Original Paper

Interferon Regulatory Factor 1 Activates Autophagy to Aggravate Hepatic Ischemia-Reperfusion Injury by Increasing High Mobility Group Box 1 Release

Zilin Cui^{a,b} Shipeng Li^c Zirong Liu^a Yamin Zhang^a Haiming Zhang^b

^aDepartment of Hepatobiliary Surgery, Tianjin First Center Hospital, Tianjin, ^bOrgan Transplant Center, Key Laboratory of Organ Transplantation of Tianjin, Tianjin First Center Hospital, Tianjin, ^cDepartment of General Surgery, Jiaozuo People's Hospital, Xinxiang Medical University, Jiaozuo, China

Key Words

IRF-1 • Autophagy • HMGB1 release • Hepatic ischemia-reperfusion injury

Abstract

Background/Aims: Interferon regulatory factor 1 (IRF-1) and high mobility group box 1 (HMGB1) have been independently identified as being key players in hepatic ischemia-reperfusion injury (IRI). We attempted to determine whether IRF-1 activates autophagy to aggravate hepatic IRI by increasing HMGB1 release. **Methods:** The hepatic IRI model was generated in C57BL/6 mice, euthanized at 2, 6, 12 or 24 h after reperfusion. To examine the effects of HMGB1 release inhibition, Glycyrrhiza acid (GA) was administered to the mice and at six hours after injection. AML12 cells were immersed in mineral oil for 90 min and then cultured in complete Dulbecco's Modified Eagle's Medium (DMEM)/F12 to simulate IRI. AML12 cells were treated with IRF-1 siRNA, Ad-IRF-1 or GA. The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as histological changes were examined. Next, autophagic vacuoles were detected by transmission electron microscopy (TEM) or LC3 dots. The expression of IRF-1 and HMGB1 mRNA were measured by real-time polymerase chain reaction. The expression of IRF-1, microtubule-associated protein 1 light chain 3 (LC3), Bcl-2, Beclin 1, HMGB1 were detected by western blotting or immunohistochemistry. **Results:** The levels of hepatic IRF-1, mRNA and protein were significantly increased in livers after exposure to IRI, together with, IRI-induced increase of HMGB1 mRNA and release of HMGB1 in liver tissue. Knockout of IRF-1 decreased expression and release of HMGB1 in liver, and inhibiting the release of HMGB1 could alleviate hepatic IRI. In addition, knockout of IRF-1 downregulated LC3II and Beclin1, while number of autophagosomes or LC3 dots were increased. Up-regulating IRF-1 expression could increase the levels of LC3 II expression in AML12 cells after exposure to IRI. The levels of HMGB1 in Ad-IRF-1 transfected AML12 cell supernatants increased, together with number of LC3 dots increasing. However, GA could inhibit both Ad-IRF-1 induced HMGB1 release and the increase in the number of LC3 dots. **Conclusions:** IRF-1 activates autophagy to aggravate hepatic IRI by increasing HMGB1 release.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Haiming Zhang

Organ Transplant Center, Key Laboratory of Organ Transplantation of Tianjin, Tianjin First Center Hospital, No. 24 Fukang Road, Nankai District, Tianjin 300192 (China)
Fax +86 2223626600, E-Mail mdzhanghaiming@qq.com

Introduction

Hepatic ischemia-reperfusion injury (IRI), a condition in which hypoxia is accentuated following the ensuing reperfusion of blood flow and oxygen delivery, is an inevitable complication associated with liver transplantation, partial hepatectomy and hypovolemic shock [1-3]. This pathogenesis predisposes grafts to both short- and long-term dysfunction and contributes to poor prognosis and patient survival. Many attempts have been undertaken to ameliorate hepatic IRI, including ischemia preconditioning, pharmacological and surgical manipulations and gene therapies. However, despite advances in graft managements, the mechanisms of IRI remain largely unknown [4].

Autophagy is a highly conserved intracellular process in which impaired proteins or organelles are engulfed by double-membraned autophagosomes and transported to lysosomes for degradation. The presence of basal levels of autophagy ensures that cells are capable of digesting cytoplasmic materials to meet energy demands in stressful conditions such as starvation or hypoxia. However, undisciplined autophagy, which can occur under extreme conditions such as following acute organ injury or reperfusion insult, can produce an accumulation of autophagic vacuoles, which may then predispose the cells to death [5]. It has been hypothesized that excessive autophagy, due to its relatively non-specific degradative actions, may devour organelles that are essential for protection against cell failure. Of particular relevance to the present report are findings suggesting that increased autophagy may lead to an increased susceptibility of livers to IRI. Such findings indicate a potentially novel strategy to ameliorate the effects of IRI, as could be achieved by modulating levels of autophagy [6].

