

## Full-length article

# Glycine blunts transplantative liver ischemia-reperfusion injury by downregulating interleukin 1 receptor associated kinase-4<sup>1</sup>

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## Key words

liver transplantation; reperfusion injury; glycine; lipopolysaccharides; IRAK-4

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## Abstract

**Aim:** To determine whether glycine could downregulate interleukin 1 receptor associated kinase-4 (IRAK-4) expression to interfere with lipopolysaccharides (LPS) signal transduction and blunt transplantative liver ischemia-reperfusion injury (I/RI). **Methods:** SD rats were randomly divided into two groups: donor animals of the glycine group ( $n=40$ ) were given glycine (1.5 mL; 300 mmol/L, iv) 1 h before harvest, and the control group were treated with 1.5 mL physiological saline ( $n=40$ ). Orthotopic liver transplantation was then performed according to the Kamada technique. Ten animals in each group were followed up for 7 d after surgery to assess survival. The remaining animals in each group were divided into 3 subgroups ( $n=10$ ) at 1h, 2 h and 6 h after portal vein reperfusion. Levels of LPS, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin in portal circulation, as well as IRAK-4 and TNF- $\alpha$  expression, NF- $\kappa$ B transcriptional activity and morphological study of liver tissues were analyzed. **Results:** Reperfusion resulted in a significant elevation of LPS concentrations in each group persisting to the end of our study. However, glycine, which led to improved survival rate and liver function, significantly alleviated liver parenchyma cell damage by downregulating IRAK-4, TNF- $\alpha$  expression and NF- $\kappa$ B transcriptional activity compared with the control group. **Conclusion:** Glycine can attenuate hepatic I/RI by downregulating IRAK-4 to interfere with LPS signal transduction.

## Introduction

Despite intense research on the development of novel therapeutic strategies, primary graft dysfunction caused by ischemia-reperfusion injury (I/RI) still represents a major problem in clinical liver transplantation<sup>[1-3]</sup>. The most common candidates for liver transplantation are patients with all kinds of end-stage liver diseases combined with high levels of endotoxin or lipopolysaccharides (LPS), an integral component of the cell wall of gram-negative bacilli. LPS, a potent stimulus for Kupffer cells (KC), is involved in priming and deterioration in I/RI<sup>[4-6]</sup>. Substantial evidence indicates that KC, the resident macrophages of the liver, play a central role in the pathogenesis of liver parenchyma cell damage during the reperfusion phase, because activated KC are capable of

releasing numerous mediators for regulating hepatic microcirculation<sup>[7]</sup>, and microcirculatory disturbance is a key factor in enhanced susceptibility of the donor liver to reperfusion<sup>[8]</sup>.

As a consequence, blockade of LPS-induced KC activation with glycine, has been shown to reduce hepatic I/RI in animal models and humans<sup>[8-11]</sup>. Glycine is a nontoxic non-essential amino acid and inhibitory neurotransmitter in the central nervous system<sup>[12]</sup>. It prevents the increase of intracellular Ca<sup>2+</sup> concentration by activating a glycine-gated chloride channel. Increases of intracellular Ca<sup>2+</sup> concentration in KC are essential for the release of prostanoids and inflammatory cytokines in response to stimuli such as LPS<sup>[8,13]</sup>. Furthermore, Glycine can also inhibit the expression of LPS membrane-bound receptor CD14 on KC<sup>[14]</sup>. However, little

has so far been documented about the relationship between glycine and the mediators of LPS signaling transduction. It's well known that toll-like receptor (TLR) dependent pathways are critical for receiving LPS signals to initiate inflammation responses. Recently, a key breakthrough has been made in the area of TLR signaling with the discovery of interleukin 1 receptor associated kinase-4 (IRAK-4). In IRAK-4-deficient mice, the LPS signals mediated by TLR are severely impaired, indicating that IRAK-4 plays an essential role in these signaling pathways<sup>[15]</sup>. The aim of this study was to determine whether brief intravenous infusion of glycine to donors would downregulate IRAK-4, interfering with LPS signal transduction and blunt I/RI after liver transplantation.

