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ORIGINAL ARTICLE

Remote ischemic preconditioning prevents lipopolysaccharide-induced liver injury through inhibition of NF-κB activation in mice

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Abstract
Purpose Remote ischemic preconditioning (RIPC) is a potent preconditioning stimulus that may confer subsequent protection to organs subjected to potentially lethal injury. The aim of this study was to investigate the effect of RIPC on nuclear factor (NF)-κB activation, tumor necrosis factor (TNF)-α release, and hepatic injury in lipopolysaccharide (LPS)-induced sepsis.
Methods This randomized experimental animal study was performed using 8-week-old mice weighing 35–40 g. Mice were randomized ($n = 13$ per group) to four groups. RIPC was induced with three 10-min cycles of hind limb ischemia by placing an elastic rubber band tourniquet on the proximal part of the limb, with each ischemia cycle followed by 10 min of reperfusion. The groups were treated as follows: (1) the control group received an injection of saline [intraperitoneally (i.p.)]; (2) the RIPC group was subjected to RIPC, followed immediately by an injection of saline (i.p.); (3) the LPS group received an injection of LPS (20 mg/kg, i.p.); (4) the RIPC/LPS group was subjected to RIPC, followed immediately by an injection of LPS (20 mg/kg, i.p.). TNF-α, NF-κB, and IκB-α levels, neutrophil accumulation, and microabscess formation in the liver were evaluated after LPS injection.
Results Among our treatment groups, RIPC significantly attenuated TNF-α release in response to endotoxin and inhibited NF-κB activation, neutrophil accumulation, and microabscess formation in the liver.
Conclusion The results demonstrate that RIPC has protective effects in liver injury via attenuation of TNF-α production in LPS-induced sepsis. The suppressive effect on TNF-α production may be mediated through inhibition of NF-κB activation.

Keywords Remote ischemic preconditioning · NF-κB · TNF-α · Sepsis

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Introduction

Severe sepsis and septic shock are associated with a very high mortality rate, making it a leading cause of death, especially in intensive care units. The mechanisms of severe sepsis and septic shock are complex and numerous [1–3]. One well-established mechanism activates the transcription of nuclear factor (NF)-κB. This factor is normally kept inactive through binding with its inhibitor, IκB, but in cells stimulated with proinflammatory substances, IκB is phosphorylated and subsequently degraded. NF-κB is then activated and translocated to the nucleus, where it facilitates the transcription of inflammation-associated target genes [4, 5]. NF-κB activation mediates the expression of numerous cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-1beta (IL-1β), IL-6, and IL-8 [2]. TNF-α expression is increased in the very early stages of inflammation, and this cytokine plays important roles in inflammation throughout the body [6].

The liver is a major plays a central role in the inflammatory responses to sepsis and endotoxemia [7]. In the liver, NF-κB activation induces an increase in the expression of acute phase proteins and proinflammatory cytokines [8]. This key role of NF-κB in the regulation of inflammation has led to it becoming the target of therapeutic agents for reducing tissue and organ injury.

The protective effect of ischemic preconditioning (IPC) in liver ischemia/reperfusion (I/R) injury is well-known [9]. Peralta et al. [10] demonstrated that IPC prevents the release of TNF-α from Kupffer cells during the reperfusion phase following hepatic ischemia. This attenuation of TNF-α production was associated with protection against liver injury.

Remote ischemic preconditioning (RIPC), first described in 1993 by Przyklenk et al. [11], has been reported to have a protective effect on I/R injury and also to down-regulate systemic inflammatory activation through significant modulation of neutrophil recruitment [12]. We hypothesized that RIPC attenuates TNF-α release in response to endotoxin treatment and that this effect could be modulated by inhibiting the activation of NF-κB. In this study, we investigated the effects of RIPC on TNF-α release and liver injury induced by endotoxin treatment. We also examined whether RIPC attenuates the activation of NF-κB in endotoxin-induced sepsis.