Interferon regulatory factor-1 (IRF-1) is one of a family of highly conserved transcriptional factors that regulates the expression of certain genes involved in innate and acquired immunity. IRF-1 as related to hepatic IRI and found that IRF-1 exerts a detrimental role in hepatic IRI by modulating the expression of multiple inflammatory mediators [7]. The significance of these findings was the identification of a novel target for use in protection of livers against IRI. Originally identified as a transcriptional activator of IFN- β and IFN- α genes, IRF-1 was subsequently demonstrated to play a critical role in the development of IRI [8]. Complementing these findings were results from IRF-1 gene knockout mice, which showed their livers to be protected from IRI.

High mobility group box-1 (HMGB1) is a nuclear non-histone chromatin-binding protein, which maintains nucleosomal structure and stability, and regulates gene transcription [9]. In mammalian cells, HMGB1 can be actively secreted extracellularly in response to external stimuli, such as lipopolysaccharide (LPS) and TNF- α , or it can be passively released from necrotic cells. Intracellular HMGB1 plays a role in a number of fundamental cellular processes such as transcription, replication, DNA repair, and recombination [10]. When secreted extracellularly, HMGB1 is associated with increased mortality in animal models of sepsis and in septic patients, while inhibition of HMGB1 activity protects animals from lethal doses of LPS and reduces injurious ventilation-induced lung inflammation [11]. It has also been reported that extracellular HMGB1 may interact with TLR and or receptors for advanced glycation end products (RAGE), leading to activation of the MAPK p38 signaling pathway.

Under normal conditions, autophagy clears long-lived proteins, dysfunctional organelles and generates substrates for adenosine triphosphate production during periods of starvation and other types of cellular stress. Interestingly, HMGB1 has been shown to be a critical regulator of autophagy [12]. Stimuli that enhance reactive oxygen species promote cytosolic translocation of HMGB1 and thereby enhance autophagic flux. HMGB1 directly interacts with the autophagy protein Beclin1, and displaces Bcl-2. Mutation of cysteine 106 (C106), but not the vicinal C23 and C45 of HMGB1, promotes cytosolic localization and sustained autophagy. Further evidence demonstrating a role for HMGB1 in autophagy has been provided from a study showing that a pharmacological inhibition of HMGB1 cytoplasmic translocation, as achieved with the use of ethyl pyruvate, limits starvation-induced autophagy [13].

HMGB1 and IRF-1 have been independently identified as being key players in IRI. IRF-1, which contributes to hepatocellular release of HMGB1, is a necessary component for HMGB1 release in response to hypoxia or after liver IRI. Taken together, these findings study suggest that IRI-induced release of acetylated HMGB1 is a process that is dependent on TLR4-mediated upregulation of IRF-1[14].

In this study, we attempt to determine whether IRF-1 modulates autophagy and thus promotes hepatic IRI. Specifically, IRF-1 expression was either upregulated or downregulated to examine its effects on HMGB1 release as related to the regulation of autophagy in hepatic IRI. Our data indicate that IRF-1 activates autophagy to aggravate hepatic IRI by increasing HMGB1 release.

Materials and Methods

Animals

Both C57BL/6 wildtype (WT) and C57BL/6 background IRF-1-deficient (IRF-1 KO) mice were purchased from CasGene Biotech. Mice were maintained under conditions of laminar-flow and specific pathogen-free atmosphere within the Laboratory Animal Center of Tianjin First Central Hospital. Animal experiments were conducted in adherence to the guidelines of the China Association of Laboratory Animal Care. The protocols were approved by the Committee on the Ethics of Animal Experiments of the Tianjin First Central Hospital.

Reagents

Dulbecco's modified eagle's medium/F-12 (DMEM/F-12) and fetal bovine serum (FBS) were purchased from Gibco. Glycyrrhizin Acid (GA) was obtained from Xi'an Haoxuan Biotechnology Co. Ltd., (Xi'an, China) IRF-1 siRNAs and siRNA-NC (negative control) were purchased from RiboBio Co., Ltd.. Trizol and lipofectamine™ 3000 were obtained from Invitrogen. Rabbit Abs specific for IRF-1, microtubule-associated protein 1 light chain 3 (LC3), Bcl-2, Beclin 1, HMGB1, β -actin and horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody were purchased from Cell Signaling Technology Inc.

