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Original Article

Glycyrrhizin protects rat heart against ischemia-reperfusion injury through blockade of HMGB1-dependent phospho-JNK/Bax pathway

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Aim: Glycyrrhizin (GL) has been found to inhibit extracellular HMGB1 cytokine's activity, and protect spinal cord, liver and brain against I/R-induced injury in experimental animals. The purpose of this study was to investigate the protective effect of GL in rat myocardial I/R-induced injury and to elucidate the underlying mechanisms.
Methods: Male adult Sprague-Dawley rats underwent a 30-min left coronary artery occlusion followed by a 24-h reperfusion. The rats were treated with glycyrrhizin or glycyrrhizin plus recombinant HMGB1 after 30 min of ischemia and before reperfusion. Serum HMGB1, TNF-α and IL-6 levels, and hemodynamic parameters were measured at the onset and different time points of reperfusion. At the end of the experiment, the heart was excised, and the infarct size and histological changes were examined. The levels of Bcl2, Bax and cytochrome c, as well as phospho-ERK1/2, phospho-JNK and phospho-P38 in the heart tissue were evaluated using Western blot analysis, and myocardial caspase-3 activity was measured colorimetrically using BD pharmingen caspase 3 assay kit.
Results: Intravenous administration of GL (10 mg/kg) significantly reduced the infarct size, but did not change the hemodynamic parameters at different time points during reperfusion. GL significantly decreased the levels of serum HMGB1, TNF-α and IL-6. GL changed the distribution of Bax and cytochrome c expression between the mitochondrial and cytosolic fractions in the heart tissue, resulting in inhibition of myocardial apoptosis. Moreover, expression of phospho-JNK, but not ERK1/2 and P38 was decreased by GL in the heart tissue. All of the effects produced by GL treatment were reversed by co-administration with the recombinant HMGB1 (100 μg). Intravenous administration of SP600125, a selective phospho-JNK inhibitor (0.5 mg/kg), attenuated HMGB1-dependent Bax translocation and the subsequent apoptosis.
Conclusion: These results demonstrate that GL alleviates rat myocardial I/R-induced injury via directly inhibiting extracellular HMGB1 cytokine activity and blocking the phospho-JNK/Bax pathway.

Keywords: glycyrrhizin; heart; ischemia-reperfusion injury; apoptosis; high-mobility group box 1 (HMGB1); cytokine; phospho-JNK; SP600125; Bax

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Introduction

Although in preclinical models, therapeutic neovascularization for cardiovascular ischemia with a specific combination of angiogenic and arteriogenic factors to promote the formation of stable vascular networks is a promising avenue for the treatment of patients with ischemic heart disease[1–4], the potential risk of complications followed by the reperfusion injury remains a major threat. Numerous mechanisms of I/R injury have been revealed including the involvement of nitric oxide, generation of oxygen-derived free radicals, complement activation and infiltration of neutrophils into the ischemic area[5]. Most of these mechanisms will ultimately produce irreversible apoptotic injury to the myocardial tissues. Based on recent studies, it is now believed that high-mobility group box 1 (HMGB1) plays a major role in early I/R-induced apoptotic injury by binding to the receptor for advanced glycation end-products (RAGE)[6, 7], which results in the activation of pro-inflammatory pathways and enhanced myocardial injury. Therefore, the identification of HMGB1 inhibitors or antagonists is of great potential therapeutic interest for reducing myocardial I/R injury.

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Accordingly, we have focused our attention on studying glycyrrhizin (GL), a natural triterpene glycoconjugate that is derived from the root of licorice (*Glycyrrhiza glabra L*), as another HMGB1 inhibitor. In Japan, GL is administered at high dosages to treat hepatitis B and C[8, 9]. A recent study reported that GL could exert protective effects against transient spinal cord ischemic injury in rats by reducing the generation of inflammatory cytokines and inhibiting the release of HMGB1 *in vivo*[10]. GL directly binds to both HMG boxes in HMGB1 without significantly affecting their secondary structure and inhibits its chemo-attractant functions on fibroblasts and smooth muscle cells[11]. Therefore, because it inhibits the chemotactic and mitogenic functions of HMGB1, GL may possess great therapeutic potential to prevent I/R injury in various organs.

Three major mitogen-activated protein kinases (MAPKs) signaling pathways, including c-Jun NH_2-terminal protein kinases (JNKs)/stress-activated protein kinases (SAPKs), p38 MAPK and extracellular signal-related protein kinases (ERK1/2), are activated in response to myocardial I/R[12, 13]. The activation of p38 MAPK and JNK exerts deleterious effects compared to ERK1/2 activation, which exerts beneficial effects on post-ischemic myocardial apoptosis and cardiac function recovery[14, 15]. Treatment with HMGB1 box A, a functional antagonist of extracellular HMGB1 cytokine activity, significantly protects against myocardial I/R injury and suppresses the activation of the inflammatory cascade through blockade of JNK and ERK1/2 pathways without any effect on the p38 signaling pathway[16].

GL has attracted great attention for protecting several organs including the spinal cord, liver and brain from I/R-induced injury in experimental animals[10, 17, 18]. However, to date, there are no studies that have investigated the role of GL in preventing myocardial I/R injury and the potential protective molecular mechanism(s), specifically related to MAP kinase activation. Therefore, the aim of this study was to investigate the protective effects and the mechanism of action of GL during I/R injury in the rat heart by examining the following: (1) measurement of cardiac function, infarct size and area at risk, and histological examinations; (2) concentrations of the released HMGB1 and related cytokines in the rat serum; (3) the effect of GL on the alleviation of apoptosis caused by I/R injury; (4) the involvement of a certain MAPK pathway and its possible downstream molecules, which are modulated by GL.

Materials and methods

Animals and groups

All of the procedures and studies in this article conformed to the "Guide for the Care and Use of Laboratory Animals" published by the US NIH (National Institutes of Health Publication No 85–23, revised 1996) and were approved by the Committee for Animal Experiments at the Zhejiang University. Male Sprague-Dawley rats weighing 325±25 g were obtained from the Experimental Animal Center of Zhejiang University (China) and were allowed free access to laboratory chow and tap water in day-night quarters at 25 °C.

Rats were randomized into four experimental groups with eight animals in each group: (1) healthy control, sham-operated rats (sham group); (2) I/R rats pretreated with saline (NS group); (3) I/R rats pretreated with GL at a dose of 10 mg/kg (GL group); (4) I/R rats pretreated with GL and recombinant HMGB1 (100 μg per rat). In the pretreatment groups, NS, GL, or GL and rHMGB1 were administered intravenously in the tail vein in a volume of 0.5 mL after 30 min of ischemia and before reperfusion, followed by a 24-h reperfusion.