Materials and methods

Animals and the lipopolysaccharide-induced sepsis model

This study was approved by the Ethical Committee on Animal Research at the Faculty of Medicine, Korea University, Seoul, South Korea. All animals were treated humanely as described in the "Guide for the care and use of laboratory animals" issued by the Korea University School of Medicine. Eight-week-old male mice (Hanlim Co. Ltd., Hwasung, South Korea) weighing 35–40 g were maintained in a temperature-controlled environment and fasted (with access to water ad libitum) for 16 h before use in the study. The lipopolysaccharide (LPS)-induced sepsis model was established by injecting mice intraperitoneally (i.p.) with 20 mg/kg LPS (*Escherichia coli* O127: B8; Sigma, St. Louis, MO) dissolved in 0.5 mL of normal saline. In our preliminary study, serum TNF-α levels ($n = 5$) were measured at 0.5, 1, 2, 3, 4, 5, 6, 12, and 24 h after LPS injection for the time-course analysis. The expression of IκB-α and NF-κB activity in the liver ($n = 5$) was measured at 0.5, 1, 2, and 3 h after LPS injection. Liver histopathological findings ($n = 5$) were recorded at 0.5, 1, 2, 3, 4, 6, and 12 h after LPS injection. Based on the results of these preliminary studies, in subsequent analyses we measured the levels of serum TNF-α and liver IκB-α and NF-κB activity at 1 h after LPS injection and examined liver histopathological findings at 6 h after LPS injection.

Experimental protocols

Remote ischemic preconditioning was induced with three 10-min cycles of hind limb ischemia by placing an elastic rubber band tourniquet on the proximal part of the limb, with each ischemia cycle followed by 10 min of reperfusion. Mice were randomly assigned to one of the following four groups: (1) the control group ($n = 13$), where the mice received 0.9 % NaCl solution (0.5 mL, i.p.); (2) the RIPC group ($n = 13$), where the mice underwent RIPC, followed by injection of 0.9 % NaCl solution (0.5 mL, i.p.); (3) the LPS group ($n = 13$), where the mice were injected with LPS (20 mg/kg) dissolved in normal saline (0.5 mL, i.p.); (4) the RIPC/LPS group ($n = 13$), where the mice underwent RIPC, followed by injection of LPS (20 mg/kg, i.p.) (Fig. 1). The RIPC protocol was based on evidence that three 10-min cycles of I/R injury is more effective than a single cycle [13].

Cytokine immunoassays

After blood sampling via cardiac puncture, the blood samples were immediately mixed with an anticoagulant and then centrifuged for separation of the serum. Thereafter, samples were frozen until subsequent examination. TNF-α secretion was evaluated using an enzyme-linked immunosorbent assay (ELISA) (Komabiotech, Seoul, South Korea) sandwich method. Ninety-six-well plates were precoated with monoclonal antibodies specific to mouse TNF-α. The secreted factors were detected according to the manufacturer's protocol.

Western blot analysis of IκB-α and NF-κB

Cytoplasmic protein extracts from the liver tissue were heated in equal volumes of 2× sample buffer [250 mM Tris–HCl, pH 6.8, 4 % sodium dodecyl sulfate (SDS), 10 % glycerol, 2 % β-mercaptoethanol, and 0.003 % bromophenol blue] for 5 min at 99 °C. Protein samples (30 μg) were separated on denaturing 10 % SDS–polyacrylamide gels and transferred to a polyvinylidene fluoride membrane. The gels were stained with a Coomassie stain to confirm that equal amounts of proteins had been loaded. The membranes were blocked for 1 h with blocking buffer at room temperature (1× phosphate-buffered saline with 0.1 % Tween 20 and 5 % nonfat-dry milk), then washed and incubated overnight at 4 °C with a