## Materials and methods

**Experimental animals and treatment** Male Sprague-Dawley rats (250–270 g) were purchased from the Chongqing Experimental Animal Center of Chongqing University of Medical Sciences and randomly divided into the glycine group and control group. Donor animals of the glycine group ( $n=40$ ) were given glycine by infusion (1.5 mL; 300 mmol/L) through the femoral vein 1 h before harvest, and the control group were treated with 1.5 ml physiological saline ( $n=40$ )<sup>[8]</sup>. Experimental procedures were approved by the Institutional Animal Care and Use Committee.

**Transplantation procedure** Liver transplantation was performed according to the Kamada technique<sup>[6]</sup>. Donor livers were harvested in a standardized fashion, including freeing the organ from ligaments and cannulation of the bile duct, retracting, and moving the liver lobes *in situ* continuously to minimize the harvest-dependent graft injury<sup>[2]</sup>. Cuffs were attached in the infrahepatic vena cava and portal vein of the donor liver, which were stored at 0 °C–4 °C UW solution for 1 h. Orthotopic liver transplantation was then performed in hepatectomized recipient rats by connecting the suprahepatic vena cava with a running 7/0 Prolene suture, inserting cuffs into the corresponding vessels and anastomosing the bile duct over an intraluminal polyethylene splint, without reconstruction of the hepatic artery. During this time the recipient portal vein was clamped for 15–17 min.

**Determination of survival rate and experimental design** After the abdominal incision was closed the rats were provided with food and water. We followed up 10 animals of each group for 7 d after surgery to assess survival. The other animals of each group were divided into 3 subgroups (10 animals of every subgroup) at 1 h, 2 h and 6 h after portal vein reperfusion, blood from portal vein blood and liver

tissues were sampled respectively.

**LPS determination**<sup>[16]</sup> The plasma of portal vein blood was obtained and subjected to a deproteination and neutralization process by acid base precipitation using perchloric acid and sodium hydroxide, and then, the LPS levels were assayed by the chromogenic limulus amoebocyte lysate (LAL) test with a kinetic modification according to the test kit procedure.

**Enzyme assays** ALT and AST activity of serum was determined by standard enzymatic methods, while total bilirubin (TB) was detected by direct spectrophotometry at 454 nm.

**Reverse transcription-polymerase chain reaction (RT-PCR)** Total RNA was extracted from liver tissues using TRIzol reagent (Life Technologies, New York, USA) according to the manufacturer's instructions. Total RNA was quantified with the ratio of absorption values of RNA samples at 260 nm and 280 nm. Each total RNA sample was reverse-transcribed to complementary DNA (cDNA) using RT-PCR kit (Roche, USA) and stored at -70 °C until PCR.  $\beta$ -actin was used as an internal positive control. Specific primer sequences of  $\beta$ -actin, IRAK-4, TNF- $\alpha$ <sup>[17]</sup> were as follows and the sizes of production were 300, 597, and 694 bp respectively: IRAK-4: 5'-CGGCGACGACAGATAACAATC-3' and 5'-TGC TGACACGTTGUATTAC-3';  $\beta$ -actin: 5'-CATTGTGATGGA CTCCGGAG-3' and 5'-ATAGTGATGACCTGGCCGTC-3'; TNF- $\alpha$ : 5'-CCCAGACCCTCACACTCAGA-3' and 5'-GCCACTACTTCAGCATCTCG-3'. All PCR products were electrophoresed on 2% agarose gels. The relative expression of mRNAs were assessed by taking the ratio of the intensity of the DNA bands of IRAK-4 or TNF- $\alpha$  to  $\beta$ -actin band using the Bio-Image analysis system (Bio-Rad Gel Doc 2000, Hercules, CA, USA) and expressed as arbitrary units.