Hepatic IR model

Mice were permitted free access to water but were food restricted on the night before surgery. Following anesthesia with sodium chloral hydrate (30 mg/kg, ip), a non-lethal model of segmental (70%) hepatic warm ischemia and reperfusion was administered as described previously. After laparotomy, an atraumatic vascular clip was used to interrupt blood supply to the median and left lateral lobes of the liver, resulting in a 70% hepatic ischemia for 90 min. Reperfusion was initiated by removal of the clip. Sham control mice underwent the same procedure without vascular occlusion. Mice were euthanized at 2, 6, 12 or 24 h after reperfusion. Blood and liver tissues were collected for analysis.

To examine the effects of HMGB1 release inhibition, GA (30 mg/kg, i.p.) was administered to the mice and at six hours after injection, mice were subjected to the IR treatment.

Serum ALT, TNF- α and HMGB1 levels

Blood samples obtained from IRI mice were centrifuged at 3000 rpm for 15 min. Supernatants were collected and assayed for serum alanine aminotransferase (ALT) and aspartate transaminase (AST) levels using a standard automatic biochemistry analyzer. Serum samples were also analyzed for TNF- α levels using an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience). An ELISA was also used to determine serum HMGB1 according to the manufacturer's protocol (R&D Systems).

Liver histopathology

Liver parenchymas were fixed in formalin, embedded in paraffin, sectioned into 4- μ m-thick sections, and stained with hematoxylin-eosin (H&E).

Cell culture and treatment

The α -mouse liver 12 (AML12) cell line was purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences. Cells were cultured in DMEM/F-12, supplemented with 10%

FBS plus 100 U/ml penicillin and 100 µg/ml streptomycin (Solarbio). The GA was used to inhibit HMGB1 release in AML12 cells. For IRF-1 down-regulation, siRNA (F: 5'-GUAAGGAGGAGCCAGAAAU dTdT-3', R: 3'-dTdT CAUUC CUCCUCGGUCUUUA-5') was transfected into AML12 cells. Moreover, AML12 cells were transfected with Ad-IRF-1 or Ad-GFP (Hanbio) according to the instruction manual. After transfection, cells were transferred to a sealed pneumatoresis incubator to simulate IR conditions. The ischemia time was 90 min followed by a 12 h period of reperfusion.

Immunohistochemistry

Sections (4-µm-thick) were deparaffined and hydrated before antigen retrieval in 10 mM citric acid buffer. AML12 cells were inoculated in coverslips fixed in twelve-well plates. After rinsing with PBS, cells were fixed in 10% formalin for 30 min and incubated in 1% triton X-100 for 15 min. After eliminating endogenous peroxidase activity with 3% hydrogen peroxide in deionized water for 15 min, specimens were rinsed with PBS, then incubated with primary rabbit anti-HMGB1 (1:200) at 4°C overnight. Specimens were washed with PBS and incubated with HRP-conjugated secondary antibody for 1 h on the following day. The conjugation reaction was facilitated by HRP-conjugated streptavidin and then visualized by diaminobenzidine (DAB, ZSGB-BIO). Images were obtained using bright-field microscopy.

Transmission Electron Microscopy (TEM)

Livers and cell samples were placed in 1% glutaraldehyde and post-fixed with 2% osmium tetroxide. Sections or cell pellets were embedded in epon resin. The data were quantified by counting the number of autophagosomes per cross-sectioned cell.

Immunofluorescence

AML12 cells were cultured in 6-well plates to 60-70% confluence. To further detect autophagy induction, cells were transfected with tandem GFP-LC3 adenovirus (Hanbio) according to the instruction manual.

QRT-PCR

Total RNA was extracted by RNAiso Plus (Takara). The PrimeScript 1st Strand cDNA Synthesis Kit (Takara) was used to synthesize total cDNA in a 20 µl reaction system. According to the 2×Taq PCR MasterMix real-time PCR kit (Aidlab), 1 µg cDNA was recommended as a template in the 25 µl reaction system of the PCR. The specific primers (Sangon) used were as follows: IRF-1 WT (F: 5'-TCCCATGTTCCAATGCTCGGT-3', R: 5'-GCCCTTGT TCCTACTCTGATCCTTC-3'), IRF-1 KO (F: 5'-TCCCATGTTCCAATGCTCGGT-3', R: 5'-AG GCATCCTTGTGATGTCCCA-3'), HMGB1 (F: 5'-GCATCCTGGCTTATCCATTGG', R: 5'-GGCTGCTTGTCATCTGCTG-3'), GAPDH (F: 5'-AGGTCGGTGTGAACGGATTG-3', R: 5'-GTAGACCATGTAGTTGAGGTCA-3'). For real-time PCR, the reactions were performed on the 7000 Sequence Detection System (Applied Biosystems). Relative expressions were calculated as ratios normalized by GAPDH using the $2^{-\Delta\Delta Ct}$ method. The experiments were repeated at least three times.