Experimental I/R injury model

The myocardial I/R model was established according to a method reported by Oozawa *et al*[19]. Each rat was anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Supplemental doses of anesthesia were given as required. The animal was placed in the supine position, and tracheal intubation was performed. During the surgery, body temperature was monitored using a rectal probe and was maintained at 35.5–37.5 °C with a heat lamp. A catheter for intravenous (iv) injection was inserted into the right internal inguinal vein. An interarterial catheter was inserted into the femoral artery for measuring arterial blood pressure and heart rate (HR). The chest cavity was entered through a left sternotomy. An 8–0 nylon suture was passed under the left anterior descending coronary artery, and a balloon occluder was applied on the artery. Myocardial I/R was induced by inflating and deflating the balloon occluder. The model of I/R injury was developed within 30 min of left coronary artery occlusion followed by a 24-h reperfusion.

Determination of JNK activity

The JNK activity was measured with immune complex protein kinase assays according to the manufacturer's protocol (Cell Signaling Technology) as reported by Shuzo *et al*[20]. Briefly, cells were lysed in ice-cold cell lysis buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L glycerol phosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride).

Equal volumes of cell lysates (300 μg) were incubated with c-Jun antibody beads at 4 °C overnight. After centrifugation, the pellets were suspended in kinase buffer (25 mmol/L Tris, pH 7.5, 5 mmol/L glycerol phosphate, 2 mmol/L dithiothreitol, 0.1 mmol/L Na_3VO_4, 10 mmol/L $MgCl_2$, and 200 μmol/L ATP) and subsequently immunoprecipitated with c-Jun, a specific fusion protein, at 30 °C for 30 min. Then, JNK activity was measured by Western blotting with a 1:1000 dilution of primary antibodies [rabbit polyclonal phospho-c-Jun (Ser 63)] as described in the Western blot analysis protocol.

JNK activity inhibition

To investigate the role of JNK pathway in myocardial I/R-induced apoptosis, a specific JNK inhibitor was injected. SP600125, the inhibitor, was purchased from Biomol Research Laboratories (Plymouth Meeting, PA) and was dissolved in dimethylsulfoxide and PBS. SP600125 (0.5 mg/kg in 25%

dimethylsulfoxide in PBS) was administered intravenously in the tail vein 30 min before the I/R process.

Serum detection

Blood samples (0.5 mL) were collected from the femoral vein by using the arterial catheter at the onset of reperfusion and at 0, 30 min, 1, 2, 6, 12, and 24 h after reperfusion. The serum was isolated after centrifugation at 5000×*g* for 20 min at 4 °C. After centrifugation, the serum was frozen at -80 °C until enzyme-linked immunosorbent assay (ELISA) analysis was performed. HMGB1 concentration and levels of the inflammatory mediators (TNF-α and IL-6) in the serum were quantified using specific ELISA kits for rats according to the manufacturer's instructions (Biosource International Inc, USA).

Measurement of cardiac function

An interarterial catheter was inserted into the femoral artery for measuring arterial blood pressure and heart rate (HR). The indices of cardiac function including HR and mean arterial blood press ure (MBP) were monitored using two models of blood pressure amplifiers, AP-601G and AP-641G, and analyzed using a cardiograph (Power lab, ADInstruments, Otago, New Zealand).

Determination of infarct size and area at risk

At the end of the experiment, the heart was excised, the blood was flushed out with normal saline, and the heart was perfused with a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) at 37 °C. The infarcted area remained unstained, whereas the non-infarcted area stained red. Furthermore, the coronary artery was retied at the site of previous occlusion, and the heart was perfused with a 2% solution of Evans blue dye to delineate the ischemic area (area at risk). The atrial and right ventricular tissues were then excised, and the heart was cut into five transverse slices, fixed in 10% neutral buffered formaldehyde, weighed and digitally photographed. The areas of infarction including ischemic and nonischemic myocardium were measured and calculated using NIH image analysis, and based on these measurements, infarct size was calculated as a percentage of the area at risk (AAR).

Estimation of plasma troponin-T (TpT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH)

Blood samples (1 mL) were collected from the femoral vein via the arterial catheter at the onset of reperfusion and at 0, 30 min, 1, 2, 6, 12, and 24 h after reperfusion. Serum was isolated after centrifugation at 5000×*g* for 20 min at 4 °C. TpT, AST and LDH levels were analyzed using standard methods established by the SRL Corp (Tokyo, Japan).

Subcellular fractionation of cytoplasmic and mitochondrial fractions

For subcellular fractionation, lysates were produced using a glass tissue grinder (Wheaton, Millville, NJ). The lysates were centrifuged at 750×*g* for 10 min at 4 °C and subsequently at 8000×*g* for 20 min at 4 °C. The 8000×*g* pellets were used to obtain the mitochondrial fraction. The supernatant was centrifuged further at 100 000×*g* for 60 min at 4 °C and was used to analyze the cytosolic fraction.

Western blot

Total protein extracts or isolated subcellular fractions from the rat heart tissue were prepared as previously described[20]. The antibodies and dilutions were as follows: phosphorylated SAP/Jun NH_2-terminal kinase (JNK) (No 9255, 1:2000), SAP/JNK (No 9252, 1:1000), phosphorylated extracellular signal-regulated kinase (ERK) [1/2] (No 9101, 1:1000), ERK [1/2] (No 4695, 1:1000), phosphorylated p38 (No 9211, 1:1000), p38 (No 9212, 1:1000) (Cell Signaling Technology, Danvers, MA), Bcl-2 (1 μg/mL, No ab7973, Abcam Plc, Cambridge, UK), Bax (1 μg/mL, No ab7977, Abcam Plc, Cambridge, UK), cytochrome *c* (1 μg/mL, No ab90529, Abcam Plc, Cambridge, UK) and HMGB1 (1 μg/mL, No ab18256, Abcam Plc, Cambridge, UK). Horseradish peroxidase-coupled rabbit or mouse IgG (1:2000) were used as secondary antibodies. Proteins were separated on 10%–20% SDS polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes, which were subsequently incubated with the primary antibody diluted in blocking solution overnight at 4 °C. After the membranes were washed, they were treated with horseradish peroxidase-conjugated secondary antibodies and subsequently with enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). The film was scanned with a GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA), and the results were quantified with Multi-Analyst software (Bio-Rad Laboratories).