**Western blot** Protein extracts of the liver tissues were obtained by homogenizing samples in a cell lysis buffer containing 20 mmol HEPES (pH 7.9), 25% glycerol, 0.42 mmol NaCl, 15 mmol MgCl<sub>2</sub>, 0.2 mmol EDTA, 0.5 mmol phenylmethylsulfonyl fluoride (PMSF) and 0.5 mmol dithiothreitol (DTT), then by two cycles of centrifugation at 12 000×g for 15 min. Protein concentration was determined by Bradford assay kit (Bio-Rad, Hercules, CA, USA). Extracted protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes (Dupont, Boston, MA, USA). Membrane was washed with 0.1% Tween20-PBS and incubated with 5% dry non-fat skimmed milk powder in 0.1% Tween20-PBS, pH 7.4 for 1 h, then with rabbit anti-mouse IRAK-4 polyclonal antibody (diluted 1:200, IMG-441, Imgenex, San Diego, CA, USA) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, CA, USA) for 1h. Finally,

the membranes were developed with diaminobenzidine reagent. The relative amount of IRAK-4 protein was quantified from relative optical density of the band by Bio-Image analysis system (Gel Doc 2000, Bio-Rad, Hercules, CA, USA).

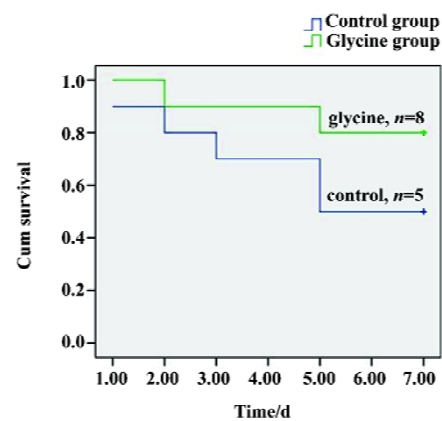
**Analysis of nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcriptional activity** Electrophoretic mobility shift assay (EMSA) was performed for analysis of NF- $\kappa$ B transcriptional activity of liver as described in a previous study<sup>[15]</sup>. Nuclear extracts were prepared and protein concentrations were determined by assay kit (Bio-Rad, Hercules, CA, USA). The double-stranded consensus-binding sequences for the EMSA comprised the oligonucleotide 5'-AGTTGAGGGGACTTCCC-AGG-3' for NF- $\kappa$ B and for the mutant NF- $\kappa$ B 10  $\mu$ g of nuclear extract was incubated with an end-labeled, double-stranded, NF- $\kappa$ B oligonucleotide probe. The reaction was performed in a total of 20  $\mu$ L binding buffer (5 mmol/L HEPES pH 7.8, 50 mmol/L KCl, 0.5 mmol/L dithiothreitol and 10% glycerol) for 20 min at room temperature. After incubation, samples were fractionated on a 5% polyacrylamide gel and complex formation was visualized by autoradiography film (Eastman Kodak, Belgium). At the same time, NF- $\kappa$ B DNA-binding activity in nuclear extracts was measured using the Trans-AM NF- $\kappa$ B p65 ELISA kit (Active Motif Europe, Rixensart, Belgium) according to manufacturer's instructions<sup>[18]</sup>. Five  $\mu$ g nuclear extract were added to a 96-well plate to which oligonucleotide containing NF- $\kappa$ B consensus-binding site had been immobilized. The NF- $\kappa$ B complex bound to the oligonucleotide was detected by adding a specific mAb for p65 subunit. A secondary horseradish peroxidase-conjugated mAb was added and developed with tetramethylbenzidine substrate. After an optimal development time, the reaction was stopped using H<sub>2</sub>SO<sub>4</sub> 0.5 mol/L, and absorbance was measured at 450 nm.

**Morphological study by light and electron microscopy** Liver biopsies for light microscopy were cut into blocks of <1 mm diameter and immediately stored in formalin. Paraffin sections were stained with hematoxylin and eosin. The specimens for electron microscopy were immediately cut into 1 mm cubes and fixed in 2.5% glutaraldehyde in sodium cacodylate-hydrochloride buffer overnight at 4 °C for electron microscopy section. The sections were examined under a transmission electron microscope (Philips EM208, Eindhoven, Holland).

**Data analysis** Results were expressed as mean $\pm$ SD. Statistical difference was calculated by analysis of Independent-Samples *t*-test using SPSS10.0 software. A *P* value <0.05 was considered significant.

## Results

**Survival** A Kaplan–Meier model was constructed from the data to compare overall survival rates between the two groups. Seven-day survival rates were 80% (mean survival time, 6.3 $\pm$ 0.49 d) in the glycine group, whereas the control group had a significantly lower survival rate of 50% (mean survival time, 5.1 $\pm$ 0.7 d), (*P*<0.05, Figure 1). Therefore, our direct comparison indicates that glycine is effective in preserving long-term survival.



**Figure 1.** Survival rates after transplantation. Kaplan-Meier estimations, *P*<0.05.

**Ischemia-reperfusion induced a significant increase in portal vein blood LPS concentrations** Results presented (Table 1) indicated that LPS concentrations in portal vein blood kept significantly increasing to the end of our observation period after reperfusion (6 h, *P*<0.01). However, the difference between the two groups at the same point was not significant (*P*>0.05).

**Glycine alleviates hepatic I/RI after liver transplantation** Hepatocellular injury, reflected by serum AST, ALT and TB, was significantly reduced in the glycine group at 6 h

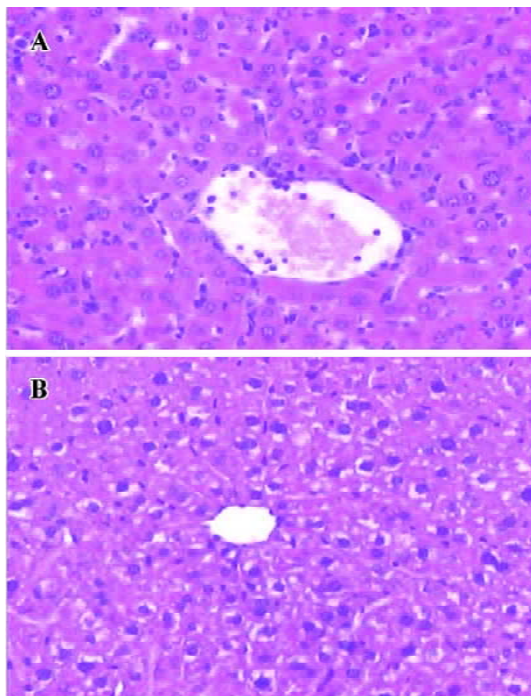
**Table 1.** Changes in portal blood LPS concentrations after I/RI Mean $\pm$ SD. <sup>a</sup>*P*>0.05 vs control group. <sup>f</sup>*P*<0.01 vs the treatment with 1 h.

Groups (n = 10)	LPS (EU/L)		
	1 h	2 h	6 h
Control group	852 $\pm$ 105	1145 $\pm$ 237 <sup>f</sup>	1865 $\pm$ 183 <sup>f</sup>
Glycine group	877 $\pm$ 124 <sup>a</sup>	1180 $\pm$ 264 <sup>af</sup>	1940 $\pm$ 201 <sup>af</sup>

after reperfusion (Table 2). Glycine pretreated livers had less severe signs of I/RI compared with the control group, as reflected by numbers of necrotic cells, infiltrating leukocytes and vacuoles (Figure 2). Furthermore, the hepatic ultrastructural features were significantly different between the two groups. The hepatic sinusoid was well maintained in the glycine group compared to the obvious disruption of sinusoidal lining cells in the control group, in which degeneration of cytoplasm and tremendous mitochondria swelling were also found (Figure 3).

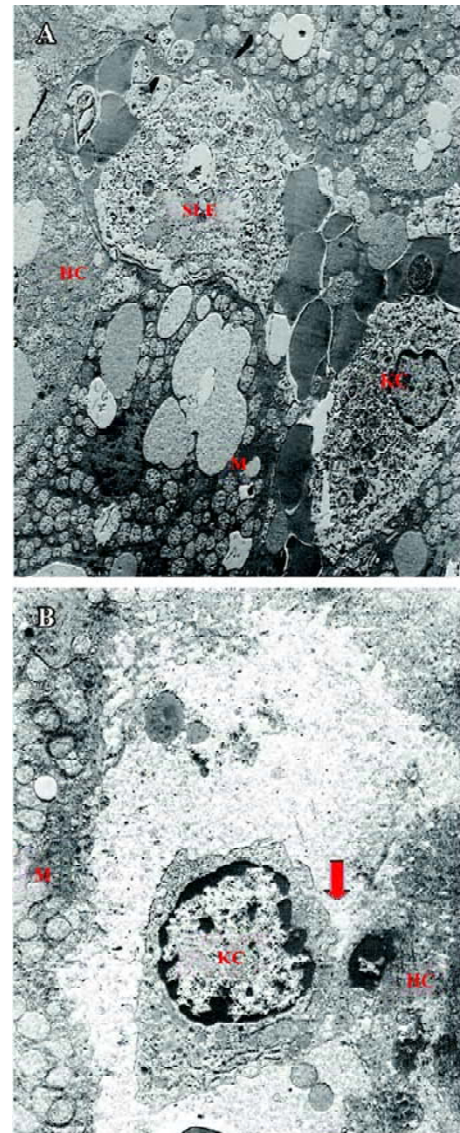
**Table 2.** Effects of glycine on hepatic I/RI after liver transplantation. Mean±SD. <sup>c</sup>*P*<0.01 vs control group.

Groups ( <i>n</i> =10)	AST (U/I)	ALT (U/I)	TB (g/100mg)
Control group	320.37±37.24	280.17±45.26	0.34±0.05
Glycine group	245.42±32.50 <sup>c</sup>	220.38±25.64 <sup>c</sup>	0.18±0.03 <sup>c</sup>



**Figure 2.** Hepatic architecture after reperfusion. (A) The glycine group at 6 h after reperfusion (HE stain, ×400); (B) the control group at 6 h after reperfusion (HE stain, ×400).

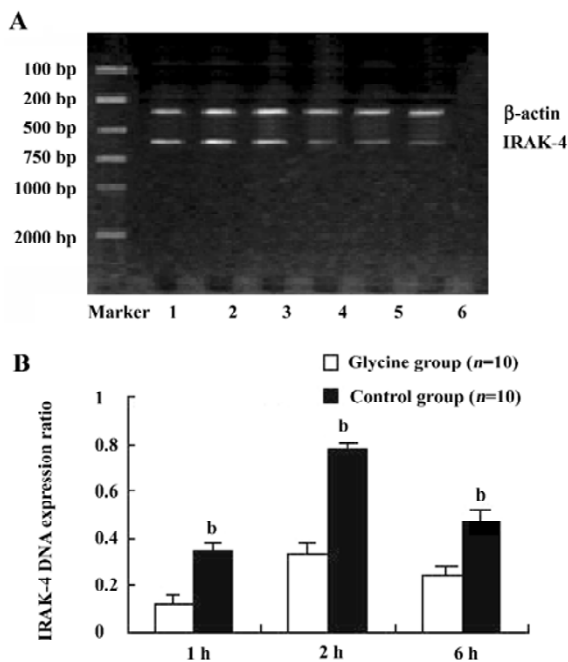
**Glycine downregulates IRAK-4 after reperfusion** To examine the interference effects of glycine on LPS signal transduction during reperfusion phase, IRAK-4 gene expression changes on reperfusion livers were examined with RT-



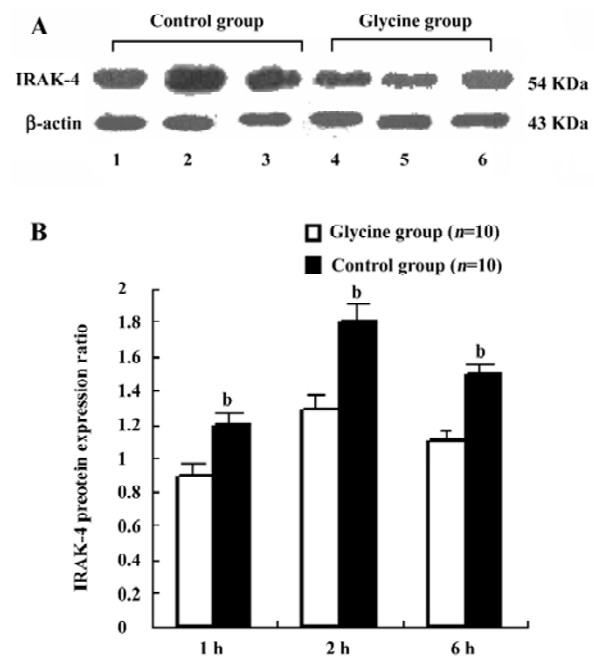
**Figure 3.** Hepatic ultrastructure at 6 h after reperfusion. (A) Glycine treatment group at 6 h after reperfusion (×7000); (B) the control group at 6 h after reperfusion (×4200). M: Mitochondria; SLE: sinusoidal lining cell; KC: Kupffer cell; HC: hepatic cell; arrow: Place where sinusoidal lining cells disappeared and KC contacted with hepatic cell directly.

PCR and Western-blot (Figure 4 and 5). After reperfusion, the expression of IRAK-4 were dramatic increased in control group and reached a peak value 2 h after reperfusion, strongly indicating that LPS induced dramatic inflammation after reperfusion, however, Glycine significantly diminished the expression of IRAK-4, supporting the hypothesis that downregulation LPS signal transduction mediator's expression is a mechanism of glycine-mediated protection.

**Glycine depresses NF-κB activation** To further examine



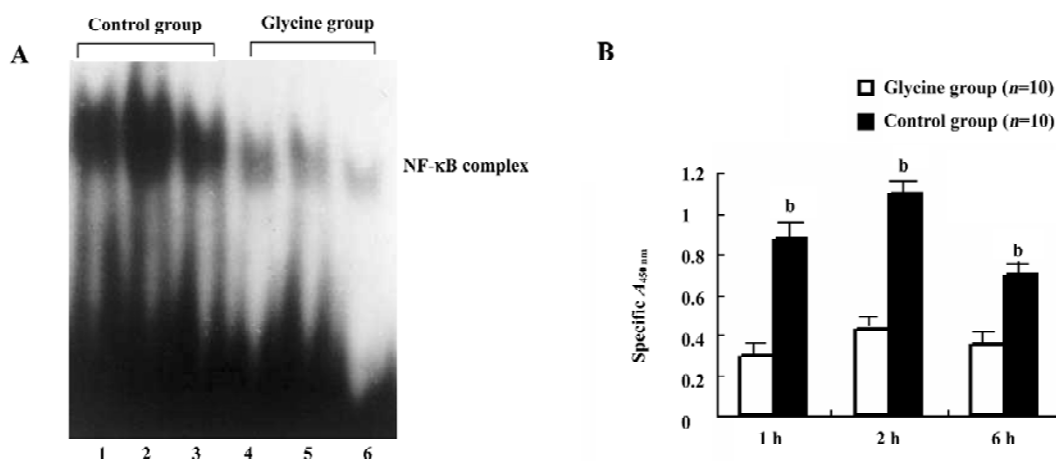
**Figure 4.** The influence of glycine on IRAK-4 gene expression (mRNA levels) at different time after liver transplantation. (A) The mRNAs isolated from the control group and the glycine group are shown on lanes 1–3, 4–6 at 1 h, 2 h, 6 h after reperfusion respectively. (B) Gels were photographed and digitized, and optical densities of IRAK-4 mRNA bands were normalized using optical densities of the  $\beta$ -actin mRNA bands. <sup>b</sup> $P < 0.05$  vs glycine group at same point.



**Figure 5.** The influence of glycine on IRAK-4 protein expression at different times after liver transplantation. (A) The IRAK-4 protein isolated from the control group and the glycine group are shown on lanes 1–3, 4–6 at 1 h, 2 h, 6 h after reperfusion respectively. (B) Densitometry of IRAK-4 was performed as described in the legend to Figure 4. <sup>b</sup> $P < 0.05$  vs glycine group at same point.

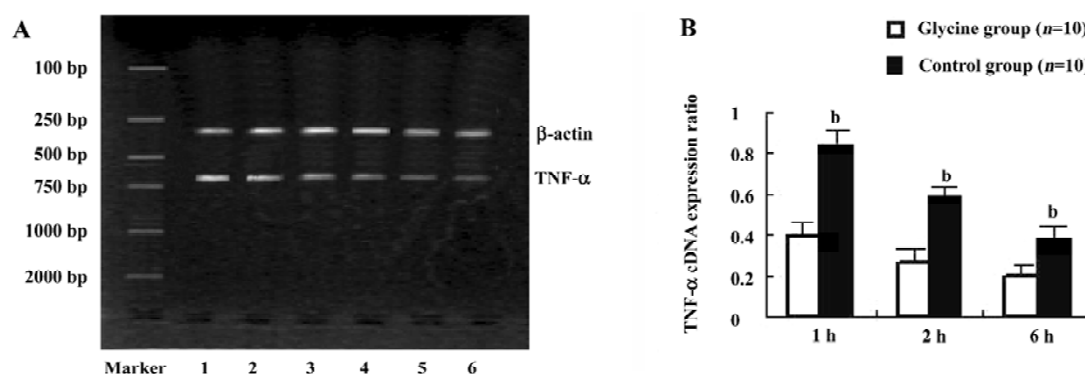
the interference effect of glycine on LPS signal transduction, NF- $\kappa$ B activation on reperfusion livers were first examined by ELISA (Figure 6A). We observed that the glycine group

expressed low NF- $\kappa$ B p65 DNA-binding activity, and the control group induced a rapid and clear increase in NF- $\kappa$ B p65 activation, which was optimal after 2 h of reperfusion. This observation was confirmed and extended in EMSA



**Figure 6.** The influence of glycine on NF- $\kappa$ B activation at different times after reperfusion. (A) Nuclear extracts were obtained from the control group and the glycine group are shown on lanes 1–3, 4–6 at 1 h, 2 h, 6 h after reperfusion, respectively. (B) Time course determination of NF- $\kappa$ B DNA-binding activity using ELISA. Optical density values ( $A_{450\text{ nm}}$ ) were corrected for background levels. <sup>b</sup> $P < 0.05$  vs glycine group at same point.





**Figure 7.** The influence of glycine on TNF- $\alpha$  gene expression (mRNA levels) at different times after reperfusion. (A) The mRNAs isolated from the control group and the glycine group are shown on lanes 1–3, 4–6 at 1 h, 2 h, 6 h after reperfusion, respectively. (B) Gels were photographed and digitized, and optical densities of TNF- $\alpha$  cDNA bands were normalized using optical densities of the  $\beta$ -actin bands. <sup>b</sup> $P < 0.05$  vs glycine group at same point.

experiments performed on nuclear extracts (Figure 6B). We observed a low level of NF- $\kappa$ B activation in the glycine group and a dramatic enhancement after 2 h of reperfusion in the control group.

**Glycine inhibits TNF- $\alpha$  mRNA expression** As NF- $\kappa$ B family members are known to control the expression of the TNF- $\alpha$  gene, TNF- $\alpha$  mRNA levels on reperfusion livers were also quantified using RT-PCR (Figure 7). Reperfusion markedly raised TNF- $\alpha$  mRNA levels, which reached a peak value 1 h after reperfusion. However, glycine significantly suppressed the elevation of TNF- $\alpha$  mRNA throughout the experimental period when compared with the control group ( $P < 0.05$ ).

## Discussion

The present study showed that a marked increase of LPS concentrations in portal vein persisted to the end of our study. Indeed, it is a ubiquitous phenomenon in liver transplantation for recipients of end-stage liver disease<sup>[5]</sup>. Furthermore, prospective organ donors may be exposed to various types of stress, such as trauma, hemorrhage or transient intestinal ischemia. As a result, translocation of gut-derived bacteria and LPS into body fluids is also frequent<sup>[19]</sup>. Substantial evidence indicates that recipients are susceptible to LPS with an increase in mortality in rat liver transplantation, and the dominating nosogenesis of LPS is to activate KC.

LPS acts as a potent stimulus for KC mainly through TLR signaling pathways, which results in the activation of transcription factors. LPS stimulation triggers TLR-4 aggregation that facilitates recruitment of myeloid differentiation factor 88 (MyD88) to LPS-receptor complexes via interac-

tions of the C-terminal TIR domains within these receptors and MyD88. MyD88 also interacts with IRAK-4 and IRAK-1 through its death domain, via the region of MyD88 situated between its C-terminal TIR and N-terminal death domains. Interaction of IRAK-4 with IRAK-1 induces IRAK-1 phosphorylation and activation, which also has been reported to facilitate recruitment of IRAK-1 to MyD88. Once hyperphosphorylated, IRAK-1 releases from the receptor complex and interacts with the TNF associated factor 6 complex (TRAF6) to trigger downstream signaling cascades, leading to activation of NF- $\kappa$ B and TNF- $\alpha$  expression. In contrast, in IRAK-4-deficient mice, the LPS signals mediated by TLR are severely impaired. Thus, IRAK-4, bridging the gap between the stimulated receptor complex and IRAK-1 activation, is considered to be a key mediator in the LPS signaling pathway<sup>[20–23]</sup>.

A number of experimental studies demonstrated that glycine has beneficial effects against LPS inducing hepatic injury. In the presence of glycine, a glycine-gated chloride channel is activated causing an influx of chloride, leading to hyperpolarization of cell membrane, which makes calcium channels on the plasma membrane more difficult to open and inhibits the influx of calcium. Moreover, the influx of chloride could also inactivate the IP<sub>3</sub>-gated calcium channels and block the release of calcium from intracellular stores. We reported here that pretreatment of glycine led to downregulation of IRAK-4 expression in reperfusion graft. It is well known that calcium acts as an intracellular second messenger, phosphorylation of MAP kinases and translocation of NF- $\kappa$ B to the nucleus were preceded by an increase in intracellular Ca<sup>2+</sup> levels after LPS stimulation<sup>[24]</sup>. As both NF- $\kappa$ B and MAP kinases are important mediators of intracellular LPS signal

transduction mediator and inflammatory cytokine gene expression, from this fact we could deduce that inactivation of MAP kinases and NF- $\kappa$ B also impaired the synthesis of IRAK-4 during hepatic I/RI. As a result, the downstream signaling cascades were also blunted, resulting in inactivation of NF- $\kappa$ B and depression TNF- $\alpha$  expression. TNF- $\alpha$  is a potent inflammatory cytokine mainly produced by the macrophage lineage. This cytokine exerts pleiotropic activities including the induction of adhesion molecules, complement activation, acute phase response, priming of the oxidative burst in granulocytes and promotes apoptotic or necrotic cell death in various cell types. There is increasing evidence indicating that TNF- $\alpha$  plays a key role in the initiation or progression of hepatic I/RI. Inhibition of TNF- $\alpha$  synthesis or activity attenuates this injury<sup>[25,26]</sup>. Being consistent with those reports, our current study also demonstrated that hepatic I/RI can be markedly reduced by glycine pretreatment with the inhibition of TNF- $\alpha$  synthesis, reflected by both plasma levels of AST, ALT, TB and liver histology.

In summary, we clearly demonstrated that glycine had a potent effect against LPS induced liver impairment caused by interference with the LPS signal transduction pathway through inhibition of IRAK-4 expression, prevention of NF- $\kappa$ B activation, and reduction of TNF- $\alpha$  during reperfusion phase. Given the impact of glycine on multiple factors critical to the LPS related I/RI<sup>[27]</sup>, it could be a novel compound for the development of an effective treatment for hepatic I/RI during liver transplantation.

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