Western blot analysis

Frozen liver tissue and AML12 cells were homogenized in RIPA lysis buffer and fully dissociated. The mixture was centrifuged at 12000 rpm for 20 min. The supernatant was collected. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Solarbio). Samples were mixed with loading buffer and run on a 12% sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE). The proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5% skim milk for 1 h and then incubated at 4°C overnight with primary antibodies for IRF-1, LC3, Bcl-2, Beclin 1 and β-actin. After being washed with TBST and incubated with the HRP-conjugated secondary antibody for 1 h at room temperature, membranes were washed and developed with the Super Signal detection system (Pierce Chemical) and exposed to film. The experiments were repeated at least three times.

Statistical analysis

All of the experiments were repeated at least three times. Data are presented as the mean ± SD. Analysis of variance (ANOVA) or Student's t test were used to examine potential differences performed for the parameters of ALT levels, optical density of protein and photometric values. SPSS 22.0 software

was applied in all statistical analyses. A P value less than 0.05 was required for results to be considered statistically significant. * P < 0.05, ** P < 0.01, *** P < 0.001.

Results

IR-induced an increase in IRF-1 and release of HMGB1 in mice livers

As shown in Fig. 1A, histological examination of livers revealed extensive areas of sinusoidal congestion and ballooning degeneration after exposure to IR. Severe sinusoidal stenosis and patchy necrosis were present within 12 h after reperfusion. Consistently, serum ALT and AST levels increased significantly (P < 0.05) and peaked at 12 h after reperfusion as compared with that of the sham group (Fig. 1B and C). Serum levels of TNF- α were also significantly increased (P < 0.05) after reperfusion and peaked at 6 h (Fig. 1D). Serum levels of HMGB1 increased significantly (P < 0.05) after reperfusion as compared with that of the sham group (Fig. 1E). To determine whether this injury was associated with changes in IRF-1 expression, we examined levels of IRF-1 mRNA and protein expressions. As shown in Fig. 1F, the levels of hepatic IRF-1 mRNA significantly increased (P < 0.05) after reperfusion, and IRF-1 protein was also up-regulated in livers after exposure to IR (Fig. 1G). At the same time, the levels of HMGB1 mRNA increased (Fig. 1H, P < 0.05) after reperfusion, and HMGB1 protein was also up-regulated and HMGB1 release was increasing in livers after exposure to IR (Fig. 1I). As shown in the *in vivo* mouse model, IR-induced increase of IRF-1 and HMGB1 mRNA and release of HMGB1 in liver tissue.

Knockout of IRF-1 decreases HMGB1 protein and release of HMGB1 in liver

IRI appeared to be ameliorated in IRF-1 KO mice as assessed by H&E staining (Fig. 2A). This effect was associated with significantly decreased (P < 0.01) serum ALT levels in IRF-1 KO versus WT mice (Fig. 2B and C). Compared with the WT group, levels of HMGB1 mRNA

Fig. 1. IR-induced an increase in IRF-1 and release of HMGB1 in livers. A: IR treatment increases the histopathologic changes in liver (red arrow, magnification $\times 200$); B, C, D and E: IR treatment increased serum ALT, AST, TNF- α and HMGB1 levels in mice compared with sham; F and G: The expression of IRF-1 in mouse livers subjected to IR as determined by qRT-PCR and western blot analysis; H: qRT-PCR for HMGB1 mRNA in mouse liver; I: Immunohistochemistry for HMGB1 in mouse liver; * P < 0.05, ** P < 0.01, *** P < 0.001 vs Sham group. Every experiment was repeated three times.

Fig. 2. Knockout of IRF-1 decreases HMGB1 protein and release of HMGB1 in livers. A: HE stain was used to observe the histopathologic changes in WT and IRF-1 KO mouse livers (red arrow, magnification $\times 200$); B and C: The ALT and AST levels in WT and IRF-1 KO mice; D: The expression of HMGB1 mRNA in IRF-1 KO mouse livers subjected to WT mouse as determined by qRT-PCR; E: Immunohistochemistry for HMGB1 in mouse liver; F: The HMGB1 levels in WT and IRF-1 KO mice; * $P < 0.05$, ** $P < 0.01$ vs WT group. Every experiment was repeated three times.