Histological examinations

Animals were sacrificed after 24 h of reperfusion. The hearts were dissected and cut transversely into five slices, fixed in 4% paraformaldehyde, and embedded in paraffin. Subsequently, slices with 6-μm thickness were cut during the routine follow-up procedure. The sections were stained with hematoxylin and eosin for examination with light microscope. An observer who was blinded to the experimental conditions of the animals recorded the data.

Briefly, the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed with a commercially available kit, according to the manufacturer's instructions (Roche, Switzerland). Apoptotic cells in the rat heart after I/R in each group were counted separately using Image J software.

Statistical analysis

All data were expressed as the mean±SEM (standard error of mean). SPSS 17.0 was used for statistical analysis of the data. The concentrations of serum HMGB1 and inflammatory mediators were analyzed using two-way repeated-measures (time and group) analysis of variance followed by the *post hoc* Student-Newman-Keuls test. The $P<0.05$ level of probability

was used as the criteria to determine significance.

Results

Hemodynamic values at different time points during I/R in the rat heart

There were no significant differences in the baseline hemodynamic values (HP, MBP, and rate-pressure product) between each of the groups at different time points during I/R in the rat heart (data not shown).

Effects of GL on the histologic examination, infarct size, AAR, TpT, AST, and LDH levels in rat hearts subjected to I/R

The representative micrographs of rat myocardial HE staining 24 h after reperfusion are shown in Figure 1A. The muscle fibers were disrupted, and an enlargement of the extracellular space was observed after I/R (Figure 1Ac–1Ad); however, no such damage was observed in the rat hearts that were obtained from the sham group (Figure 1Aa). Cardiac sections obtained from rats undergoing GL treatment demonstrated a relatively more intact and less disrupted myocardial fibers with transverse striations compared to those from the NS group. Furthermore, co-treatment with GL plus recombinant HMGB1 (rHMGB1) 30 min before ischemia caused significant worsening with morphological changes similar to that observed in the NS group (Figure 1Ad).

Determination of the infarct size and AAR are direct indicators of the degree of heart damage. Although there were no significant differences in the myocardial regions of area at risk (AAR) in rats that belonged to the NS, GL, or GL+rHMGB1 groups, there was a significant decrease in the size of the infarcted region in the GL group compared to the NS group (Figure 1B). Moreover, co-treatment with GL and rHMGB1 resulted in enlargement of the infarcted regions in hearts obtained from rats from the GL group (Figure 1B). Plasma troponin-T (TpT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) levels were additionally measured to evaluate the extent of cardiac injury. The plasma TpT, AST, and LDH levels were almost undetectable in the sham group. After reperfusion, the levels of TpT, AST, and LDH were markedly elevated in the NS, GL, and GL+rHMGB1 groups (Figure 1C). Compared to the NS group, treatment with glycyrrhizin in the GL group caused a significant reduction of TpT, AST, and LDH levels, which was reversed by rHMGB1 co-treatment (Figure 1C). These results demonstrated that glycyrrhizin exerts a protective effect against myocardial injury, which is caused by HMGB1 release.

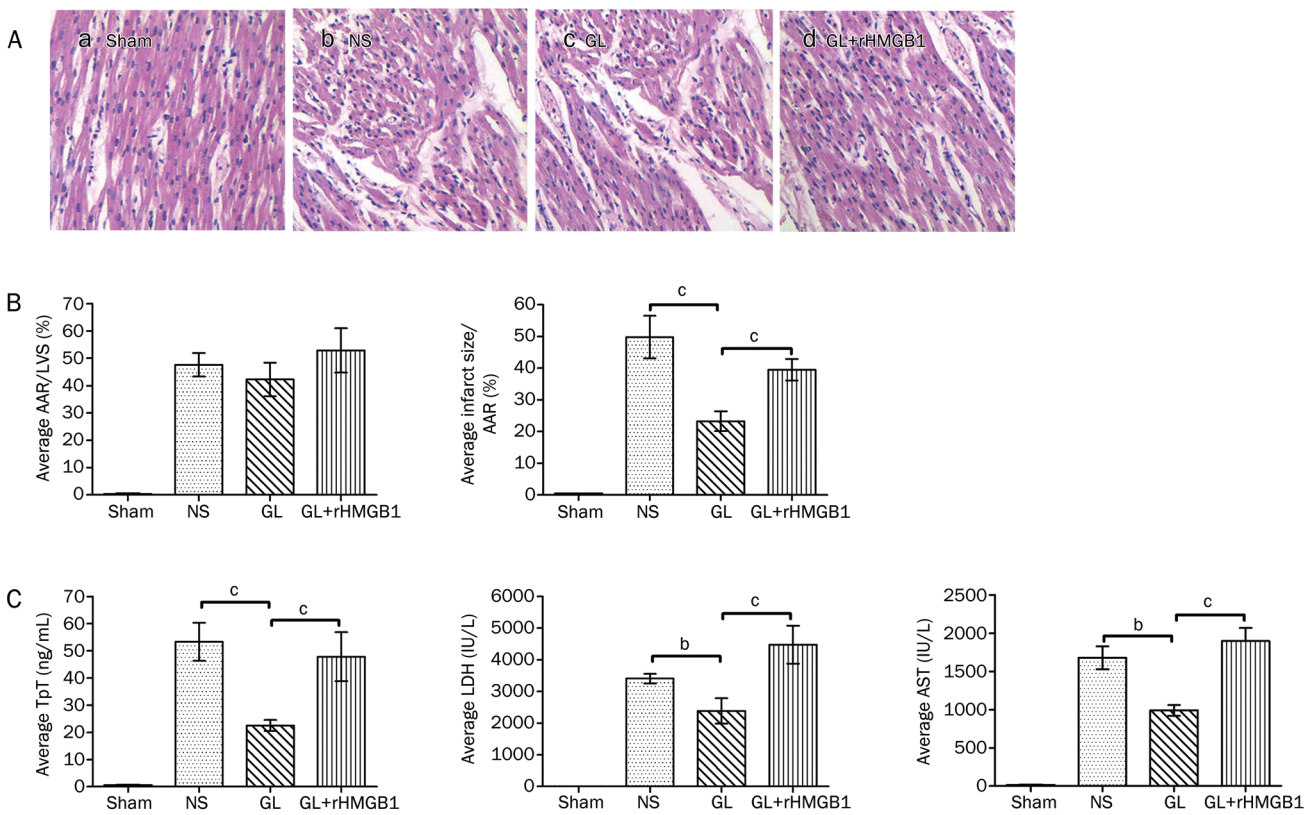

Figure 1. Effects of glycyrrhizin on the histological examination, infarct size, AAR, TpT, AST, and LDH levels in I/R rat hearts. (A: a–d) Representative micrographs of H&E staining that demonstrate histological changes in the rat heart after ischemia-reperfusion (I/R) in each group. (a) Sham group, (b) NS group, (c) GL group, (d) GL+rHMGB1 group. H&E (Haemotoxylin and Eosin) staining, magnification×200. The area at risk (AAR) and infarct size as a percentage of left ventricular size (LVS) and AAR are presented (B). Plasma troponin-T (TpT) expressed as nanogram per milliliter (ng/mL), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) levels expressed as units per liter (IU/L) were also analyzed as measures of cardiac injury (C) for each group. Mean±SEM. n=8. ^{b}P<0.05, ^{c}P<0.01. NS, normal saline; GL, Glycyrrhizin; HMGB1, high-mobility group box 1.

