Augmenter of liver regeneration ameliorates ischemia-reperfusion injury in steatotic liver via inhibition of the TLR4/NF-κB pathway

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Abstract. Hepatocytes from donors with preexisting hepatic steatosis exhibited increased sensitivity to ischemia-reperfusion injury (IRI) during liver transplantation. Augmenter of liver regeneration (ALR) protected the liver against IRI, but the mechanism was not clarified. Therefore, the hypothesis that ALR attenuated IRI in steatotic liver by inhibition of inflammation and downregulation of the Toll-like receptor 4 (TLR4)/nuclear factor-κB (NF-κB) pathway was examined. C57BL/6 mice were subjected to a methionine-choline-deficient (MCD) diet to induce liver steatosis. Mice were transferred with ALR-containing adenovirus 3 days prior to partial warm hepatic IRI. After 30 min of ischemia and 6 h of reperfusion injury, liver function, hepatic injury, the inflammatory response and TLR4/NF-κB signaling pathway activation were assessed. ALR maintained liver function and alleviated hepatic injury as indicated by the decreased levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), preserved hepatic structure and reduced apoptosis. ALR also reduced the IRI-induced inflammatory response by suppressing Kupffer cell activation, inhibiting neutrophil chemotaxis and reducing inflammatory cytokine production. Further investigation using reverse transcription-quantitative PCR, western blotting and immunohistochemistry revealed that ALR reduced TLR4/NF-κB signaling pathway activation, which led to a decreased synthesis of inflammatory cytokines. ALR functioned as a regulator of the IRI-induced inflammatory response by suppressing the TLR4/NF-κB pathway, which supports the use of ALR in therapeutic applications for fatty liver transplantation.

Introduction

Hepatic ischemia-reperfusion injury (IRI) is the phenomenon in which hepatocellular damage is aggravated after the reperfusion of blood flow in the liver (1). Hepatic IRI occurs in numerous clinical situations, such as liver transplantation, liver resection, trauma, shock and hemorrhage (2,3). The incidence of nonalcoholic fatty liver disease (NAFLD) has markedly increased and is currently estimated at approximately 20-30% in the general population and up to 70-80% in the obese population (4). An increasing trend of IRI in fatty liver is predicted in the near future (3). Clinical studies and animal experiments revealed that steatotic livers are particularly susceptible to IRI (5). Fatty liver grafts are associated with a primary non-function rate of 60% compared to 5% for non-steatotic grafts and increase the risk of postoperative morbidity and mortality after fatty liver surgery (6,7). However, there are few effective methods to reduce the susceptibility of fatty liver to IRI. Recent studies demonstrated that inflammation played an important role in the process of fatty liver IRI, including more obvious inflammatory cell infiltration and inflammatory factor release (8-10).

Toll-like receptors (TLRs) form a part of the host innate immune system that triggers the innate immune response and production of inflammatory mediators (11). TLR4 is a key TLR family member that is an essential participant in hepatic IRI. TLR4 is ubiquitously expressed in hepatocytes and nonparenchymal cells (12,13). TLR4 activation initiates signal transduction pathways. Then, transcription factors such as NF-κB are activated and cytokines are produced (14). TLR4-deficient mice have decreased levels of inflammatory cytokines and are significantly protected against hepatic IRI (15).

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Abbreviations: ALR, augmenter of liver regeneration; IRI, ischemia-reperfusion injury; MCD, methionine-choline-deficient; TLR4, Toll-like receptor 4; NF-κB, nuclear factor-κB; MPO, myeloperoxidase; F4/80, mouse EGF-like module-containing mucin-like hormone receptor-like 1

Key words: augmenter of liver regeneration, ischemia-reperfusion injury, Toll-like receptor 4, NF-κB, fatty liver
Augmenter of liver regeneration (ALR), also referred to as hepatic stimulator substance (HSS), was first identified for its promotion of liver regeneration after partial hepatectomy (16). ALR plays an important role in protecting hepatocytes against various injuries by reducing endoplasmic reticulum stress and improving mitochondrial function (17,18). A previous study has revealed that ALR also reduced the inflammatory response. ALR reduced the infiltration of CD4+ T cells and neutrophils to alleviate normal liver IRI (19). However, the relationship between ALR and the inflammatory response in fatty liver IRI and the underlying mechanisms remain unclear. It was hypothesized that ALR protects against fatty liver IRI via inhibition of the TLR4/NF-κB pathway to reduce inflammatory cell infiltration and inflammatory factor release.