Fig. 3. Effects of IRF-1 deficiency on autophagy. A: IR treatment increases the LC3 II detected by western blot in liver relative to the sham group; B: TEM images of mouse hepatocytes after ischemia followed by reperfusion (autophagosomes, red arrow); C: The data were quantified by counting the number of autophagosomes per cross-sectioned cell, *** $P < 0.01$ vs sham group; D: The expression of HMGB1 mRNA in IRF-1 KO mouse livers subjected to WT mouse as determined by qRT-PCR; E: TEM images of IRF-1 KO mouse hepatocytes after ischemia followed by reperfusion (autophagosomes, red arrow); F: The data were quantified by counting the number of autophagosomes per cross-sectioned cell; *** $P < 0.001$ vs WT group. Every experiment was repeated three times.

were decreased in the IRF-1 KO group (Fig. 2D). Knockout of IRF-1 decreased HMGB1 protein and release of HMGB1 in liver tissues (Fig. 2E). Serum level of HMGB1 were significantly decreased in the IRF-1 KO group ($P < 0.05$) after reperfusion as compared with that of the WT group (Fig. 2F).

Effects of IRF-1 deficiency on autophagy

As shown in Fig. 3A, the expression of LC3II was up-regulated as a function of time following reperfusion. Next, we evaluated autophagic vacuoles using TEM. Autophagosomes, which contained partially degraded cytoplasmic material, were clearly observed with TEM (Fig. 3B and C). The basal number of autophagosomes within the IR group was increased relative to the sham group ($P < 0.001$). Results from TEM revealed a decrease in autophagic signaling, with bare autophagic vacuoles being present in livers of IRF-1 KO mice after exposure to IR ($P < 0.001$, Fig. 3E and F).

Inhibiting release of HMGB1 alleviates hepatic IRI

Treating mice with GA, inhibited IR-induced hepatocyte edema, congestion and apoptosis as compared with that of the non-GA treated IR group (Fig. 4A). Moreover, serum AST and ALT levels in the GA-treated group were decreased relative to that observed in the

Fig. 4. Inhibiting the release of HMGB1 alleviates hepatic IRI. A: HE stain was used to observe the histopathologic changes in GA mouse livers (red arrow, magnification $\times 200$); B and C: The ALT and AST levels in mice; D: The expression of HMGB1 mRNA in IRF-1 KO mouse livers subjected to WT mouse as determined by qRT-PCR; E: Immunohistochemistry for HMGB1 in mouse liver; F: The HMGB1 levels in mice; * $P < 0.05$, ** $P < 0.01$ vs IR group. Every experiment was repeated three times.

IR group (Fig. 4B and C). Compared with IR group, levels of HMGB1 mRNA were unchanged in GA group (Fig. 4D). GA treatment resulted in an increase in HMGB1 protein, while inhibiting the release of HMGB1 in liver tissues (Fig. 4E). Moreover, serum levels of HMGB1 were significantly decreased significantly ($P < 0.001$) in the GA group after reperfusion as compared with that observed in the IR group (Fig. 4F).

IRF-1 activates autophagy by increasing HMGB1 release

The presence of LC3 II immunostaining indicates that IRF-1 expression was down regulated in these IRI livers. Associated with this effect was a down-regulation in levels of LC3 II and Beclin1 expression, but an up-regulation in the levels of Bcl-2 expression (Fig. 5A). IRF-1 siRNA pretreatment also decreased the levels of LC3 II expression in AML12 cells after exposure to IR. In accordance with these findings, images from immunofluorescence confirmed that decreased levels of autophagosomes were present in the presence of IRF-1 siRNA as compared with the siRNA-NC condition ($P < 0.001$, Fig. 5B and C). Moreover, levels of HMGB1 in GA treated AML12 cell supernatants decreased significantly ($P < 0.001$) after reperfusion as compared with that of the IR group (Fig. 5D). The images from immunofluorescence confirmed that a decreased amount of autophagosomes were present in GA treated AML12 cells as compared with those exposed to IR alone ($P < 0.001$, Fig. 5E and F). For showing a direct link between IRF-1 and HMGB1 in activating autophagy, we transfected AML12 cells with Ad-IRF-1 or Ad-GFP, and The GA was used to inhibit HMGB1 release in AML12 cells. Up-regulating IRF-1 expression could increase the levels of LC3 II expression in AML12 cells after exposure to IR. The levels of HMGB1 in Ad-IRF-1 transfected AML12 cell supernatants increased significantly ($P < 0.001$), with the number of LC3 dots increasing significantly ($P < 0.001$) at same time. However, GA could inhibit both Ad-IRF-1 induced HMGB1 release and the increase in the number of LC3 dots ($P < 0.001$).

