1 protein mediated organ damage in hepatic IRI through TLRs [23,27]. The release of HMGB1 also depends on the intracellular ROS levels [28,29]. These initiators trigger release of chemokines [30]. In our study, we found that simvastatin pretreatment down-upregulated the mRNA expression of HMGB1, CD68 and TLR4 compared with IRI-Control group (Fig. 4D—F). Oxidative stress damage of DNA was detected in the serum by 8-OHdG. We found that simvastatin pretreatment reduced oxidized nuclear DNA damage in total hepatic IRI (Fig. 4C). In accordance with HMGB1, the serum of TNF-α and IL-6 levels and the mRNA expression of IL-6, IL-1β and ICAM-1 levels were lower in the simvastatin pretreatment group compared to IRI-Control group. Indeed, pro-inflammatory cytokines such as TNF-α and IL-1β also have been demonstrated to inhibit KLF2 expression [31].These observations demonstrated that simvastatin pretreatment may have an anti-pro-inflammatory protective

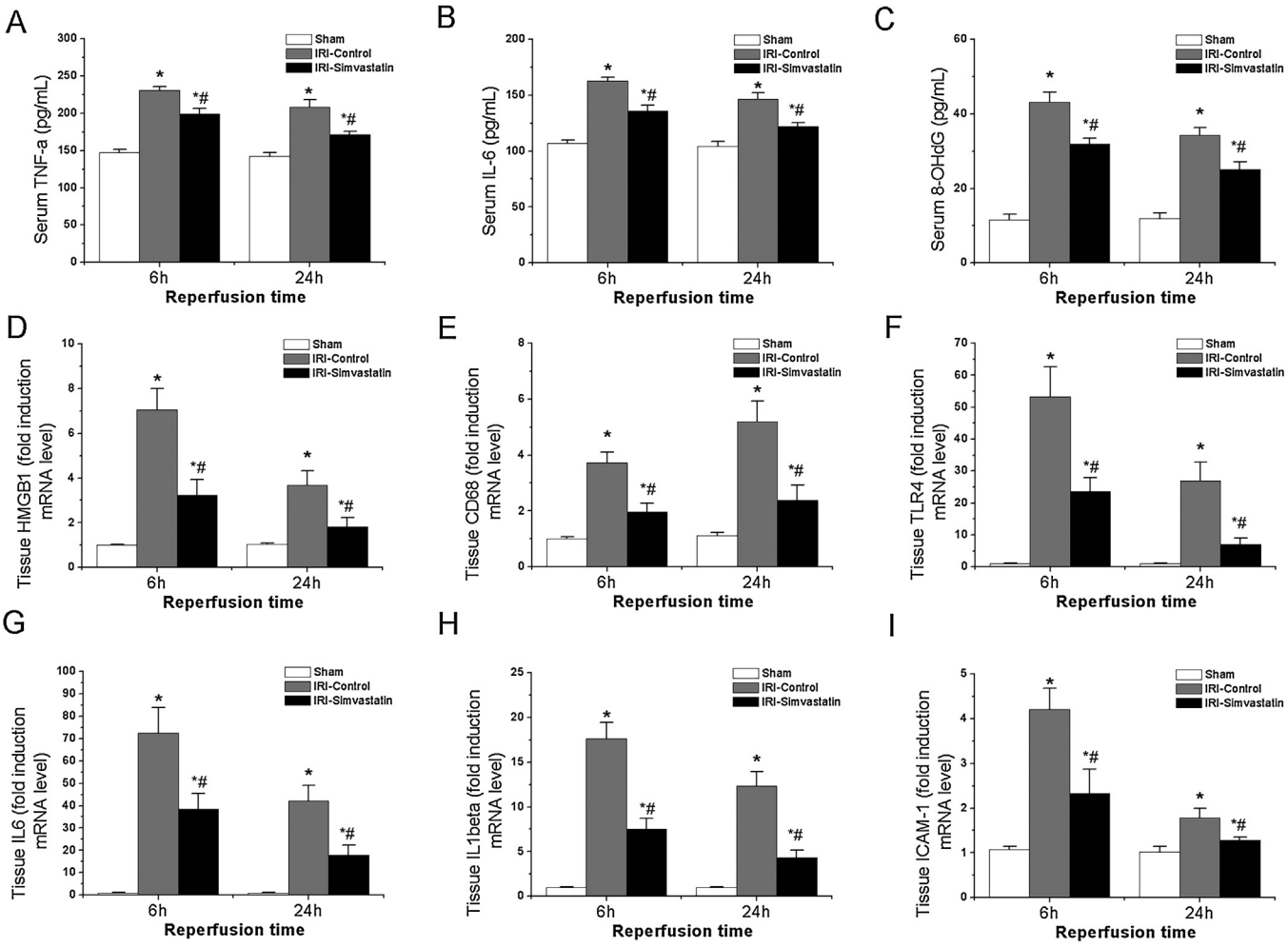

Figure 4 Simvastatin pretreatment inhibits the levels of inflammatory cytokines. (A) Serum TNF-α, (B) IL-6 and (C) 8-OHdG levels were expressed as the mean $\pm$ SD ($n = 6$; $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. IRI-Control group). The mRNA expression of HMGB1 (D), CD68 (E), TLR4 (F), IL-6 (G), IL-1β (H), and ICAM-1(I) were assessed by RT-PCR. The experiments were repeated by three times and all results are shown as the mean $\pm$ SD ($n = 6$; $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. IRI-Control group).

effects in total hepatic IRI which in turn may be dependent on the expression of KLF2.

There are certain limitations in our study: firstly, the role of KLF2 in the protection against total hepatic IRI should be further investigated using genetic tools where the expression of KLF2 would be either upregulated or knocked-down specifically. Next, we need further to explore the effects of simvastatin pretreatment on total hepatic IRI in vitro using a KLF2-inhibitor.

In conclusion, the present study demonstrates that simvastatin pretreatment ameliorates total hepatic IRI in rats. The mechanisms by which simvastatin reduced total hepatic IRI and protects the tissue from damage may be KLF2-mediated. As of now, it is not determined yet whether this protective effect is due to its vasodilatory, anti-thrombotic, anti-oxidative, anti-apoptotic or anti-pro-inflammatory properties. In addition, the true long-term survival of the animals has yet to be investigated. Overall, simvastatin may be used as a potential treatment strategy for clinical trials against hepatic IRI.

Disclosure of interest

The authors declare that they have no competing interest.

Authorship

L Z and L C: designed and performed the research, analyzed the data, and wrote the article. X Y and W Y: performed the research, contributed ideas, and helped write the article. LZZ and Z X: helped establish the model in vivo. Senninger Norbert: provided guidance and revised the article. Y Q and Y S: designed the experiments, provided overall guidance, and helped with the manuscript.

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