Concentrations of serum HMGB1 and inflammatory cytokines

As shown in Figure 2A, serum HMGB1 concentrations in the sham group remained unchanged during the experimental procedure. However, the concentration of serum HMGB1 in the NS, GL, and GL+rHMGB1 groups was significantly increased at 1 h after reperfusion and peaked at 6 h after reperfusion; thereafter, the serum HMGB1 levels remained high. Moreover, at a certain time point from 1 h to 24 h after reperfusion, serum HMGB1 concentrations after treatment with GL were significantly lower than those observed in the NS group. TNF-α and IL-6 are described as key inflammatory cytokines in the pathophysiology of cardiac I/R injury[21, 22]. The concentrations of TNF-α and IL-6 were observed to be relatively constant and low in the serum of rats belonging to the sham group (Figure 2B–2C). Compared to the sham group, serum levels of the two inflammatory cytokines were greatly enhanced from 2 h to 24 h after reperfusion in the experimental groups. Compared to the NS group, administration of GL before I/R caused a significant decrease in the TNF-α and IL-6 concentrations, whereas co-treatment with rHMGB1 enhanced their concentrations; however, the concentrations were still lower than those observed in the NS group. There are several lines of evidence that demonstrate that HMGB1 could modulate inflammatory responses by inducing the release of inflammatory cytokines, such as TNF-α and IL-6[23], and that GL could inhibit the activity of HMGB1 by directly binding to it[24]. Hence, our results suggest that GL may reduce the I/R injury by inhibiting HMGB1-mediated cytokine release and their activity.

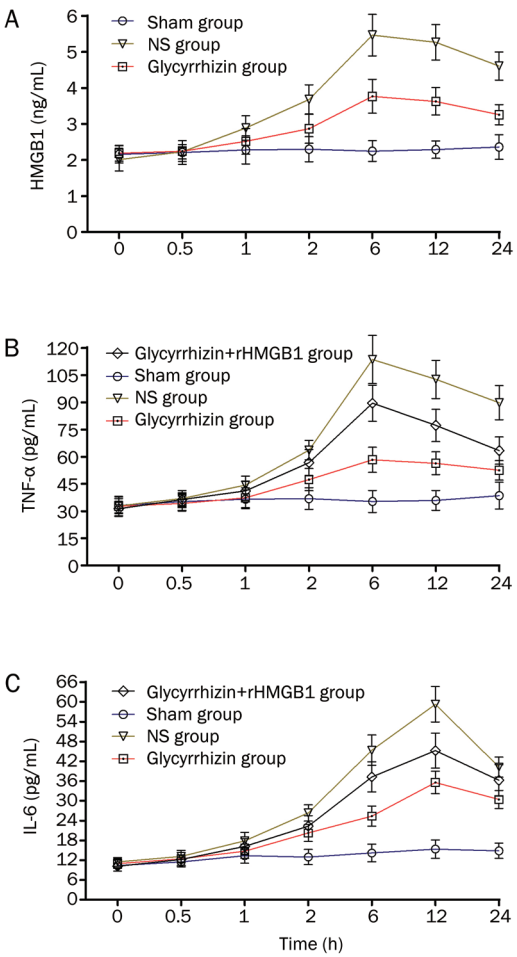

Figure 2. Serum concentrations of HMGB1 and inflammatory cytokines (TNF-α, IL-6) at different time points of reperfusion. (A) HMGB1, (B) TNF-α, (C) IL-6.

Ischemia-reperfusion-induced apoptosis is mitochondria dependent and is alleviated by pre-treatment with GL

Specifically, inflammation is considered to be a major cause of I/R-induced tissue injury due to apoptosis. Next, we investigated whether the anti-inflammatory effect of GL can alleviate I/R-induced apoptosis and whether apoptosis-related Bcl-2 protein family and cytochrome *c*, the two most important players, are involved in this process. TUNEL staining identified programmed cell death, as depicted in Figure 3A. In the sham group, negligible positive cell staining was observed. In contrast, numerous cells were observed to be strongly positive for TUNEL staining in the NS group. However, in the samples obtained from the GL group, only a few cells were positive for TUNEL staining, and the average intensity of nuclear staining was relatively lighter compared with the NS group. Moreover, as expected, co-treatment with rHMGB1 increased the number of TUNEL-positive cells.

Regulation of mitochondrial dysfunction and membrane permeabilization by proteins of the Bcl-2 family including Bcl-2, Bcl-x_L, Bax, and Bim plays an important role in I/R-induced injury of the cardiac myocyte via apoptosis[25]. To identify whether glycyrrhizin can influence the Bcl-2 protein family, we performed immunoblotting analysis to detect Bax, Bcl-2, and cytochrome *c* protein expression in the cytosolic and mitochondrial fractions (Figure 3B). No significant differences in Bcl-2 protein expression were observed in the cytosolic and mitochondrial fractions between these groups (data not shown). In the sham group, we observed that under basal conditions, Bax was predominantly expressed in the cytosolic fraction. When compared to the sham group, a significant decrease in Bax expression was detected in the cytosolic fraction, whereas an increase in the mitochondrial fraction was observed in the NS group. Compared to the NS group, pre-treatment with glycyrrhizin resulted in a significance increase in bax expression in the cytosolic fraction and a decrease in the mitochondrial fraction; however, it still did not reach the level observed in the sham group. The change in cytochrome *c* protein expression was the opposite to that observed with bax. Moreover, co-treatment with recombinant HMGB1 could reverse the effect of glycyrrhizin on the subcellular distribution of Bax and cytochrome *c* proteins between the cytosolic and mitochondrial fractions. There was no significant difference in the total expression of bax and cytochrome *c* protein between the groups.