Discussion

Autophagy is regarded as a natural and essential defense mechanism against inflammation, damnification and oncotherapy [15]. Hence activation of autophagic pathways have been implicated in the pathogenesis of numerous human diseases. Recently, a considerable amount of attention has been focused on autophagy and liver diseases as related to liver ischemia-reperfusion [16]. Our previous research was directed toward examining the role of IRF-1 in regulating autophagy to aggravation on hepatic IRI as coupled with IRF-1. We also found that IRF-1 was up-regulated during hepatic IRI and was associated with an activation of the autophagic signaling [17]. In this study, we report that IRF-1 was

Fig. 5. IRF-1 activates autophagy by increasing HMGB1 release. A: The expression of IRF-1, Beclin1, LC3 II and Bcl-2 was detected by western blot in AML12 cells; B: Immunofluorescence was used to detect LC3 dots in AML12 cells; C: The data were quantified by counting the number of autophagosomes per cross-sectioned cell; $***P < 0.001$ vs siRNA-NC group. D: The levels in levels of HMGB1 in AML12 cell supernatants; E: Immunofluorescence was used to detect LC3 dots in AML12 cells; F: The data were quantified by counting the number of autophagosomes per cross-sectioned cell; $***P < 0.001$ vs IR group. G: The levels in levels of HMGB1 in AML12 cell supernatants; H: Immunofluorescence was used to detect LC3 dots in AML12 cells; I: The data were quantified by counting the number of autophagosomes per cross-sectioned cell; $**P < 0.01$, $***P < 0.01$ vs Ad-IRF-1 group. siRNA-NC: negative control siRNA. Every experiment was repeated three times.

upregulated in mouse livers subjected to IR, an effect which was accompanied by a significant increase in serum HMGB1 levels. In this vivo mouse model, we now show that IR-induced an increase in HMGB1 mRNA and release of HMGB1 in liver tissues. These data reveal a clear relationship between IRF-1 and HMGB1 in hepatic IRI.

HMGB1, a non-histone nuclear protein, is mainly expressed in the nuclei of eukaryotic cells and plays an important role in the regulation of transcription [3, 5, 18, 19]. HMGB1 can be either actively or passively released into the extracellular milieu, where it acts as an essential damage-associated molecular pattern (DAMP) molecule. In this state, it can activate proinflammatory signaling pathways by interacting with certain pattern recognition receptors, such as Toll-like receptor and the receptor for advanced glycation end-products (RAGE) [20]. Recent evidence has provided support of the notion that HMGB1 is an early critical mediator of injury and inflammation following IR [14]. For example, HMGB1 can function as an autophagy sensor in the presence of oxidative stress. Moreover, hydrogen peroxide and loss of superoxide dismutase 1 (SOD1)-mediated oxidative stress promotes cytosolic HMGB1 expression and extracellular release [21, 22]; while inhibition of HMGB1 release or loss of HMGB1 decreases the number of autolysosomes and autophagic flux in human and mouse cell lines under conditions of oxidative stress. Additional evidence in support of a critical role for HMGB1 in autophagy have been provided from several sources [21]. Stimuli that enhance reactive oxygen species promote cytosolic translocation of HMGB1 and thereby enhance autophagic flux [13], and HMGB1 directly interacts with the autophagy protein Beclin1 and displaces Bcl-2. Mutation of cysteine 106 (C106), but not the vicinal C23 and C45, of HMGB1 promotes cytosolic localization and sustained autophagy [23]. This absence of an effect upon vicinal C23 and C45 of HMGB1 appears to be a critical factor in this process, as the intramolecular disulfide bridge (C23/45) of HMGB1 is required for binding to Beclin1 and sustaining autophagy [24]. Further evidence demonstrating a role for HMGB1 in autophagy has been provided from a study showing that a pharmacological inhibition of HMGB1 cytoplasmic translocation, as achieved with the use of ethyl pyruvate, limits

starvation-induced autophagy [13]. Thus, endogenous HMGB1 is a critical pro-autophagic protein that enhances cell survival and limits programmed apoptotic cell death.