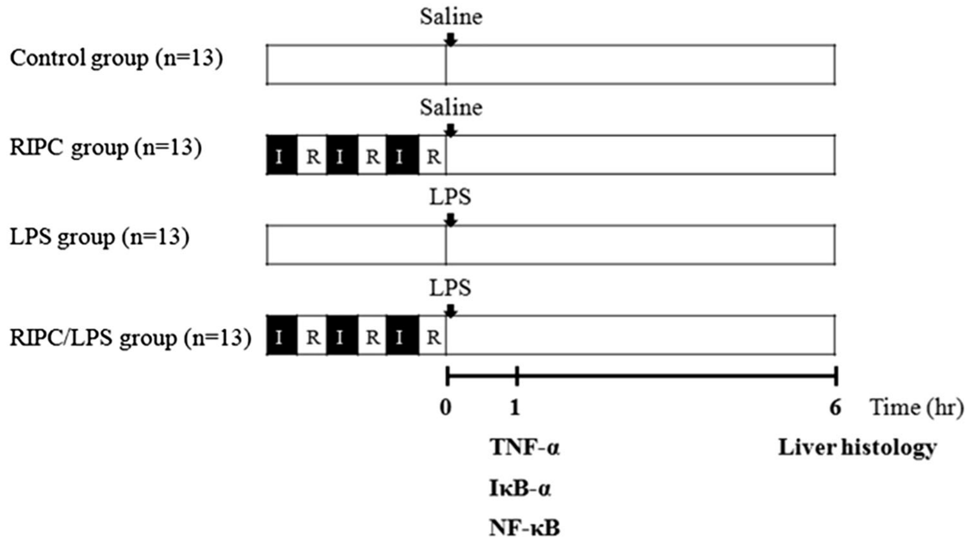

Fig. 1 Experimental protocol. The control group ($n = 13$) received 0.9 % NaCl solution [0.5 mL, intraperitoneally (i.p.)]. In the remote ischemic preconditioning (*RIPC*) group ($n = 13$), RIPC [three 10-min cycles of complete hind limb ischemia (*I*), each followed by 10 min of reperfusion (*R*)] was performed, followed by injection of 0.9 % NaCl solution (0.5 mL, i.p.). The lipopolysaccharide (*LPS*) group ($n = 13$) received LPS (20 mg/kg) dissolved in normal saline (0.5 mL, i.p.). In the RIPC/LPS group ($n = 13$), RIPC was performed, followed by injection of LPS (20 mg/kg, i.p.). *TNF-α* Tumor necrosis factor alpha, *NF-κB* nuclear factor κB, *IκB-α* inhibitor of NF-κB

polyclonal rabbit anti-IκB-α antibody (Abcam, Cambridge, UK; 1:2000 dilution) and a polyclonal rabbit anti-NF-κB p65 antibody (Abcam; 1:2000 dilution) in the blocking buffer. Next, the membranes were washed and incubated for 1 h with an appropriate secondary antibody at room temperature [anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (1:5000 dilution) in Tris-buffered saline with Tween 20 (TBS-T)], followed by six washes in TBS-T (5 min/wash). The membranes were then developed with the enhanced chemiluminescence system (Millipore Corp., Billerica, MA) according to the manufacturer's protocol and exposed to X-ray films.

NF-κB p65 activity assay

Cytoplasmic protein (10 μg) extracted from liver tissue was used for assessing NF-κB p65 activation by using the NF-κB p65 assay kit (Trans^{AM} p65; Active Motif, Carlsbad, CA) according to the manufacturer's instructions.

Histological examination

At 6 h after the LPS injection, the animals were sacrificed under anesthesia following i.p. injection of zoletil (20 mg/kg) for removal of the liver tissue. The right lobe of the liver was quickly removed, fixed with 10 % neutro-formalin, embedded in paraffin, and cut into 4-μm-thick sections. Tissue sections were then stained with hematoxylin and eosin or with naphthol AS-D chloroacetate esterase (Sigma) to assess neutrophil counts. Neutrophil accumulation was quantified by counting neutrophil in ten random high power field under the microscope at 400× magnification. Microabscess formation was quantified by counting microabscesses of the liver samples in ten random high power fields under the microscope at 400× magnification. Microabscesses were composed of several (>4) neutrophils and necrotic hepatocytes.

Statistical analysis

All data were expressed as the mean ± standard error of the mean. The Kruskal–Wallis test was used to compare cytokine levels in the four groups, and the Mann–Whitney *U* test was used for comparisons between twp independent groups. For all tests, a p value <0.05 was considered to be statistically significant. Analyses were performed utilizing SPSS, version 12.0 (SPSS®, Chicago, IL).

Results

RIPC inhibits TNF-α production in serum after LPS injection

Our preliminary analysis showed that TNF-α peaked at 1 h after LPS injection. Therefore, TNF-α levels were measured at this time in the four groups in subsequent experiments. Low-dose TNF-α was detected in the RIPC group

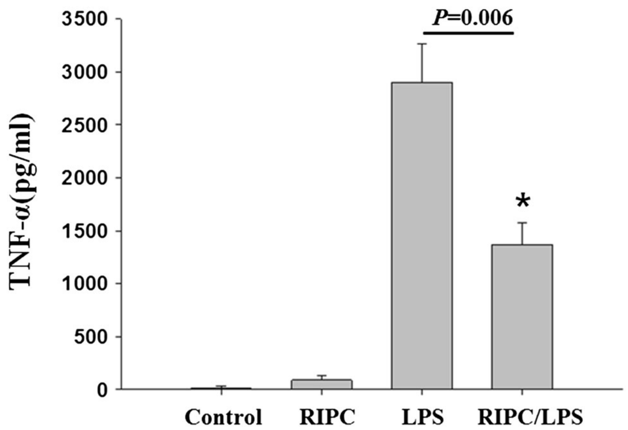

Fig. 2 Effects of RIPC on levels of TNF-α in mouse serum. Protein levels of TNF-α were analyzed in the serum of mice 1 h after LPS injection using an enzyme-linked immunosorbent assay (ELISA). The mice groups (treatments) are as defined in caption to Fig. 1. Data are expressed as mean ± standard error of the mean (SEM). $*p < 0.05$ vs. LPS group