An executioner caspase, caspase-3, is activated in the apoptotic cell by an intrinsic (mitochondrial) pathway, and the caspase-3 zymogen is virtually inactive until it is cleaved by

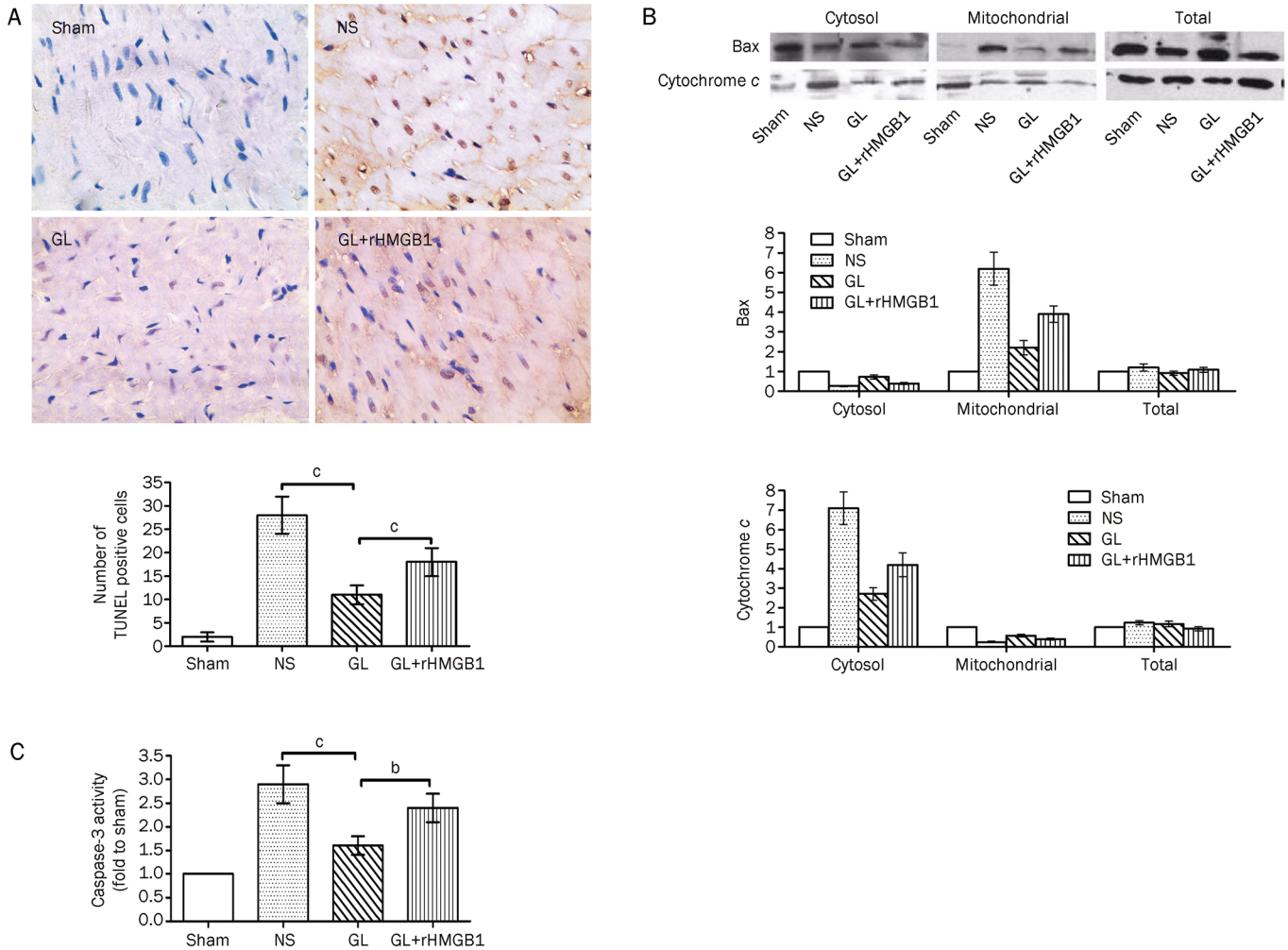

Figure 3. (A) Representative photomicrographs show TUNEL staining for apoptotic cells in the rat heart at 24 h after reperfusion in sham, NS, GL, and GL+rHMGB1 groups. Effects on the severity of cardiac apoptosis are demonstrated as an average quantitative analysis of the number of TUNEL-positive cells. Data are represented as the mean±SEM. n=8 for each group. ^{b}P<0.05, ^{c}P<0.01. (B) Bax was predominantly expressed in the cytosol while cytochrome c was predominantly in the mitochondria of the non-I/R heart (sham). After the onset of ischemia–reperfusion and treatment with different interventions, Bax and cytochrome c successively translocated between the cytosolic and mitochondrial fractions during the experiment. Semi-quantitative densitometric analysis (n=8) clearly revealed a reciprocal relationship between Bax and cytochrome c expression between the cytosolic and mitochondrial fractions 24 h after reperfusion. (C) Effects of GL with or without rHMGB1 treatment on caspase-3 activity in area at risk zone of the cardiac tissues at 24 h after reperfusion. The Bax and cytochrome c bands were observed at 21 kDa and 14 kDa, respectively. Results are represented as the mean±SEM from three separate experiments. ^{b}P<0.05, ^{c}P<0.01.

an initiator caspase after the apoptotic signaling events have occurred. As shown in Figure 3C, the caspase-3 activity was significantly enhanced when treated with I/R compared to the sham group. Pre-treatment with GL significantly reduced caspase-3 activity compared to that observed in the I/R group, which was again reversed in the GL+rHMGB1 group. These results demonstrate that the administration of GL significantly alleviates injury due to apoptotic events caused by I/R in the myocardial cell of the rat by antagonizing the function of HMGB1 and the mechanism involved in the regulation of Bax translocation and cytochrome c release.

GL modulates phospho-JNK but not phospho-ERK and p38 signaling pathways

The mitochondria-dependent apoptosis pathway is tightly regulated by the mitogen-activated protein (MAP) kinase family, and among these, JNK, ERK1/2, and p38 have been observed to be activated during I/R injury[12, 13]. Treatment with HMGB1 box A significantly reduced the phosphorylation of ERK1/2 and JNK, but did not affect the phosphorylation of phospho-p38 MAPK[16]. To study whether glycyrrhizin can modulate MAP kinase activity, we analyzed these MAP kinases by performing Western blot analysis. As shown in

Figure 4A, I/R-induced phosphorylation of JNK, ERK1/2, and p38 in the left ventricles isolated from rats. In rats pretreated with glycyrrhizin, the phosphorylation level of JNK was decreased compared to the NS-treated group. Co-treatment with rHMGB1 enhanced the phosphorylation level of JNK compared to the glycyrrhizin group, but the level was still lower than that observed in the NS group. Notably, activation of p38 MAPK and ERK1/2 due to I/R injury, as determined by immunoblotting for phosphorylated Tyr182 and Thr202/Tyr204, was not affected by treatment with glycyrrhizin. Co-treatment with rHMGB1 did not affect the phosphorylation level of p38 MAPK; however, it slightly enhanced the phosphorylation level of ERK1/2. Pre-treatment with glycyrrhizin alone or with rHMGB1 did not affect total levels of JNK, ERK1/2, and p38 proteins.