The findings that interferon regulatory factor-1 (IRF-1) mediates the release of HMGB1 in endotoxemia as shown in mice, encouraged us to examine this factor in our study. In IRF-1 KO mice, IRI appeared to be ameliorated as assessed by H&E staining. As compared with IR (WT) group, the levels of HMGB1 mRNA were decreased in IRF-1 KO group. Knockout of IRF-1 can decrease of HMGB1 protein and release of HMGB1 in the liver tissue. IRF-1 KO mice experienced less mortality, and released less systemic HMGB1 compared to their WT counterparts. Exogenous administration of recombinant HMGB1 to IRF-1 KO mice returned the mortality rate to that seen originally in IRF-1 WT mice. In cultures of peritoneal macrophages or RAW264.7 cells, an *in vitro* administration of LPS induced HMGB1 release in an IRF-1 dependent manner. The JAK-IRF-1 signal pathway appeared to participate in the signaling mechanisms of this LPS-induced HMGB1 release by mediating acetylation of HMGB1. IRF-1 plays a role in LPS induced release of HMGB1 and therefore may serve as a novel target in sepsis [25]. IRF-1 contributes to caspase-1 activation and apoptosis-associated speck-like protein containing a caspase activation and recruitment domain pyroptosome formation in AMs and leads to downstream inflammatory cytokine release, including that of IL-1 β , IL-18, and HMGB1. The nuclear translocation of IRF-1 is linked to the presence of TLR4. Our findings suggest that pyroptosis and the downstream inflammatory response in AMs induced by LPS is a process that is dependent on TLR4-mediated up-regulation of IRF-1. In this way, IRF-1 plays a key role in controlling caspase-1-dependent pyroptosis and inflammation [26].

Treatment of mice with GA resulted in inhibition of IR induced hepatocyte edema, congestion and apoptosis as compared with that of the non-GA treated IR group. Moreover, serum AST and ALT levels were decreased relative to that observed in the IR group. While levels of HMGB1 mRNA were unchanged in response to GA, as compared with that of the IR group. GA was effective in increasing HMGB1 protein levels and inhibiting release of HMGB1 in liver tissue.

It is well known that excessive release of HMGB1 in cancer leads to unlimited replication potential, an ability to develop blood vessels (angiogenesis), evasion of programmed cell death (apoptosis), self-sufficiency in growth signals, insensitivity to inhibitors of growth, inflammation, tissue invasion and metastasis [27-29]. In reviewing these effects, we considered some of the mechanisms by which HMGB1 regulates apoptosis and autophagy in glioma [30]. Treatment with 58-F effectively rescued hepatocytes by suppressing the PLCgamma1-IP3R-SOC signalling pathway and decreasing the calcium concentration in cells, thus reducing HMGB1 release [22]. HMGB1 plays a critical role in the development of ICH-induced secondary injuries through the amplification of plural inflammatory responses; and, an intravenous injection of neutralizing anti-HMGB1 mAb has been shown to have potential as a novel therapeutic strategy for ICH[31]. As pro-inflammatory signaling pathways are activated by the interaction of released HMGB1 with its receptors, a disruption of this process represents an essential component for mitigating tissue damage after organ IRI[3].

In conclusion, we have demonstrated that inhibition of IRF-1 or nuclear HMGB1 release by GA significantly protects the liver against IRI by directly reducing HMGB1 release. Moreover, the use of this strategy for therapy would most likely avoid the increased susceptibility to cellular death that results from the complete deletion of

Fig. 6. Model of IRF-1 activation of autophagy to aggravate hepatic ischemia-reperfusion injury by increasing HMGB1 release.

nuclear HMGB1 in hepatocytes. Therefore, IRF-1 activates autophagy to aggravate hepatic ischemia-reperfusion injury by increasing HMGB1 release, which provide new insights for potential therapeutic approaches in the treatment of hepatic IRI (Fig. 6).

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 81370576).

Disclosure Statement

The authors declare to have no competing interests.