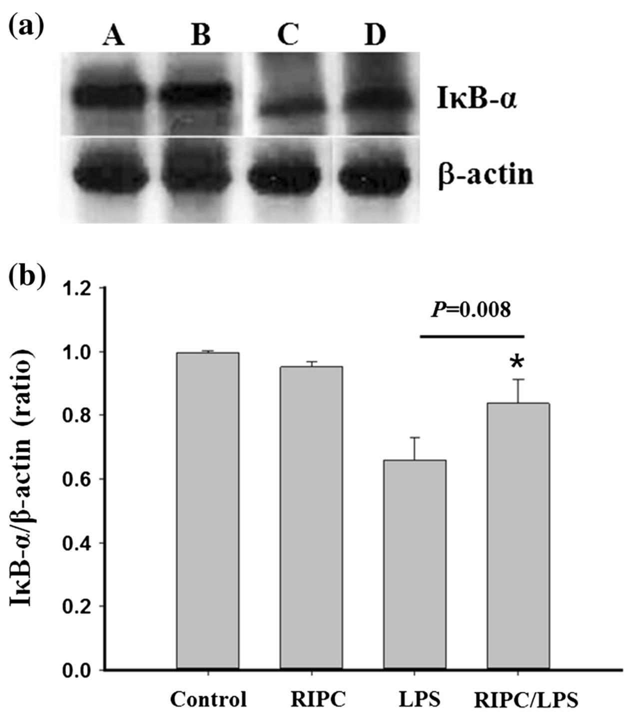

Fig. 3 RIPC inhibits LPS-induced IκB-α degradation in the liver. **a** Western blot analysis with an anti-IκB-α antibody was performed with cytoplasmic protein extracts from the liver tissue. Mice were treated with saline (control group, *A*), RIPC + saline (RIPC group, *B*), LPS (20 mg/kg, i.p.; LPS group, *C*), and RIPC + LPS (20 mg/kg, i.p.; RIPC/LPS group, *D*). **b** Densitometric analysis of the western blot of IκB-α levels in the liver. $*p < 0.05$ vs. LPS group

(Fig. 2), and serum TNF-α levels were significantly lower in the RIPC/LPS group than in the LPS group ($p = 0.006$). These data suggest that RIPC inhibits TNF-α production in serum after LPS injection.

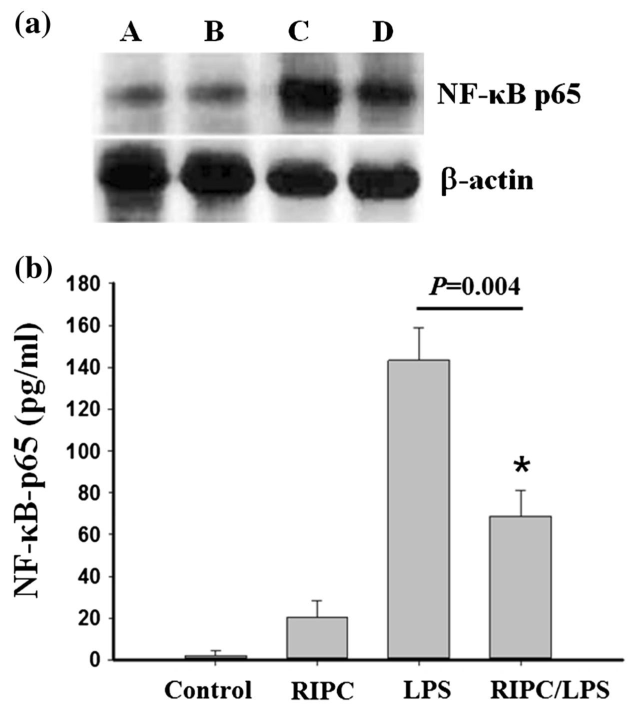

Fig. 4 RIPC inhibits LPS-induced NF-κB activation in the liver of mice. **a** Western blot analysis with an anti-NF-κB p65 antibody was performed with cytoplasmic protein extracts from liver tissue. Mice were treated with saline (control group, *A*), RIPC + saline (RIPC group, *B*), LPS (20 mg/kg, i.p.; LPS group, *C*), and RIPC + LPS (20 mg/kg, i.p.; RIPC/LPS group, *D*). **b** Quantification of total p65 levels in liver tissues. The levels of activated NF-κB, as assessed by the total p65 levels, were analyzed using ELISA. $*p < 0.05$ vs. LPS group