Materials and methods

Animals and model of fatty liver IRI. Experiments were performed on male C57BL/6 mice (age ~6-8 weeks; weight ~18-22 g) that were purchased from the Academy of Military Sciences (Beijing, China). All animals were treated humanely according to the Guidelines for Animal Care and Use in Medical Research (20) and the experimental methods are approved by The Ethics Committee of Capital Medical University (Beijing, China; approval no. AEEI-2016-094). A total of 30 male mice were housed at a constant room temperature (22-25°C), a relative humidity of 40-60% under a 12/12 h light-dark cycle with access to water and food ad libitum. Mice were fed a methionine-choline-deficient (MCD) diet (Beijing HFK Bioscience Co., Ltd.) for 2 weeks, and then underwent warm liver IRI (21,22), while the control mice were fed an isocaloric control diet. Briefly, mice were anesthetized with 4% chloral hydrate intraperitoneally (320 mg/kg), and the artery/portal vein blood flow to the left and middle lobes of the liver was temporarily interrupted using an atraumatic clip, which induced ~70% liver ischemia. Liver ischemia was maintained for 30 min, and the clip was loosened to allow reperfusion for 6 h. Mice in the sham-operated group underwent the same surgical procedure without clamping of the hepatic vessels. At the end of the experiments, mice were sacrificed by inhalation of anesthetics in a closed chamber (e.g. an overdose of 5% isoflurane). Mice continued to be exposed to isoflurane until they stopped breathing for 1 min, and then they were removed from the chamber to confirm death through the absence of a heartbeat and nerve reflexes. Blood samples were collected from the inferior vena cava, and liver tissues were prepared for further analysis.

Gene transfer. Human ALR cDNA was cloned into a replication-deficient adenoviral vector with the flag tag as previously described (21). Three days before IRI, mice subjected to an MCD diet were randomly divided into 4 groups: 3 groups were injected with Adnull (control virus, 1x10⁹ PFU/mouse), AdALR (1x10⁹ PFU/mouse) or normal saline (NS) via the tail vein. Sham-operated mice served as controls.

Biochemical assays and triglyceride determination. The activities of serological alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and the concentrations of serological triglyceride (TG) were analyzed using an automatic biochemical analyzer (Hitachi, Ltd.) at the Clinical Laboratory Center of Capital Medical University. The TG content in liver tissue was determined using a TG kit (cat. no. K622; Biovision, Inc.) according to the manufacturer's protocol.

Enzyme-linked immunosorbent assay (ELISA). The levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) in murine serum were assessed using commercially available ELISA kits (cat. nos. MLB00C, M6000B, MTA00B, respectively; R&D Systems, Inc.) according to the manufacturer's protocol based on the quantitative sandwich enzyme immunoassay technique. Briefly, 50 µl of murine serum was incubated in ELISA plates at room temperature for 2 h. After washing with washing buffer, 100 µl of conjugated antibody was added and incubated at room temperature for another 2 h. The substrate solution was added and incubated for 20 min in the dark, followed by the addition of a stopping solution. The optical density (OD) was measured at 450 nm, and the concentrations of the cytokines were calculated by reference to the standard curves.

Histology and Oil Red O staining. Liver tissues from the ischemic lobes were fixed in 10% buffered formalin for 24 h at room temperature and embedded in paraffin. For hematoxylin and eosin (H&E; 200X) staining, sections of 5-µm thickness were stained in hematoxylin for 5 min at room temperature, then transferred to 1% hydrochloric acid alcohol for differentiation and washed in running water for 2 min. After soaking in the eosin stain for 3 min at room temperature, sections were washed in running water. The Suzuki score was used to evaluate liver injury based on 3 indices: sinusoidal congestion, hepatocyte necrosis and ballooning degeneration (23). To evaluate the intracellular lipid distribution in the liver, liver samples were fixed in 4% paraformaldehyde for 24 h at room temperature frozen in liquid nitrogen and sectioned into 6-µm slices. Sections were stained in Oil Red O solution for 15 min at room temperature, rinsed with 60% isopropanol, stained with hematoxylin at room temperature for 30 sec, and images were captured using light microscopy (magnification, x100).