References

- 1 Zhang Y, Zhu J, Guo L, Zou Y, Wang F, Shao H, Li J, Deng X: Cholecystokinin protects mouse liver against ischemia and reperfusion injury. *Int Immunopharmacol* 2017;48:180-186.
- 2 Du Y, Li D, Han C, Wu H, Xu L, Zhang M, Zhang J, Chen X: Exosomes from Human-Induced Pluripotent Stem Cell-Derived Mesenchymal Stromal Cells (hiPSC-MSCs) Protect Liver against Hepatic Ischemia/Reperfusion Injury via Activating Sphingosine Kinase and Sphingosine-1-Phosphate Signaling Pathway. *Cell Physiol Biochem* 2017;43:611-625.
- 3 Leventhal JS, Schroppel B: Toll-like receptors in transplantation: sensing and reacting to injury. *Kidney Int* 2012;81:826-832.
- 4 Gong J, Wang J, Tian Y, Zhang J, Liang W, Li Z, Yu J, Tang B, He S: Expression of tubulin folding cofactor B in mouse hepatic ischemia-reperfusion injury. *Biomed Rep* 2017;6:525-531.
- 5 Yu C, Yu X, Zhu HW, Li X, Huang LH, Li ZQ, Han D, Huang H: Expression pattern of HMGB1 and its association with autophagy in acute necrotizing pancreatitis. *Mol Med Rep* 2016;14:5507-5513.
- 6 Thorburn J, Frankel AE, Thorburn A: Regulation of HMGB1 release by autophagy. *Autophagy* 2009;5:247-249.
- 7 Yokota S, Yoshida O, Dou L, Spadaro AV, Isse K, Ross MA, Stolz DB, Kimura S, Du Q, Demetris AJ, Thomson AW, Geller DA: IRF-1 promotes liver transplant ischemia/reperfusion injury via hepatocyte IL-15/IL-15R α production. *J Immunol* 2015;194:6045-6056.
- 8 Castellaneta A, Yoshida O, Kimura S, Yokota S, Geller DA, Murase N, Thomson AW: Plasmacytoid dendritic cell-derived IFN- α promotes murine liver ischemia/reperfusion injury by induction of hepatocyte IRF-1. *Hepatology* 2014;60:267-277.
- 9 Cheng Y, Wang D, Wang B, Li H, Xiong J, Xu S, Chen Q, Tao K, Yang X, Zhu Y, He S: HMGB1 translocation and release mediate cigarette smoke-induced pulmonary inflammation in mice through a TLR4/MyD88-dependent signaling pathway. *Mol Biol Cell* 2017;28:201-209.
- 10 Di Candia L, Gomez E, Venereau E, Chachi L, Kaur D, Bianchi ME, Challiss RAJ, Brightling CE, Saunders RM: HMGB1 is upregulated in the airways in asthma and potentiates airway smooth muscle contraction via TLR4. *J Allergy Clin Immunol* 2017;140:584-587 e588.
- 11 Lv H, Liu Q, Wen Z, Feng H, Deng X, Ci X: Xanthohumol ameliorates lipopolysaccharide (LPS)-induced acute lung injury via induction of AMPK/GSK3 β -Nrf2 signal axis. *Redox Biol* 2017;12:311-324.
- 12 Ouyang F, Huang H, Zhang M, Chen M, Huang H, Huang F, Zhou S: HMGB1 induces apoptosis and EMT in association with increased autophagy following H/R injury in cardiomyocytes. *Int J Mol Med* 2016;37:679-689.
- 13 Tang D, Kang R, Livesey KM, Cheh CW, Farkas A, Loughran P, Hoppe G, Bianchi ME, Tracey KJ, Zeh HJ, 3rd, Lotze MT: Endogenous HMGB1 regulates autophagy. *J Cell Biol* 2010;190:881-892.
- 14 Dhupar R, Klune JR, Evankovich J, Cardinal J, Zhang M, Ross M, Murase N, Geller DA, Billiar TR, Tsung A: Interferon regulatory factor 1 mediates acetylation and release of high mobility group box 1 from hepatocytes during murine liver ischemia-reperfusion injury. *Shock* 2011;35:293-301.

- 15 Tang D, Kang R, Cheh CW, Livesey KM, Liang X, Schapiro NE, Benschof R, Sparvero LJ, Amoscato AA, Tracey KJ, Zeh HJ, Lotze MT: HMGB1 release and redox regulates autophagy and apoptosis in cancer cells. *Oncogene* 2010;29:5299-5310.
- 16 Li S, Zhang J, Wang Z, Wang T, Yu Y, He J, Zhang H, Yang T, Shen Z: MicroRNA-17 regulates autophagy to promote hepatic ischemia/reperfusion injury via suppression of signal transductions and activation of transcription-3 expression. *Liver Transpl* 2016;22:1697-1709.
- 17 Yu Y, Li S, Wang Z, He J, Ding Y, Zhang H, Yu W, Shi Y, Cui Z, