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Protective Effects the Akt Activator SC79 in Hepatic Ischemia-Reperfusion Injury

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Data Collection B
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Literature Search F
Funds Collection G

AE 1 **Hui Zhou**
E 2 **Ying Yu**
F 2 **Jinna Zhang**
B 3 **Yunfang Zhang**
CF 4 **Qi Luan**
G 1 **Gongming Wang**

1 Department of Anesthesiology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, P.R. China
2 Department of Surgery, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, P.R.
3 Department of Neurology, Qingdao West Coast New District People's Hospital, Qingdao, Shandong, P.R.
4 Department of Anesthesiology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, P.R.

Corresponding Author: Gongming Wang, e-mail: luolei1012@126.com
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Background: SC79 has been reported to protect against experimental ischemia-elicited neuronal death and brain injury and to protect myocardiocytes from hypoxia/reoxygenation (H/R) injury. Here, we investigated the effects of SC79 in primary hepatocytes *in vitro* and in rat liver *in vivo* following hypoxia-reoxygenation (H/R) and hepatic I/R injury.





Material/Methods: The livers of Sprague-Dawley rats were subjected to 45 min of ischemia followed by 2–24 h of reperfusion. The primary hepatocytes were subjected to hypoxia for 6 h and for 2–24 h. The hepatocytes cells or the hepatic I/R injury model livers were treated with SC79 or/and LY294002 at different times and concentrations. The serum ALT, AST, histologic examination, cellular viability, and cell apoptosis were assessed. The levels of phospho-Akt, Bad, Bim, Bax, Bcl-2, and Bcl-XL were determined by Western blot analysis.

Results: SC79 improved viability and inhibited apoptosis in hepatocytes following H/R. SC79 decreased serum AST and ALT, markedly improved pathology, and decreased cell apoptosis in livers following I/R. In addition, SC79 promoted the expression of phospho-Akt, Bcl-2, and Bcl-XL, and decreased the expression of Bid, Bax, and Bim. PI3K inhibitor (LY294002) pre-treatment completely abolished the above-mentioned effects of SC79.

Conclusions: The protective role of SC79 against H/R of hepatocytes or hepatic I/R injury is related to activation of phosphorylation of Akt, resulting in the decrease of pro-apoptotic protein of Bim, Bax, and Bad, and increase of the anti-apoptotic protein Bcl-2 and Bcl-xL induced by cell H/R and hepatic I/R injury.

MeSH Keywords: **Adjuvants, Anesthesia • Liver Failure, Acute • Oncogene Protein v-akt**

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Background

Ischemia/reperfusion injury (I/R-I) is an important cause of liver damage occurring during liver surgery, such as hepatic resection (HR) and liver transplantation (LT), or trauma and other surgical procedures when the liver is transiently deprived of oxygen and subsequently re-oxygenated [1]. The identification of effective pharmacological agents could expand the available options for surgeons and allow for the use of HR and LT. Although animal models are frequently used for the purpose of attenuating I/R-I in LT, many of the pharmacological agents used have not become part of clinical routine [2–4]. Furthermore, the possible adverse effects of some drugs frequently limit their use in human LT [5]. The development of protective strategies using pharmacological agents to reduce the negative effects of hepatic I/R-I is urgently needed.

Recent progress in molecular biology provides a new research direction for gene therapy of liver I/R-I [6–8]. However, the toxicity of gene vectors, the low efficiency of gene transfection, and the uncertainty of protein expression after transfection limit the development of gene therapy. Although non-viral vectors may have fewer toxic or immunological problems, the bottleneck of inefficient gene transfer has not been solved [9].

The PI3K/Akt pathway has important biological functions in cell proliferation, survival, and apoptosis. Activation of PI3K/Akt-dependent signaling has been demonstrated to result in attenuation of damage to the liver and other organs suffered from I/R injury [10,11]. Activation of PI3K/Akt signaling enhances anti-apoptotic Bcl-2 and Bcl-xL protein expression, inhibits the pro-apoptotic proteins such as Bax and Bad, and protects cells against apoptosis [12]. Despite the great demand for Akt activators for various therapeutic applications, efforts to identify the true activators of Akt were ultimately unsuccessful.

SC79, a small-molecule Akt activator, specially suppresses Akt membrane translocation while activating Akt in the cytosol [13]. Cytoprotective effects have been shown in experimental ischemia-elicited neuronal death [13], early brain injury [14], retinal pigment epithelium cells from UV radiation [15], and myocytes from oxygen and glucose deprivation (OGD)/reoxygenation *in vitro* [16], but fails to reduce myocardial I/R injury [17], which was contrast to previous studies with genetic models of cardiac Akt overexpression [18,19]. It is unclear whether SC79 has a protective effect on hepatocytes or liver tissues after ischemia-reperfusion injury.