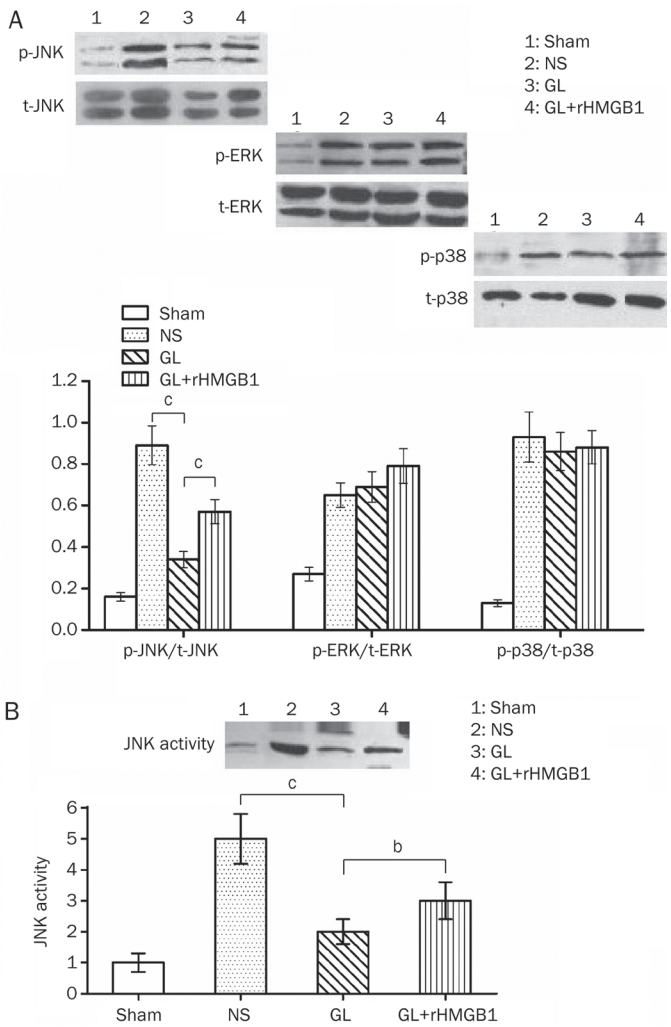

Figure 4. (A) Effects of GL with or without rHMGB1 treatment on phosphorylated (p) and total (t) ERK1/2, JNK, and p38 expression. Each blot is a representative of three experiments with similar results. The bar graph demonstrating semi-quantitative densitometric analysis summarizes the fold change in phosphorylated to total ERK1/2, JNK, and p38 in each of the groups. Data are represented as the mean±SEM. n=8 for each group. (B) JNK activity assay in the whole-cell fraction isolated from rats pretreated with GL with or without rHMGB1. Each blot is a representative of three experiments with similar results. The phospho-JNK bands were observed at 46 and 54 kDa, and the change in these two bands was parallel to JNK activity. The phospho-ERK1/2 bands were observed at 42 and 44 kDa. The phospho-p38 band was observed at 41 kDa. Data are represented as the mean±SEM. n=8 for each group. $^bP<0.05$, $^cP<0.01$.

Furthermore, to detect the effect of GL on JNK signaling pathway, JNK activity was examined by performing the immunocomplex assay. The level of JNK activity in the NS group was significantly increased (4.8 fold) compared with that observed in the sham group. Treatment with glycyrrhizin produced a decrease in JNK activity by 60% compared to that observed in the NS group. Co-treatment with rHMGB1 produced an increase in JNK activity by 30% compared with that observed in GL group. These results demonstrate that the change in JNK activity is most likely due to the regulation of phospho-JNK expression.

Selective inhibition of JNK activity by SP600125 attenuates HMGB1-dependent Bax translocation and subsequent apoptosis

To further explore whether activated JNK contributes to HMGB1-induced Bax translocation and subsequent apoptosis, we measured the distribution of Bax and cytochrome *c* between the cytosolic and mitochondrial fractions, caspase 3 activity and performed TUNEL analysis in each of the groups. The JNK activity was additionally determined by immunoblotting. As demonstrated in Figure 5A, treatment with SP600125, a highly selective inhibitor of JNK1, -2, -3, produced a significant decrease in JNK activity; however, SP600125 had no effect on phospho-JNK expression level (data not shown). This result may be attributed to the ability of SP600125 to compete with ATP and selectively inhibit its kinase activity, without any effect on JNK phosphorylation[20, 26]. Co-treatment with rHMGB1 enhanced JNK activity, which was reduced by SP600125. There was a reduction in the number of TUNEL-positive cells after treatment with SP600125, and this number was significantly increased with rHMGB1 co-treatment (Figure 5B). Treatment with SP600125 increased the expression of Bax and decreased the expression of cytochrome *c* in the cytosolic fraction; however, it decreased the expression of Bax and increased the expression of cytochrome *c* in the mitochondrial fraction, which were partly reversed by rHMGB1 (Figure 5C). Moreover, treatment with SP600125 significantly reduced caspase-3 activity compared to the NS group, and as expected, caspase-3 activity was partly reversed in the NS+SP600125+HMGB1 group (Figure 5D). These results demonstrate that SP600125 has an inhibitory effect on Bax and cytochrome *c* translocation between the cytosolic and mitochondrial fractions and that subsequent apoptosis induced by I/R is HMGB1 dependent.