RIPC inhibits LPS-induced IκB-α degradation in the liver

The preliminary western blot analysis of cytoplasmic fractions revealed a significant decrease in the intensity of the IκB-α band 1 h after LPS injection. Therefore, in subsequent studies IκB-α expression levels were measured at this time (Fig. 3a). The ratio of IκB-α/β-actin in the liver was significantly higher in the RIPC/LPS group than in the LPS group ($p = 0.008$) (Fig. 3b), suggesting that RIPC inhibits LPS-induced IκB-α degradation.

RIPC inhibits LPS-induced NF-κB activation in the liver

The preliminary analysis showed that NF-κB activity in the liver peaked 1 h after LPS injection. Therefore, in subsequent studies we measured NF-κB activity at 1 h in all four groups. The small preactivation of NF-κB following repeated limb reperfusion was measured in the RIPC group (Fig. 4b), and the results revealed that the intensity of the NF-κB band had decreased in the RIPC/LPS group compared with the LPS group (Fig. 4a). Total p65 levels,

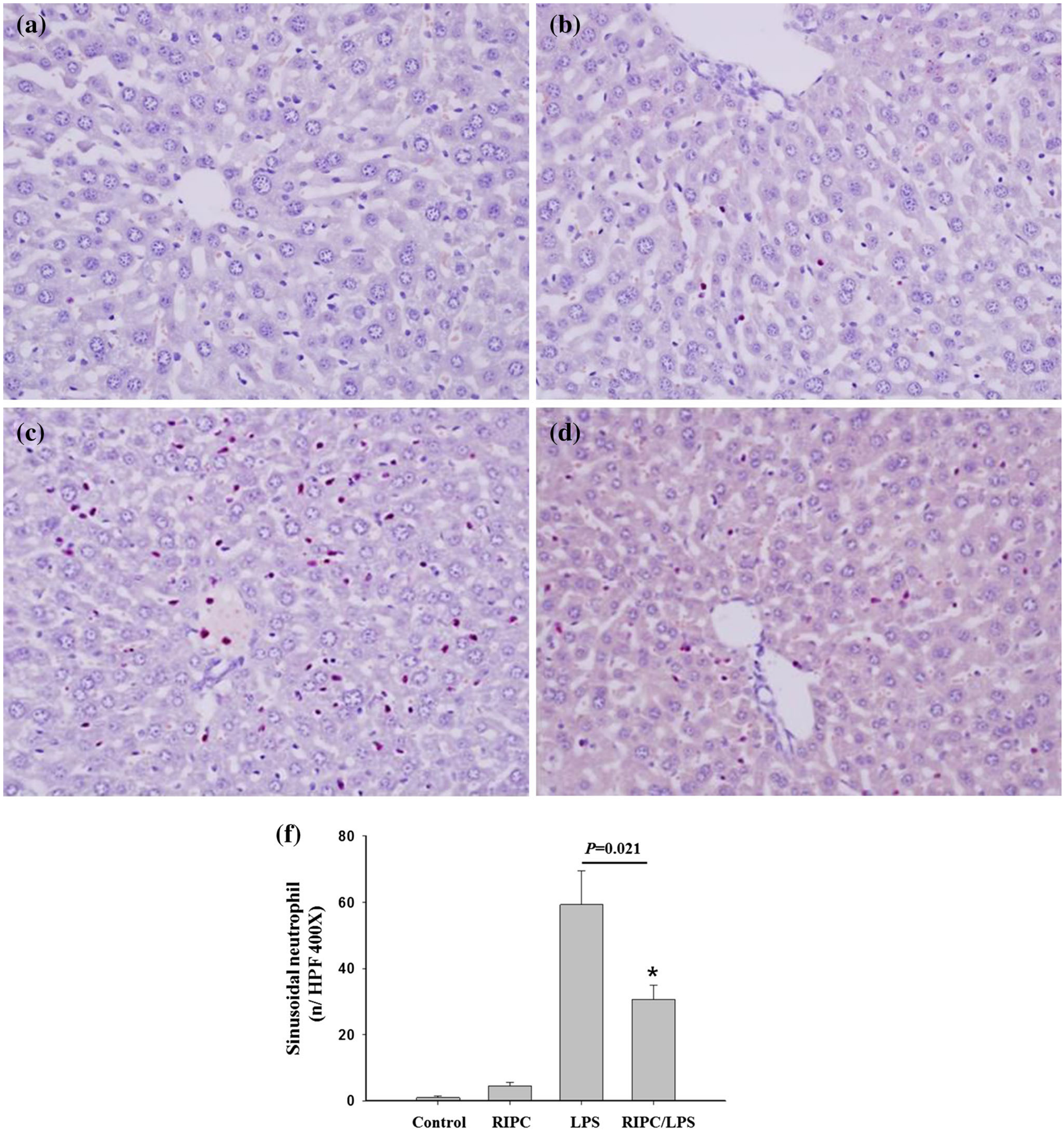

Fig. 5 RIPC prevents LPS-induced hepatic neutrophil accumulation. The liver sections were subjected to naphthol AS-D chloroacetate esterase staining in order to determine the prevalence of *red-colored* esterase-stained neutrophils in the liver. **a**–**d** Representative naphthol AS-D chloroacetate esterase-stained liver tissue sections of mice that were treated with saline (control group, **a**), RIPC + saline (RIPC group, **b**), LPS (20 mg/kg, i.p.; LPS group, **c**), and RIPC + LPS (20 mg/kg, i.p.; RIPC/LPS group, **d**). Magnification ×400. **e** Intrahepatic sinusoidal neutrophils were quantified by counting neutrophils in ten random high power field under the microscope (magnification ×400). Data are expressed as mean ± SEM. *$p < 0.05$ vs. LPS group

indicative of activated NF-κB, exhibited a significant decrease in the RIPC/LPS group compared with the LPS group ($p = 0.004$) (Fig. 4b). Together, these data suggest that RIPC inhibited the LPS-induced NF-κB activity.

RIPC prevents LPS-induced liver injury

The preliminary analysis showed that liver injury peaked 6 h after LPS injection. Therefore, histological findings were