Immunohistochemistry. Immunohistochemical staining was performed as previously described (21). Briefly, the liver tissue sections (5-µm) were dewaxed, hydrated and pretreated using the heat-induced antigen retrieval technique. Sections were performed as previously described (21). Briefly, the liver tissue sections (5-µm) were dewaxed, hydrated and pretreated using the heat-induced antigen retrieval technique. Sections were treated sequentially with a 3% H₂O₂ solution and 5% BSA to block endogenous peroxidase activity and nonspecific antibody binding, respectively. Primary antibodies directed against ALR (1:500; cat. no. 11293-1-AP; Proteintech Group, Inc.), mouse EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80; 1:200; cat. no. ab6640; Abcam), myeloperoxidase (MPO; 1:200; cat. no. ab90810; Abcam), proliferating cell nuclear antigen (PCNA; 1:500; cat. no. 13110; Cell Signaling Technology, Inc.), TLR4 (1:100; cat. no. sc-293072; Santa Cruz Biotechnology, Inc.) and NF-κB p65 (1:200; cat. no. 8242; Cell Signaling Technology, Inc.) were incubated with the sections overnight at 4°C, followed by HRP-conjugated IgG incubation (1:200; cat. no. SA0001-2; Proteintech Group, Inc.) Sections were visualized with diaminobenzidine (DAB) and counterstained with hematoxylin. The positively stained areas and positive cells were evaluated using

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ALR expression is downregulated during IRI in steatotic liver. After 2 weeks of MCD diet feeding, mice were subjected to hepatic IRI, i.e., 30 min of ischemia followed by 6 h of reperfusion. The expression of endogenous ALR was evaluated using western blot analysis. The results demonstrated that IRI decreased the expression of ALR (Fig. 1A), which indicated that ALR is involved in IRI. Adenovirus-mediated gene transfer of ALR was performed in mice 3 days before IRI. Exogenously delivered ALR was expressed efficiently within the liver, as determined by western blotting (Fig. 1B) and immunostaining (Fig. 1C). Mice fed the MCD diet exhibited elevated TG levels in liver tissues and mild-to-moderate hepatic steatosis, as evidenced by Oil Red O staining (P<0.01; Fig. 1E and F). Adenovirus-mediated ALR gene transfer did not alter TG levels in the blood (Fig. 1D).

ALR expression maintains liver function and attenuates IRI in steatotic liver. Hepatocellular function was evaluated by measuring serum ALT and AST levels. These levels were significantly decreased following IRI in the AdALR group compared with the Adnull group (serum ALT: 395±42 vs. 1163±138 U/l, P<0.01; serum AST: 539±69 vs. 1214±145 U/l, P<0.01) (Fig. 2A and B). The liver histology was associated with the serum indices. IRI significantly induced hepatic damage, as indicated by the severe sinusoidal congestion, hepatocellular necrosis and cytoplasmic vacuolization. However, hepatic damage was significantly reduced in the AdALR group compared with the Adnull group and the NS group (Fig. 2C). The Suzuki score was used for histological evaluation of hepatic injury, and these scores also confirmed the protective role of ALR against hepatic IRI (P<0.05; Fig. 2D). A TUNEL assay was performed to evaluate cellular apoptosis. Few TUNEL-positive cells were observed in the sham group, but significantly increased numbers were observed in the Adnull and NS groups after hepatic IRI. Conversely, the number of TUNEL-positive hepatocytes was decreased in the AdALR group compared with the NS and Adnull groups, which indicated that ALR expression protected hepatocytes from apoptosis.

Statistical analysis. The experimental results are expressed as the means ± standard deviations (SDs) of three independent experiments. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was performed using GraphPad Prism 6 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.
against IRI-induced cell death (P<0.01; Fig. 2E and F). The hepatocyte proliferation is demonstrated in Fig. 2G. The number of the proliferating cells in the ALR-transfected mice were increased compared with the Adnull and NS mice, which indicated that ALR enhanced cell growth in the liver after IRI (P<0.01; Fig. 2G and H).

**ALR expression decreases the infiltration of inflammatory cells and the production of inflammatory mediators.** Kupffer cell (KCs) activation, as indicated by F4/80 staining, was the early inflammatory response during hepatic IRI. The F4/80-positive area was significantly increased in the IRI groups, and the activation of KCs was decreased in AdALR mice compared with Adnull mice after IRI (P<0.01; Fig. 3A). MPO is abundantly expressed in neutrophils and is used as a marker of neutrophil infiltration (25). The number of MPO-positive cells was increased following IRI, and neutrophil infiltration in IRI-induced fatty livers was effectively suppressed in the AdALR group compared with the Adnull and NS groups (P<0.01; Fig. 3B). Inflammatory cytokines play key roles in the pathophysiology of hepatic IRI (26). The serum levels of IL-1β, IL-6, and TNF-α were assessed using ELISA (Fig. 3C-E). After IRI, serum IL-1β, IL-6 and TNF-α levels were significantly increased in the IRI groups compared with the sham-operated group but were significantly decreased in the AdALR group compared with the Adnull and NS groups (P<0.05).

**ALR modulates the TLR4/NF-κB signaling pathway during IRI in steatotic liver.** TLR4 is ubiquitously expressed in the liver, and it initiates the sterile inflammatory response following IRI. TLR4 activation eventually leads to the activation and nuclear translocation of NF-κB (27). To further understand the inflammation-regulatory role of ALR, RT-qPCR was used to observe the expression of TLR4 and NF-κB p65 in hepatic IRI. The mRNA levels of TLR4 and NF-κB in the NS and Adnull groups after IRI compared with the sham-operated group. However, the mRNA levels of TLR4 and NF-κB in the AdALR group were significantly decreased compared with the NS and Adnull groups (P<0.01; Fig. 4A and B). ALR expression inhibited the expression of TLR4 and NF-κB p65 after IRI, as demonstrated by western blot analysis (P<0.05 and P<0.01, respectively; Fig. 4C and D). The nuclear fraction was extracted to detect the nuclear distribution of NF-κB. The results revealed that the levels of nuclear NF-κB p65 were significantly increased in the NS and Adnull groups compared with the sham-operated group. However, ALR transfection decreased the nuclear NF-κB p65 (P<0.05; Fig. 4E) compared with the NS and Adnull groups. Immunohistochemistry was also used to detect the expression of TLR4 and NF-κB during hepatic IRI. After IRI, the expression levels of these proteins were significantly increased in the NS and Adnull groups compared with the sham-operated group but were significantly decreased in the AdALR group (P<0.01; Fig. 4F and G) compared with the NS and Adnull.
groups. These results indicated that ALR suppressed the TLR4 inflammatory signaling cascade during hepatic IRI.

Discussion

The present study evaluated the effects of ALR on the inflammatory response during fatty liver IRI. The key findings are summarized as follows: i) ALR expression was reduced in steatotic livers during IRI; ii) adenovirus-mediated ALR transfection maintained liver function and alleviated IRI in steatotic livers by suppressing inflammatory cell infiltration and reducing inflammatory cytokine production; and iii) ALR played a regulatory role in the inflammatory response during fatty liver IRI via inhibition of the TLR4/NF-κB signaling pathway.

Although ALR was initially reported as a mitogenic factor that promoted liver regeneration after partial hepatectomy in rats, substantial evidence has revealed that ALR was a survival factor that protected the liver in the clinical context against damaging agents, such as ethanol, carbon tetrachloride, d-galactosamine and acetaminophen-induced liver injury (18, 28). Our previous research revealed that ALR
Figure 3. ALR administration inhibits the inflammatory response and decreases proinflammatory cytokines in steatotic livers after IRI. (A) Immunohistochemical staining of Kupffer cells was performed using an anti-F4/80 antibody in liver sections. (B) Neutrophil infiltration in liver tissues was detected using an anti-MPO antibody. (C-F) The serum levels of IL-1β, IL-6 and TNF-α were assessed using ELISA. *P<0.05 and **P<0.01; n=6 per group. ALR, augmenter of liver regeneration; IRI, ischemia-reperfusion injury; F4/80, EGF-like module-containing mucin-like hormone receptor-like 1; MPO, myeloperoxidase; IL, interleukin; TNF, tumor necrosis factor; NS, normal saline.

Figure 4. ALR administration inhibits the TLR4/NF-κB signaling pathway in steatotic livers after IRI. C57BL/6 mice were fed a methionine-choline-deficient diet for 2 weeks then subjected to 30 min of ischemia followed by 6 h of reperfusion. (A and B) The mRNA levels of TLR4 and NF-κB p65 were measured using reverse transcription-quantitative PCR, and 18S rRNA was used as the internal control. (C and D) The protein expression of TLR4 and NF-κB p65 was measured using western blotting. (E) The nuclear distribution of NF-κB p65 was detected by western blotting. (F and G) TLR4 and NF-κB levels were evaluated in liver sections using immunohistochemical staining. *P<0.05 and **P<0.01; n=6 per group. ALR, augmenter of liver regeneration; TLR4, Toll-like receptor 4; NF-κB, nuclear factor-κB; IRI, ischemia-reperfusion injury; NS, normal saline.
alleviated IRI in fatty livers by promoting mitochondrial function, reducing oxidative stress and enhancing antioxidant activity (21). Previous studies reported that ALR played an important role in the formation of fatty liver. Decreased ALR expression promoted fat accumulation in the liver of mice, and the ALR level in the serum of patients with steatohepatitis was also significantly reduced (29,30). However, the regulatory effects of ALR on the inflammatory response in fatty liver IRI are not clear.

The expression of ALR after IRI in fatty livers was initially examined and it was revealed that IRI decreased ALR expression, which indicated that ALR depletion during fatty liver IRI was associated with aggravated liver injury. Adenovirus-mediated ALR transfection was used to detect the gain of function of ALR in fatty liver IRI. Our results indicated that ALR maintained liver function and alleviated liver injury, as indicated by the decreased ALT and AST levels, preserved hepatic structure, reduced apoptosis and increased hepatocyte proliferation.

Inflammation plays pivotal roles in hepatic IRI, especially in steatotic livers (31,32). The cytosolic components and cell fragments released from the damaged liver trigger the production of IL-1β, IL-6 and TNF-α, which are key proinflammatory mediators of hepatic IRI (22). KCs are liver-resident macrophages that become activated and release inflammatory mediators during the early phase of IRI. Neutrophils and monocytes migrate to and accumulate in the injured liver at later phases, which contribute to the additional, prolonged injury. An anti-inflammatory strategy of modulating the exaggerated inflammatory response to protect the liver against IRI was reported in numerous studies (33,34).

ALR expression reduced the serum levels of inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, in the present study. Activation of KCs and neutrophil recruitment, as evaluated by staining for F4/80 and MPO, respectively, were inhibited by ALR expression in fatty liver IRI. Consistent with our results, Khandoga et al used intravital fluorescence microscopy and observed that ALR expression decreased the number of rolling and firmly adherent leukocytes in post-sinusoidal venules in normal hepatic IRI (19).

TLR4 is extensively expressed in liver cells, such as KCs and hepatocytes (13), and it is closely related to the development of hepatic IRI (35,36). TLR4 triggers the activation and nuclear translocation of the transcription factor NF-κB, which culminates in the transcription of inflammatory cytokine genes (37). Blockade of TLR4 alleviates liver IRI by reducing neutrophil sequestration, CD4+ T cell infiltration, and cytokine and chemokine production (36,38).

The anti-inflammatory effects of ALR were demonstrated previously. Yan et al reported that ALR mitigated acute renal IRI in rats by reducing the protein expression of NF-κB, but the mechanism was not clear (39). Pan et al demonstrated that ALR alleviated acute pancreatitis via inhibition of the TLR4/NF-κB pathway and reduction of the release of TNF-α and cyclooxygenase-2 (COX-2) (40). These studies indicated that ALR is closely related to TLR4. In the present study, it was determined whether the regulatory role of ALR in inflammation in fatty liver IRI was mediated via the TLR4/NF-κB pathway. Our results revealed that the TLR4/NF-κB signaling pathway was activated in fatty liver IRI, and ALR transfection provided a protective effect by decreasing the TLR4/NF-κB signaling pathway.

There are some limitations in the present study. Although this study indicated that ALR inhibited the TLR4/NF-κB signaling pathway, whether this regulation was direct or indirect remains unclear. The manner with which ALR regulates the expression of TLR4 and the identity of intermediate molecules that mediate the interaction between ALR and the TLR4/NF-κB signaling pathway are not clear. Therefore, the regulation of ALR on the TLR4/NF-κB signaling pathway in fatty liver IRI requires further in-depth research.

In conclusion, the present study demonstrated that the administration of ALR effectively alleviated hepatocyte apoptosis and preserved liver function by suppressing inflammatory cell infiltration and reducing inflammatory cytokine production. ALR played a role in the inflammatory response via the inhibition of the TLR4/NF-κB signaling pathway in fatty liver IRI. Therefore, the anti-inflammatory effect of ALR establishes a promising strategy to protect steatotic livers against IRI.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding authors on reasonable request.

Authors' contributions

JW designed and performed the experiments. JW, BX and WL analyzed and interpreted the data. XW established the mouse model and performed the in vivo experiments. BX and WL designed and supervised the study, wrote and revised the manuscript. All authors read and approved the final manuscript. JW and WL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All animal studies were approved by the Ethics Committee of Capital Medical University (Beijing, China; approval number AEEI-2016-094).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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