In the present study, we investigate the effect of SC79 on primary hepatocytes following hypoxia-reoxygenation (H/R) *in vitro* and hepatic I/R injury *in vivo*. We observed that Akt was activated early (at 2 h) following liver I/R or hepatocytes hypoxia-reoxygenation (H/R), reached the highest levels at 6 h,

and restored the original level at 24 h. SC79 significantly up-regulated anti-apoptotic protein expression and down-regulated pro-apoptotic protein expression. SC79 administration effectively and significantly reduced serum AST and ALT levels, improved pathological features, attenuated hepatic I/R injury, and protected hepatocytes from hypoxia-reoxygenation injury. SC79 is an effective drug for protection against hepatic ischemia-reperfusion injury and could be used in clinical trials.

Material and Methods

Ethics statement

The study was conducted in accordance with ethical standards and the Declaration of Helsinki and according to the national and international guidelines and was approved by Shandong Provincial Hospital Affiliated to Shandong University.

Isolation and culture of primary hepatocytes

Primary hepatocytes were isolated using a 2-step collagen-perfusion technique from female C57BL/6 mice and cultured as described previously [20]. The viability of hepatocytes was 90% as determined by trypan blue exclusion.

Hypoxia-reoxygenation experiments in primary hepatocytes

The establishment of the hypoxia-reoxygenation (H/R) model *in vitro* was performed as previous described [21]. Briefly, the primary hepatocytes were placed into serum-free DMEM medium and seeded at a density of 4×10^5 cells/well in 6-well plates, which equilibrated with 1% O₂, 5% CO₂, and 94% N₂. After hypoxia for 6 h, plates were returned to the normal incubator to start the reoxygenation for 2–24 h.

Hepatic I/R model *in vivo*

Rats were fasted for 12 h but allowed free access to water before the induction of anesthesia. A model of segmental (70%) hepatic ischemia (I/R-I) was performed according to the method previously described [22]. After 45-min hepatic ischemia and either 2, 6, 12, or 24 h of reperfusion, mice were euthanized after 2, 6, 12, or 24 h of reperfusion, and blood and liver samples were taken for analysis. After reperfusion for the indicated time, blood samples were collected and the liver tissue was harvested for further analysis. Euthanasia was performed by transecting the abdominal aorta and inferior vena cava, and blood samples and liver samples were collected as above for further analysis.

Experimental groups

SC79 or saline was applied via intraperitoneal (i.p.) injection at a concentration of 0.04 mg/g of body weight at 0.5 h prior to ischemia followed by reperfusion (n=16/per group). The rats were divided into the following groups and subgroups: (1) The hypoxia-reoxygenation (H/R) model *in vitro* groups: 1. untreated primary hepatocytes; 2. primary hepatocytes exposed to 4 µg/mL saline 0.5 h prior to hypoxia followed by reoxygenation for 2, 6, 12, and 24 h groups; 3. primary hepatocytes were exposed to 4 µg/mL SC79 0.5 h prior to hypoxia followed by reoxygenation for 2, 6, 12, and 24 h groups. (2) The *in vivo* I/R groups: 1. Saline + Sham group; 2. I/R group 2 h, 6 h, 12 h, 24 h; 3. I/R + SC79 group 2 h, 6 h, 12 h, 24 h; and 4. I/R + SC79+ LY294002 group 2 h, 6 h, 12 h, 24 h.

The PI3K inhibitor LY294002 (Sigma, Shanghai, China) (1 mg/25 g body weight) or saline was given to mice 15 min before SC79 administration and 20 µM to hepatocytes 4 h before SC79 administration. Hepatocytes or rats in the model and sham groups were injected with an equal volume of saline.

Liver function tests

Blood was obtained by cardiac puncture and was centrifuged at 4000rpm for 20 min. The serum was stored at -70°C until analysis. Serum alanine amino transferase (ALT) and aspartate aminotransferase (AST) levels were detected using a diagnosis kit by bioassay as an index of hepatocellular injury.

Histological examination and TUNEL staining

Formalin-fixed liver samples were embedded in paraffin. Five-micrometer sections were stained with H&E by standard methods. Histological examination of hepatic tissue damage was performed. Histological severity of I/R injury was graded using Suzuki's criteria as described elsewhere [23]. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed by using a fluorescence detection kit (Roche Diagnostics) following the manufacturer's instructions [24]. Briefly, the samples were fixed for 30 min at room temperature, rinsed with PBS, blocked for 10 min by 96% methanol mixed with 4% H₂O₂ at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 5 min at 4°C. The nuclei were stained with DAPI for 10 min. The numbers of TUNEL-positive hepatocyte and the total hepatocytes were photographed using a fluorescence microscope (IX71+DP72, Olympus) and cells apoptosis were determined with ImagePro Plus software.