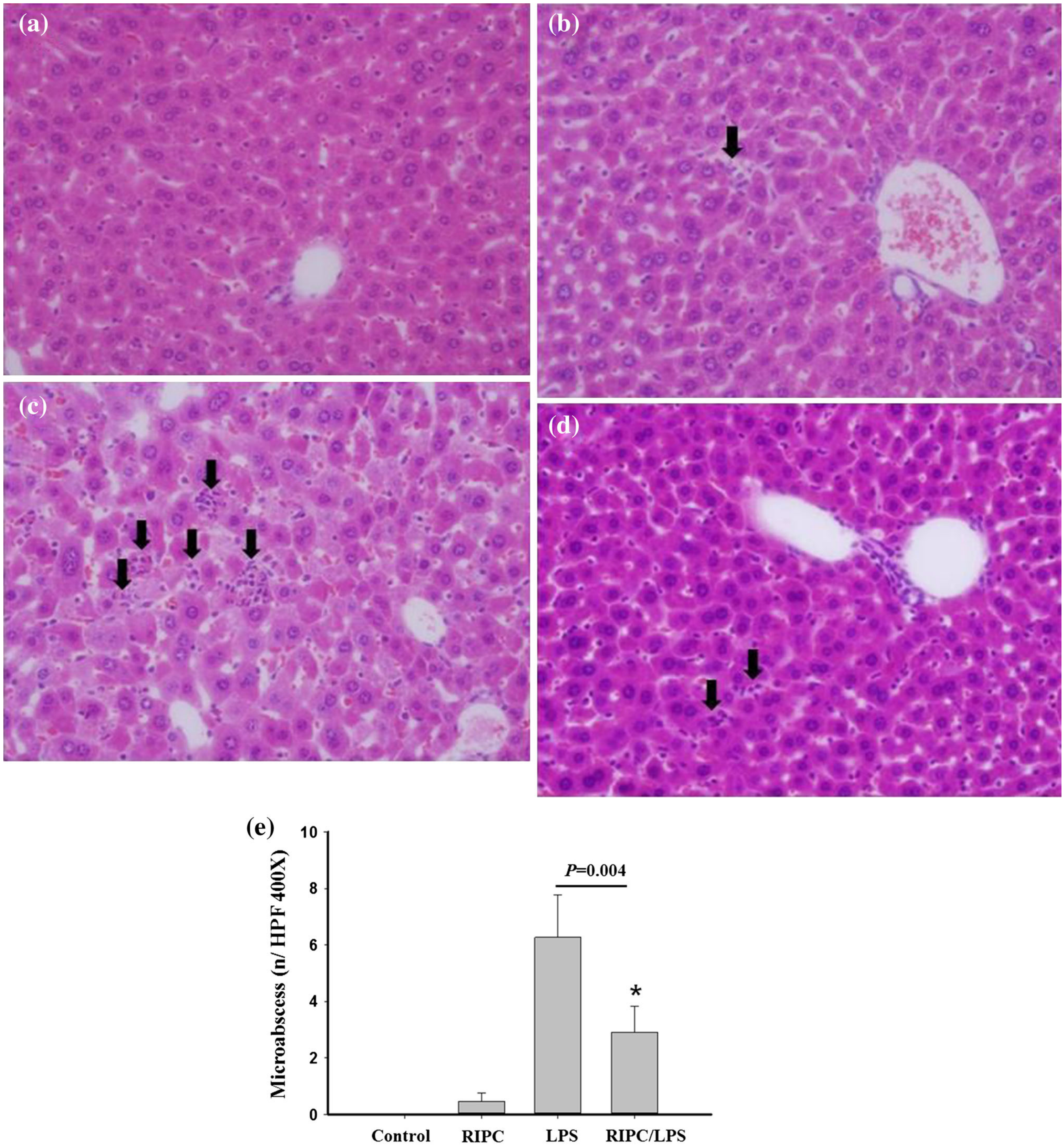

Fig. 6 RIPC prevents LPS-induced microabscess formation in the liver. Representative hematoxylin and eosin-stained liver tissue sections of mice that were treated with saline (control group, **a**), RIPC + saline (RIPC group, **b**), LPS (20 mg/kg, i.p.; LPS group, **c**), and RIPC + LPS (20 mg/kg, i.p.; RIPC/LPS group. **d**). *Arrows* Microabscesses in the liver (magnification ×400). **e** The extent of liver injury was quantified by counting microabscesses of the liver in 10 random high power fields under the microscope at ×400 magnification. Microabscesses were composed of several (>4) neutrophils and necrotic hepatocytes. Data are expressed as mean ± SEM. *$p < 0.05$ LPS group

examined at this time in the four groups. In esterase-stained slides, neutrophil accumulation within the sinusoid of the liver was reduced in the RIPC/LPS group compared with that in the LPS group (Fig. 5c, d). The intrahepatic sinusoidal neutrophils were quantified by counting neutrophils, and the data showed that RIPC induced a statistically significant reduction in neutrophil accumulation in the RIPC/LPS group compared with that in the LPS group ($p = 0.021$) (Fig. 5e).

In hematoxylin and eosin-stained slides, no histological alterations in the liver specimens were observed in the control group (Fig. 6a). However, in the LPS group, severe pathologic abnormalities were detected, including microabscesses consisting of infiltrating neutrophils and necrotic hepatocytes (Fig. 6c). These inflammatory changes were reduced in the RIPC/LPS group compared with the LPS group (Fig. 6d). Quantification of liver injury by counting microabscesses showed that RIPC induced a statistically significant reduction in the microabscess formation in the RIPC/LPS group compared with the LPS group ($p = 0.004$) (Fig. 6e).

Discussion

The data of our study demonstrate that the RIPC stimulus achieved by transient hind limb ischemia prevented LPS-induced liver injury in a mouse model. To our knowledge, this is the first report of the downregulation of NF-κB mediated by RIPC during endotoxemia in the liver.

RIPC is an interesting form of IPC, with potentially greater clinical significance. Transient tissue ischemia, such as that induced during RIPC, at a distant area may offer subsequent protection of organs subjected to potentially lethal ischemia. In a porcine model, Kharbanda et al. [14] demonstrated that RIPC induced a 50 % reduction in myocardial infarction.

TNF-α plays important roles in inflammation throughout the body [6]. However, low-dose TNF-α has been implicated in the development of ischemic tolerance [15, 16]. Teoh et al. [17] reported that low-dose TNF-α protects against hepatic I/R injury in mice. In their study, TNF-α pretreatment and ischemic preconditioning had similar effects on IκB-binding proteins and nuclear binding of NF-κB. Following the ischemic preconditioning stimulus, there was an early rise in hepatic and serum TNF-α levels, with subsequent protection against hepatic I/R injury [17]. In our study, RIPC produced low-dose TNF-α in the serum (Fig. 2). The protective effect of RIPC on hepatic injury may actually be due to the release of low-dose TNF-α preformed during limb reperfusion. We suggest that in our mouse model, low-dose TNF-α released by antecedent hind limb I/R inhibited LPS-induced IκB-α degeneration and subsequently inhibited hepatic NF-κB activation, thereby decreasing the systemic TNF-α level and ultimately resulting in liver protection.

NF-κB is also involved in ischemic preconditioning [18]. In a mouse model, Li et al. [19] demonstrated that RIPC significantly reduces the myocardial infarct size by stimulating the expression of NF-κB proteins in both limb skeletal muscle and myocardium. These authors also suggested that this mechanism may also induce protective signals in the limb that are then transferred to the heart, leading to ischemic adaptation. However, during the pathogenesis of sepsis, NF-κB is an important transcription factor and is known as a nuclear protein critical for controlling the expression of inflammation-associated factors [4]. Many researchers have studied the role of NF-κB activation during sepsis, leading to the suggestion that NF-κB activation is an early step in the pathogenesis of sepsis-induced organ injury [20, 21]. A dual role for NF-κB was demonstrated in the heart by Li et al. [19]; although excessive NF-κB activation in I/R injury had harmful effects, activation of NF-κB following limb preconditioning led to an adaptive response following sustained I/R injury. In their study, preconditioning of the hind limbs caused activation of NF-κB in both the I/R hind limbs and in the heart. When preconditioning was performed in mice with targeted deletions of the NF-κB, no adaptation to ischemia was found [19]. In our study we observed a low level of NF-κB preactivation following repeated limb reperfusion in the RIPC group (Fig. 4b). This low level of NF-κB preactivation increased IκB-α expression, which in turn attenuated NF-κB activation following LPS-induced sepsis, thereby reducing the production of TNF-α. The ultimate result was protection of the liver. The precise molecular mechanism of TNF-α- and NF-κB-induced changes in RIPC is unclear, and further studies are needed to study the mechanism and signaling pathways involved in this process.

The liver is a primary site for clearance of bacteria and bacterial products from the blood, and it plays an active role in the inflammatory response to endotoxemia and sepsis by producing proinflammatory cytokines such as TNF-α, chemokines, and adhesion molecules [7, 8]. Most of these mediators are typically controlled, at least in part, by NF-κB at the transcriptional level [22]. TNF-α is regarded as the most important early proinflammatory cytokine and has been reported to be responsible for LPS-induced liver injury [23, 24]. Increased TNF-α levels contribute to leukocyte recruitment in response to bacterial infection [25]. Fox-Robichaud and Kubes [26] demonstrated TNF-α stimulated leukocyte recruitment into murine hepatic circulation. Within the septic liver, infiltration by neutrophils contributes to significant hepatocellular damage, vascular hypoperfusion, and ultimately organ dysfunction [7]. Therefore, understanding the molecular mechanisms of neutrophil recruitment within the liver may help reveal new therapeutic strategies to prevent immune-mediated organ dysfunction during severe sepsis. In our study, RIPC suppressed LPS-induced production of TNF-α, neutrophil accumulation, microabscess formation, and liver injury in the RIPC/LPS group compared with that in the LPS group. We suggest that in our model TNF-α was typically controlled by NF-κB at the transcriptional level in the liver.

We note that this study has a number of limitations. First, RIPC was performed without any anesthesia, which could be psychologically stressful to the mice and thus result in an enhanced inflammatory response. Second, we evaluated the anti-inflammatory effects of RIPC in a mouse model of LPS-induced sepsis, which may be different from the response to sepsis in humans. Further studies addressing any potential differences in the inflammatory responses induced by RIPC with and without anesthesia will help clarify the effect of anesthesia, if any.

In conclusion, we have shown that RIPC can reduce LPS-induced liver injury through inhibition of NF-κB activation in mice. These effects are associated with a reduction of TNF-α production and hepatic neutrophil accumulation, and the prevention of LPS-induced liver injury. Our results suggest that RIPC would be effective in the clinical setting as well. RIPC thus shows potential as a novel treatment for liver injury during sepsis. Further studies are needed to identify the exact mechanisms responsible for the RIPC-mediated prevention of TNF-α production and hepatic injury and to determine whether such protection will yield an improved clinical outcome in patients with sepsis.

Conflict of interest None.

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