Discussion

Traditional Chinese medicine treatment has become increasingly important for the therapy of cardiovascular ischemia/reperfusion injury. GL is a natural anti-inflammatory compound that is commonly used in Japan to treat

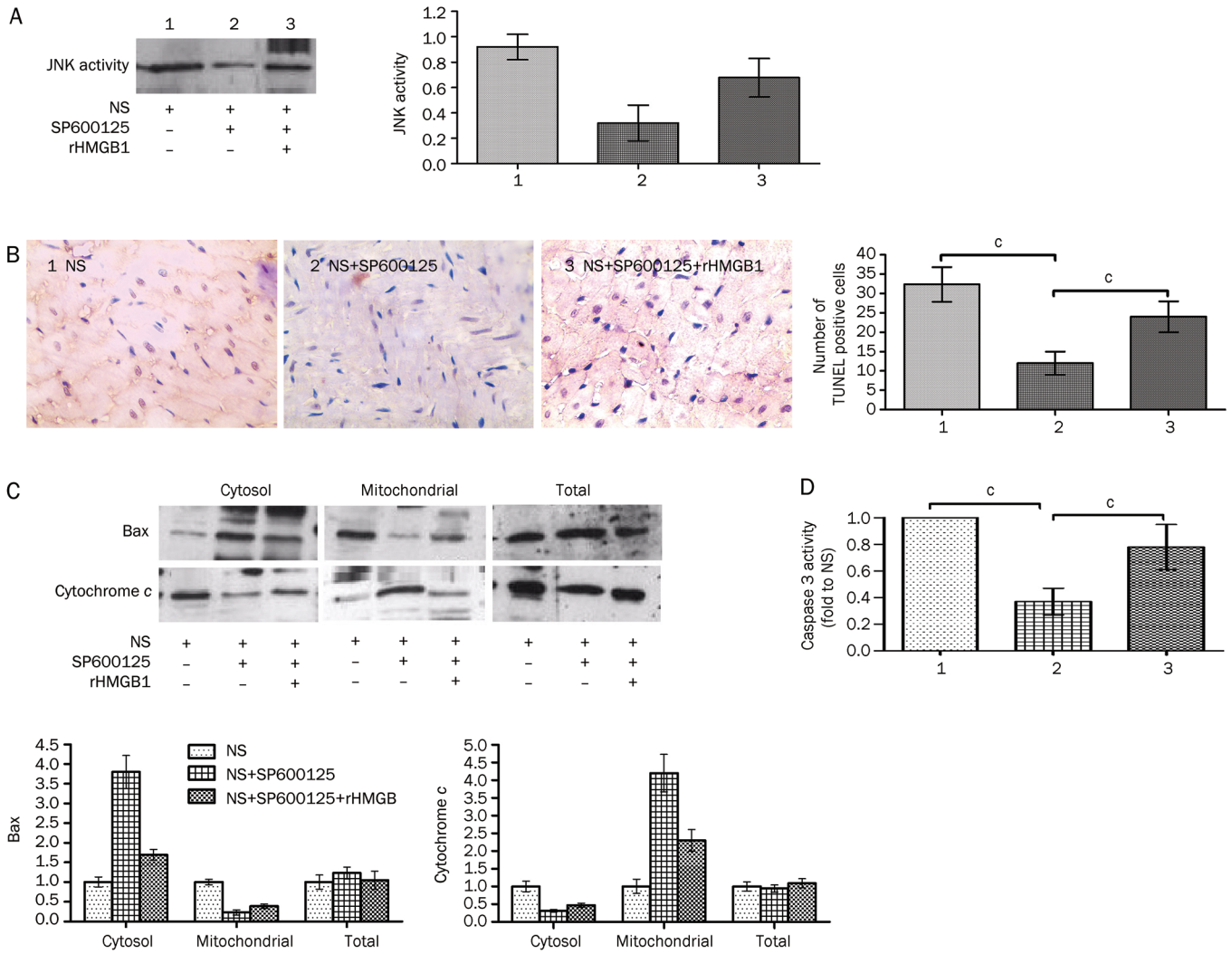

Figure 5. (A) Effects of JNK inhibitor SP600125 on JNK activity, with or without rHMGB1 treatment. Each blot shown is a representative of three experiments with similar results. The bar graph showing semi-quantitative densitometric analysis (n=4) summarizes the fold change in JNK activity in each of the groups. (B) Representative photomicrographs demonstrating TUNNEL staining for apoptotic cells in the rat heart at 24 h after reperfusion in NS, NS+SP600125 and NS+SP600125+rHMGB1 groups. Effects on the severity of cardiac apoptosis are shown in an average quantitative analysis of the number of TUNEL-positive cells. Data are represented as the mean±SEM. n=4 for each group. $^bP<0.05$, $^cP<0.01$. (C) Bax was predominantly expressed in the mitochondria while cytochrome c was predominantly present in the cytosol of the I/R heart (NS). JNK inhibitor SP600125 in the presence or absence of rHMGB1 caused the translocation of Bax and cytochrome c between the cytosolic and mitochondrial fraction during the experiment. Semi-quantitative densitometric analysis (n=4) clearly showed a reciprocal expression of Bax and cytochrome c between the cytosolic and mitochondrial fractions 24 h after reperfusion. (D) Effects of SP600125 in the presence or absence of rHMGB1 treatment on caspase 3 activity in area at risk zone of cardiac tissues at 24 h after reperfusion. Results are represented as the mean±SEM obtained from three separate experiments. $^bP<0.05$, $^cP<0.01$.

patients with chronic hepatitis[9]. As a HMGB1 inhibitor, GL directly binds to both HMG boxes in HMGB1, inhibits its chemoattractant functions and attenuates HMGB1-induced apoptotic injury in fibroblasts, smooth muscle cells and hepatocytes *in vitro*[17, 24, 27]. However, the relationship between myocardial ischemia-reperfusion injury and the possible protective effects of GL is not documented. In the present study, we demonstrated that iv pre-treatment with 10 mg/kg GL reduced the size of myocardial infarct and produced a therapeutic effect against I/R heart injury with certain evaluation indices such as TpT, AST, and LDH. This effect was accompanied by a reduction in the concentration of HMGB1 and related inflammatory factors (TNF-α and IL-6) in the rat serum. Using this dose, no significant toxicities in the effects investigated, including changes in the baseline hemodynamic values, cardiac electrophysiology, and histopathological analysis of vital organs including liver, spleen, lung, kidney, and brain, were observed (data not shown).

In this study, pretreatment with GL alleviated the apoptosis injury resulting from myocardial I/R at least in part due

to the regulation of Bcl-2/Bax ratios in the cytosolic and mitochondrial fractions, subsequent inhibition of cytochrome *c* release and caspase-3 activity. All of these protective effects of GL can be reduced by co-treatment with rHMGB1. It is well known that apoptosis is regulated by a number of factors induced by diverse apoptotic signals including the pro-and anti-apoptotic proteins of the Bcl-2 family. Bax, Bad, and Bak are the pro-apoptotic members, whereas Bcl-2, Bcl-x_L, and Bcl-w are the antiapoptotic Bcl-2 members. In particular, the balance of Bcl-2 and Bax expression in the mitochondrial fraction is an important factor in determining the extent of apoptosis in I/R hearts. It has been reported that intermittent hypoxia attenuates ischemia/reperfusion-induced apoptosis in cardiac myocytes by regulating the expression of Bcl-2/Bax in the mitochondrial membrane[28]. Ischemia–reperfusion caused the translocation of Bax from the cytosolic to mitochondrial fraction and concomitant apoptosis in the middle cerebral artery of the rats[20]. In the present study, we demonstrated that I/R can induce the translocation of Bax between cytosol and mitochondrial fractions without affecting Bcl-2, thereby resulting in a decreased ratio of Bcl-2 to Bax in the mitochondrial membrane fraction (Figure 3). Moreover, a release of cytochrome *c* from mitochondria to the cytosolic fraction was also observed. GL can reverse the disordered distribution of Bax between cytosolic and mitochondrial fractions and inhibit the release of cytochrome *c*, both of which are induced by I/R. A similar *in vitro* finding reported that GL had an anti-apoptotic effect by preventing HMGB1-induced cytochrome *c* release and caspase 3 activation in Huh-BAT cells[27]. Because the anti-apoptotic effect of GL has been linked to the inhibition of HMGB1 and caspase-dependent cytochrome *c* release *in vivo* and *ex vivo*, the question that remains now concerns how GL regulates the BAX translocation and caspase-dependent cytochrome *c* release via the inhibition of HMGB1. The exact answer is currently unknown, but we speculate that GL may modulate the activity of a particular kinase, which contributes to BAX translocation and is involved in I/R-induced HMGB1-dependent apoptosis based on the following evidence: (1) Glycyrrhizin can alleviate HMGB1-induced hepatocyte apoptosis by inhibiting the p38-dependent mitochondrial pathway in Huh-BAT cells[27]; (2) the JNK signaling pathway mediates Bax translocation and subsequent neuronal apoptosis through interaction with Bim after transient focal cerebral ischemia[20]; (3) myocyte-derived HMGB1 and TNF-α work together to promote I/R-induced myocardial apoptosis through JNK activation[29]; (4) blockade of HMGB1 using HMGB1 box A, another HMGB1 inhibitor, effectively decreased the phosphorylation of ERK1/2 and JNK, and finally reduced I/R-induced apoptosis injury[16].

The mitochondria-dependent apoptosis pathway is tightly regulated by the MAP kinase family, and JNK, ERK1/2, and p38 members of this family have been demonstrated to be activated in I/R injury[12, 13]. Treatment with HMGB1 box A, a competitive antagonist of HMGB1 cytokine, significantly reduced the phosphorylation of ERK1/2 and JNK, but did not affect the level of phospho-p38 MAPK[16]. In a Huh-BAT cell model, GL prevented HMGB1-induced cytochrome *c* release and p38 activation in a dose-dependent manner but had no effect on phospho-JNK and ERK1/2[28]. Interestingly, our studies showed that GL significantly decreased phosphorylation of JNK, but it did not affect the levels of phospho-p38 and ERK1/2 in rats that underwent I/R. This lack of clarity regarding the single class of MAPK kinase that can be modulated by GL appears to primarily depend on the difference in the levels of basic MAPK kinase *in vivo* and *in vitro*. However, these *in vivo* findings demonstrated that GL and HMGB1 box A can modulate the phosphorylation level of JNK in rats with I/R injury, despite the inconsistency in ERK1/2. The direct inhibitory effects of glycyrrhizin on extracellular HMGB1 were similar to those obtained with HMGB1 box A[24]. Hence, it is possible that upon release, HMGB1 directly or indirectly activates phospho-JNK pathway, which is supported by our results obtained due to the administration of rHMGB1 in this study. Moreover, recent studies have demonstrated that HMGB1 triggered a substantial potentiation of TNF-α-induced JNK phosphorylation and that inhibition of JNK (SP600125) prevented the myocyte apoptosis induced by a TNF-α/HMGB1 cocktail[29]; these findings support our hypotheses.

Our results demonstrated that GL, which can block and inhibit extracellular HMGB1 cytokine's activity, exerted a protective effect on I/R-induced apoptosis through blockade of JNK/Bax-mediated pathway in rats *in vivo*. However, the underlying molecular link between HMGB1, JNK, and Bax involved in the process of apoptosis is not fully understood. It has been demonstrated that there is a significant association between JNK and Bax during the regulation of apoptosis and related cytochrome *c* release after ultraviolet radiation. Bax is a potent regulator of mitochondria-dependent apoptosis in a variety of stressful conditions. In this study, inhibition of JNK activity by SP600125 attenuated HMGB1-dependent Bax translocation and subsequent apoptosis, which was reversed by the co-treatment with HMGB1 (Figure 5). Currently, there is no evidence that indicates that the activated JNK can directly phosphorylate and activate Bax, resulting in its translocation from cytosol to mitochondria fraction. However, recent studies have demonstrated that JNK might induce apoptosis by modulating BIM, a proapoptotic protein of the Bcl-2 family[30]. Moreover, the JNK signaling pathway mediates Bax translocation and subsequent neuronal apoptosis by interacting with Bim after transient focal cerebral ischemia[20]. However, in our studies, I/R-induced Bim phosphorylation was not observed (data not shown). Indeed, in addition to Bim, other members of Bcl-2 family such as Bcl-2, Bcl-x_L, Bad, and transcription factors such as c-jun, ATF2, Elk-1, p53, and c-Myc can also be phosphorylated by the activated JNK in response to a variety of extracellular stimuli[31]. Therefore, whether molecules other than Bim are phosphorylated by JNK activate Bax and cause subsequent translocation to mitochondria should be explored in future studies.

In conclusion, we have provided evidence that demonstrates that pretreatment with GL has a protective effect on ischemia-

reperfusion injury in the rat heart through the blockade of phospho-JNK/Bax pathway-mediated apoptosis. The protective effect is at least in part due to the inhibition of extracellular HMGB1 cytokine activity by treatment with GL. Bim, one of the most important downstream proteins that executes JNK-mediated Bax activation and mitochondria translocation in transient focal cerebral ischemia in rats, is not involved in this process. Considering the biological differences between species that may influence drug adsorption, metabolism, distribution and toxicity in rats and humans, it is not clear whether glycyrrhizin has similar protective effects in humans. Hence, further studies are required for investigating the lower dosage range limit that will produce a similar protective effect and for evaluating its beneficial effect in different clinical settings that are related to I/R injury.

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Author contribution

Prof Li LI designed the research and revised the manuscript; Chang-lin ZHAI conducted the research, analyzed the data and wrote the manuscript; Yun ZHANG, Hong-xia XU, Jing-min WANG, Gui-peng AN, and Yuan-yuan WANG helped with parts of the research, and Mei-qi ZHANG contributed toward writing the manuscript.

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