Viability assay

To study the viability of primary hepatocytes after the different treatments, the MTT-Based In Vitro Toxicology Assay Kit (Life

Technologies Italia, Monza; Italy) was used. Cell viability was calculated by comparing results with control cells (100% viable).

Quantification of apoptosis

Hepatocytes cultured on coverslips were treated as described above. Then, the cells were fixed with 4% paraformaldehyde (PFA) for 10 min and incubated with 50 nM NH₄Cl to extinguish PFA activity. The cell apoptosis was assessed by staining nuclei with DAPI followed by analysis using fluorescence microscopy.

Western blotting

The total cell lysates were collected from supernatants by centrifugation at 2000 × g for 5 min at 4°C. Western blot analysis was performed as described previously [25]. The respective antigens were detected by antibodies against Bcl-2, Bcl-XL, Bax, Bad, Bim, cleaved Caspase-3, anti-phospho-Akt (Thr-308/Ser-473) (sc-16646), and anti-Akt (sc-8312). β-actin was used as loading control.

Statistical analysis

All data represent the mean ± the standard error. Statistical comparisons between groups were analyzed by *t* test. P values of <0.05 were considered statistically significant.

Results

Effects of SC79 on hypoxia/reoxygenation (H/R)-induced cell death and apoptosis in hepatocytes

The results show that SC79 treatment significantly increased cell viability (Figure 1A) and decreased cell apoptosis (Figure 1B) when compared with the untreated H/R groups. We next determined whether the PI3K-Akt signaling pathway was involved in the pro-survival function of SC79. The hepatocytes were co-treated with SC79 and LY294002 subjected to H/R. The results showed that the SC79-induced protection was fully abolished when SC79 in combination with LY294002 was applied. The viability of SC79+LY294002-treated cells subjected to H/R was significantly decreased (Figure 1A), and the cell apoptosis was significantly increased when compared with the groups treated with SC79 alone (Figure 1B). LY294002 alone did not affect H/R-induced cell injury and death in primary hepatocytes (Figure 1A, 1B).

SC79 protects hepatocytes after hypoxia/reoxygenation (H/R) via Akt activation

Primary human hepatocytes expressed less detectable phospho-Akt (p-Akt), but Akt was activated early at 2 h following hepatocytes hypoxia-reoxygenation (H/R) and reached the

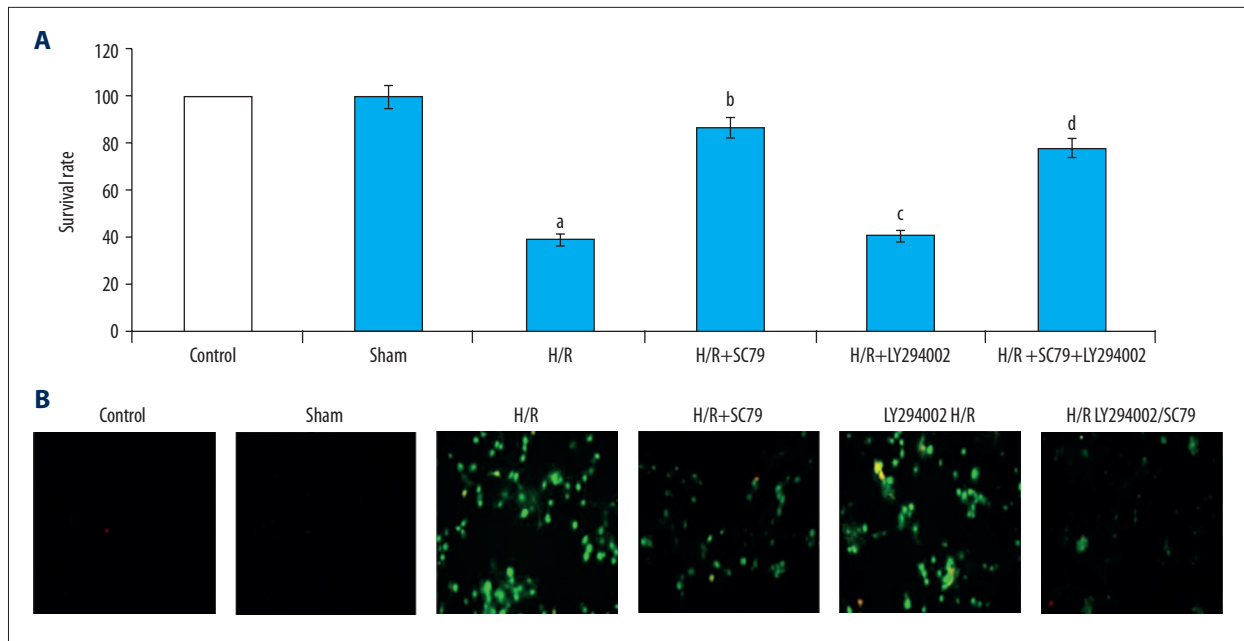


Figure 1. (A, B) SC79 protects hepatocytes from H/R injury. Hepatocytes were pretreated with 4 $\mu\text{g}/\text{mL}$ SC79 0.5 h prior to hypoxia followed by reoxygenation for 24 h or/and 20 μM to hepatocytes 4 h before SC79 administration. Cell viability was tested by MTT assay. Cell apoptosis was tested by TUNEL staining. Vs. control, ^a $p < 0.05$; vs. H/R, ^b $p < 0.05$, ^c $p > 0.05$; vs. R/H+ SC79+ LY294002, ^d $p < 0.05$.

highest levels at 6 h and was restored to the original level at 12 h (Figure 2). After the primary hepatocytes were exposed to 4 $\mu\text{g}/\text{mL}$ SC79 0.5 h prior to hypoxia followed by reoxygenation for 2, 6, 12, or 24 h, the p-Akt expression was time-dependently increased and reached the highest levels 6 h after reoxygenation and stayed at that level s to 24 h (Figure 2). However, exposure of hepatocytes to the PI3K inhibitor LY294002 (20 μM) 4 h before SC79 administration completely abolished Akt activation at 2–24 h (Figure 2).

Activation of PI3K/Akt signaling protects cells from apoptosis induced by H/R via a mechanism in which pro-apoptotic molecules Bax/Bad or Bim are phosphorylated, leading to dissociation of Bax/Bad or Bim from the anti-apoptotic molecule Bcl-2/Bcl-xL [19]. In the present study, we found that the induction of p-Akt by SC79 treatment was accompanied by activation of bcl-2 and Bcl-xL and inactivation of cleaved Caspase-3, Bim, Bax, and Bad, as evidenced by Western blot assay in H/R-treated primary hepatocytes (Figure 2). However, the activation of bcl-2 and Bcl-xL, and inactivation of cleaved caspase-3, Bim, Bax, and Bad, was fully abolished when SC79 in combination with LY294002 was applied (Figure 2).

SC79 protects the liver against ischemia/reperfusion injury in the *in vivo* rat model

To determine the effects of SC79 on hepatic I/R injury, SC79 was administered via intraperitoneal (i.p.) injection at a

concentration of 0.04 mg/g of body weight at 0.5 h prior to ischemia followed by reperfusion. Mice were killed at 2, 6, 12, or 24 h after reperfusion. The results showed that hepatic I/R exhibited significantly higher tissue injury as indicated by elevation of serum ALT and AST levels (Figure 3A, 3B) and liver histological analysis with Suzuki scores when compared with sham-treated livers (Figure 3C). The histology of liver section from the sham group appeared normal. The hepatic I/R injury groups showed marked histological evidence of tissue necrosis after reperfusion. Severe sinusoidal congestion, neutrophil infiltration, and hepatocellular necrosis were readily seen. In contrast, histological evidence of tissue damage following SC79 administration was greatly reduced and cell necrosis was not easily detected after reperfusion. However, histological changes were found in the SC79/LY294002-treated groups. The number of TUNEL-positive cells at 24 h after reperfusion was significantly increased compared with the sham-treated livers (Figure 3D).

We next sought to confirm the protective effects of SC79 on hepatic I/R injury. Treatment with SC79 followed by reperfusion for 2, 6 12, or 24 h significantly reduced ALT and AST levels compared with the untreated I/R livers (Figure 3A, 3B). Histological evidence of tissue damage after SC79 treatment was greatly reduced and the number of TUNEL-positive cells significantly decreased at 24 h after reperfusion (Figure 3D). Although LY294002 alone did not decrease liver I/R injury, it completely abrogated the liver-protective effect of SC79, as

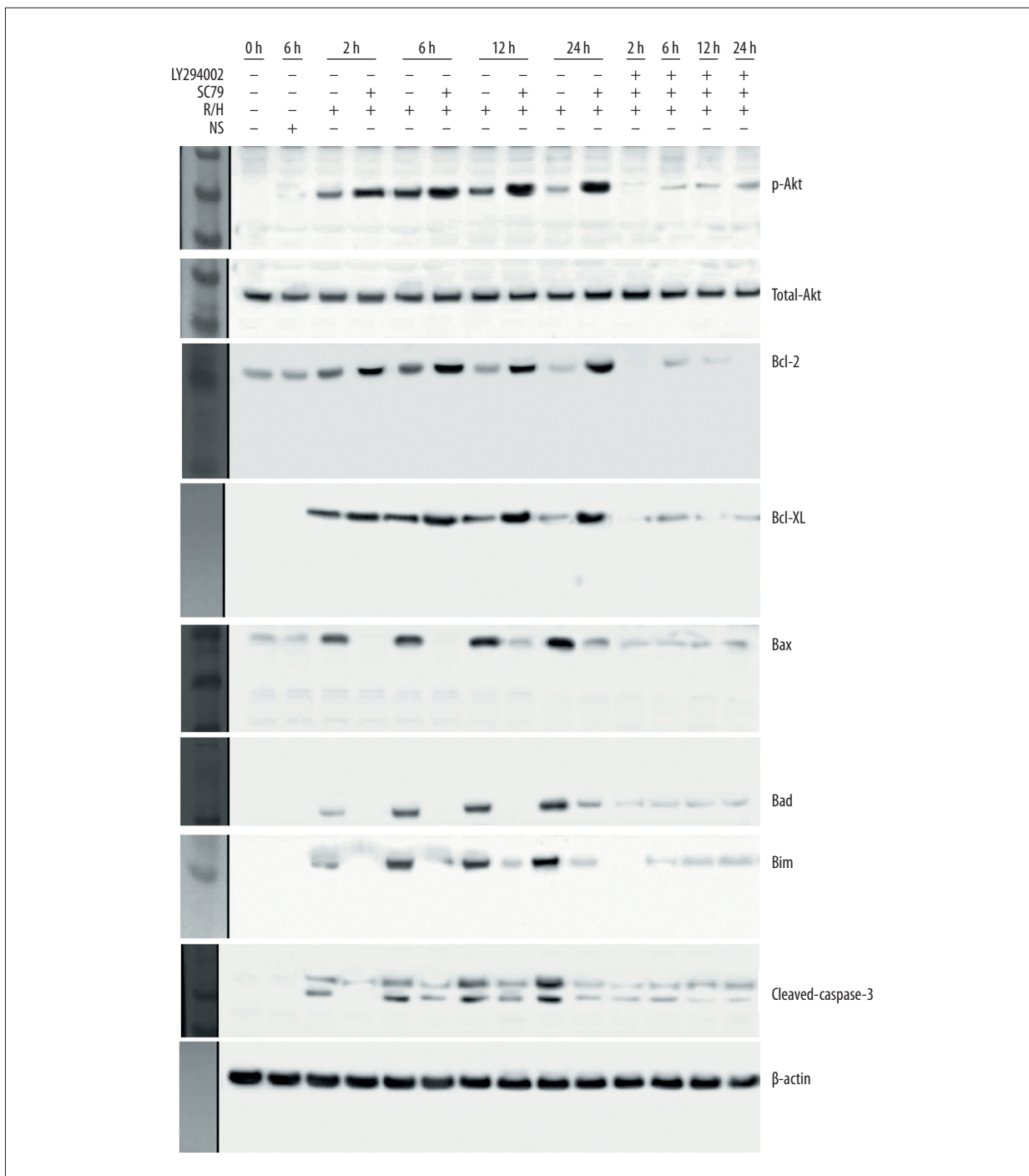


Figure 2. SC79 activated Akt in hepatocytes from H/R injury. Hepatocytes were pretreated with 4 µg/mL SC79 0.5 h prior to hypoxia followed by reoxygenation for 24 h or/and 20 µM to hepatocytes 4 h before SC79 administration. p-Akt, total-Akt, Bcl-2, Bcl-xL, Bax, Bad, Bim, and cleaved caspase-3 were assessed. β-actin was used as the control.

shown by serum ALT and AST measurements (Figure 3A, 3B), liver histological analysis (Figure 3C), and increased numbers of TUNEL-positive cells (Figure 3D).

SC79 protects liver against ischemia/reperfusion injury via Akt activation

To further reveal the mechanism of SC79 treatment in protecting against hepatic ischemia-reperfusion injury, we examined

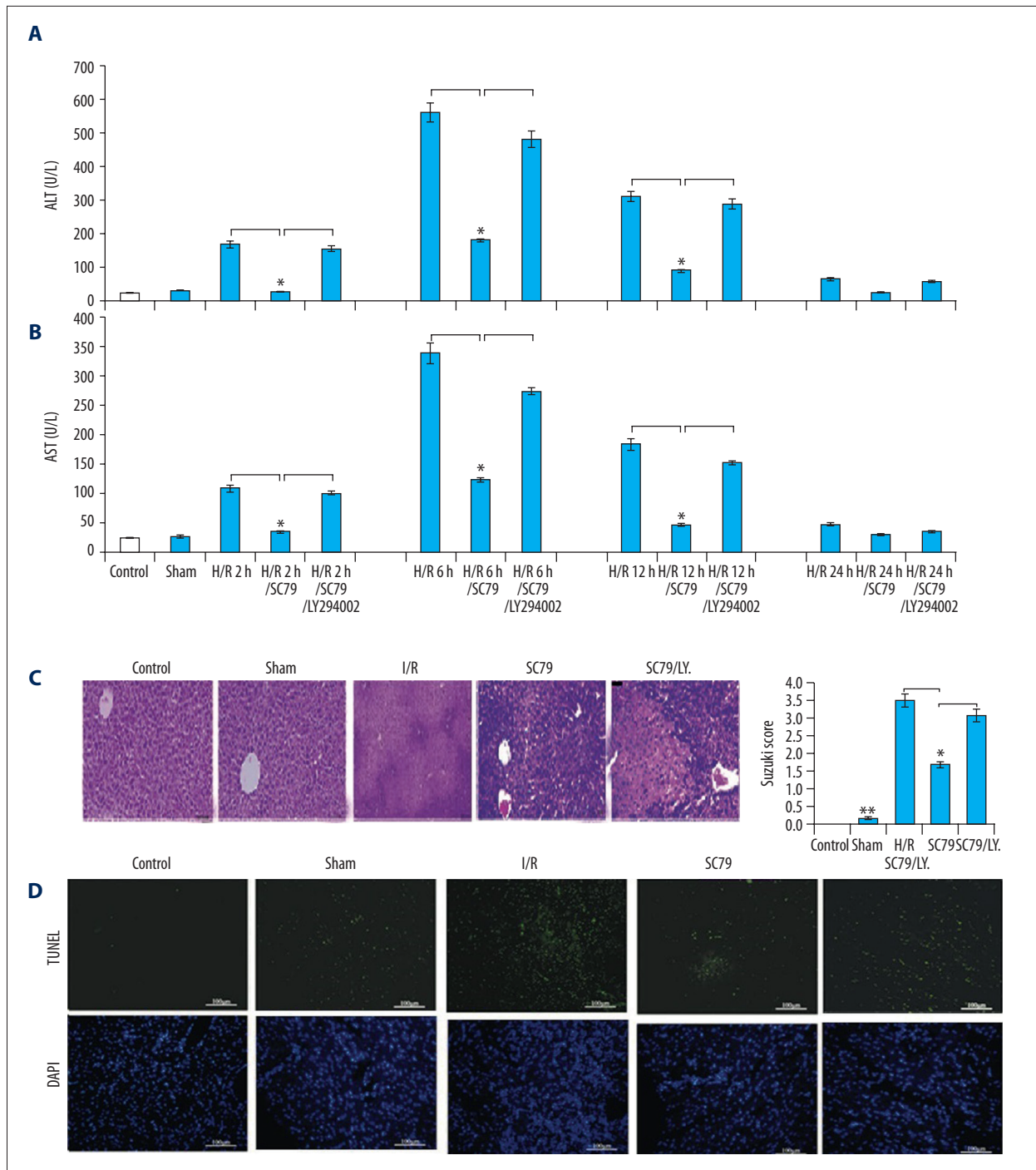


Figure 3. The effect of SC79 on ischemia/reperfusion injury in the *in vivo* rat model. After administration of SC79 (0.04 mg/g of body weight) 0.5 h prior to ischemia or LY294002 (1 mg/25 kg body weight) to mice 15 min before SC79 administration, followed by reperfusion for 2, 6, 12, and 24 h, we measured ALT alanine aminotransferase (**A**) and AST aspartate aminotransferase (**B**) (mean \pm S.D., n=16). * $p < 0.01$. Representative histological staining and Suzuki's histological score (**C**). Cell apoptosis was determined by TUNEL staining; green color is apoptotic cell stained by TUNEL and blue color is the cell nucleus stained by DAPI. ** $p < 0.01$.

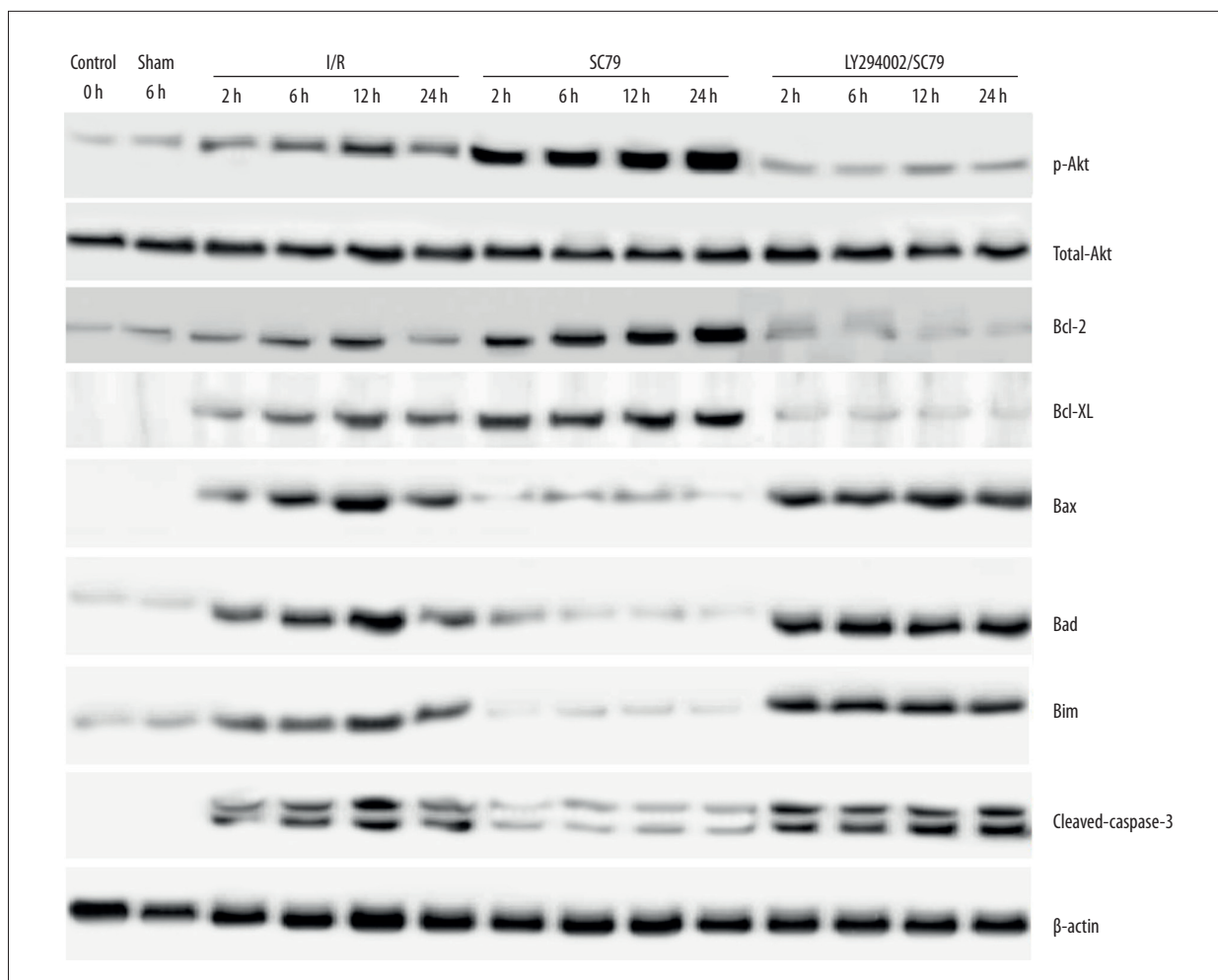


Figure 4. SC79 activated Akt in liver from H/R injury. After administering SC79 (0.04 mg/g of body weight) 0.5 h prior to ischemia or administration of LY294002 (1 mg/25 kg body weight) to mice 15 min before SC79 administration following by reperfusion for 2, 6, 12, and 24 h. We assessed p-Akt, total-Akt, bcl-2, Bcl-xL, Bax, Bad, Bim and cleaved caspase-3. β-actin was used as the control.

the phosphorylation of Akt in liver tissue. We found that within 2, 6, and 12 h of reperfusion, the level of Akt phosphorylation in tissues gradually increased, and reached the highest level at 12 h. After 12 h, the phosphorylation of Akt gradually decreased, and dropped to the lowest value at 24 h (Figure 4). However, after administration of SC79 before perfusion, the phosphorylation level of Akt gradually increased and reached the highest level at 24 h (Figure 4). After SC79 and LY294002 were co-administered, SC79-induced Akt phosphorylation was completely inhibited (Figure 4). These results indicate that preoperative SC79 treatment is an important activator of the PI3K/Akt pathway.

We next evaluated the effect of SC79 on the pro-apoptotic molecules Bax, Bad, and Bim, and the anti-apoptotic molecules Bcl-2 and Bcl-xL in hepatic tissue after ischemia-reperfusion. The results showed that hepatic tissue ischemia-reperfusion

increased the pro-apoptotic molecules Bax, Bad and Bim and decreased the anti-apoptotic molecules Bcl-2 and Bcl-xL expression, as well as increasing cleaved caspase-3 expression. SC79 administration effectively inhibited expression of Bax, Bad, Bim, and cleaved caspase-3 and increased bcl-2 and Bcl-xL expression (Figure 4). However, LY294002 completely abrogated the effect of SC79 administration on Bax, Bad, Bim, cleaved caspase-3, Bcl-2, and Bcl-xL expression (Figure 4).

Discussion

Increasing evidence shows that activation of PI3K/Akt signaling has important biological functions in protecting against H/R-induced cell injury [26]. Thus, activation of PI3K/Akt signaling becomes a primary goal of therapeutic intervention for liver protection against I/R injury. SC79 is a novel and safe

small-molecule compound, which can be used as a selective, highly-efficient, and cell-permeable Akt activator [13]. It protects against early brain injuries through the dual activities of antioxidation and antiapoptosis [26]. In addition, SC79 has proved to have no significant adverse effects in experimental studies in mice and human cells [13]. In our study, we found that SC79 protected the liver against I/R injury, as evidenced by decreased liver enzyme activities, improved hepatic morphology, and reduced hepatocyte apoptosis. The underlying mechanism of this hepatoprotective effect might involve attenuation of cell apoptosis, as shown by the *in vivo* animal I/R model and the *in vitro* cellular H/R model. More importantly, we found that Akt activation may be a mechanism involved in SC79 protection.

Although the pathophysiological roles of apoptosis and oncotic necrosis in hepatic I/R injury have not been fully elucidated, they are undoubtedly prominent features. Many investigations have shown that interventions targeting apoptosis are therapeutically effective in the inhibition of hepatic I/R injury. Activation of PI3K/Akt signaling has been reported to protect cells from apoptosis induced by I/R via mechanisms involving phosphorylation of the pro-apoptotic molecules Bad, Bax or Bim, leading to dissociation of Bad, Bax, or Bim from the anti-apoptotic molecules Bcl-2 and Bcl-XL [11]. We showed that SC79 activated Akt and protected primary hepatocytes from H/R injuries. SC79 largely attenuated H/R-induced hepatocyte apoptosis. Akt inhibition via the MEK-specific inhibitor LY294002 almost abolished SC79-induced cytoprotection against H/R. We also found a significant downregulation of the activation of caspase-3, Bad, Bax, and Bim, upregulation of bcl-2 and Bcl-xL, and fewer TUNEL-positive cells following SC79 treatment. LY294002 pre-treatment reversed this effect of SC79. These data further demonstrate that the inhibition of apoptosis by SC79 to activate Akt signals might be responsible for the protective effect of hepatocytes following H/R injury.

In the present study, we evaluated the potential role of SC79 in a model of 70% hepatic I/R. Our results showed that pre-treatment with 0.04 mg/g of body weight 0.5 h prior to ischemia following by reperfusion decreased ALT and AST levels in

the plasma and decreased the Suzuki's scores for the tissues. To evaluate the dynamics of apoptosis by I/R treatment, we examined apoptotic cells by TUNEL assay and analyzed the activation of caspase-3, a component of the enzymatic cascades that can cause apoptotic cell death. As a result, we found a significant downregulation of the activation of caspase-3, as well as a reduction in TUNEL-positive cells, in liver tissues following I/R. Interestingly, all these effects were reversed by pre-treatment with LY294002.

In our study, we found that hepatic ischemia-reperfusion injury in a mouse model was characterized by the upregulation of the pro-apoptotic proteins Bim, Bad, and Bax. In contrast, levels of the anti-apoptotic proteins bcl-2 and Bcl-xL were significantly decreased following hepatic ischemia-reperfusion injury. Administration of SC79 activated Akt and reduced the levels of the Bim, Bad, and Bax and increased the levels of bcl-2 and Bcl-xL. However, all the effect of SC79 on pro-apoptotic and anti-apoptotic protein expression was reversed by pre-treatment with LY294002. These data demonstrate that activation of Akt followed by upregulation of the anti-apoptotic protein and downregulation of the pro-apoptotic protein plays an important role in protecting the liver from I/R-induced damage.

Our study has certain weaknesses. 1. We did not explore the time- or drug-dose-dependence of cell or liver injury following I(H)/R injury. 2. It is unclear whether other signals, such as the MEK/ERK signal, are involved in the I(H)/R process and whether the drug used has an effect on MEK/ERK signaling following I(H)/R injury.

Conclusions

SC79 ameliorates pathological alterations and improves liver function following I/R injury. The mechanisms involved include suppression of cell apoptosis, which is associated with activation of PI3K/Akt signaling. Thus, SC79 may be an important therapeutic agent for treatment of hepatic ischemia-reperfusion injury.

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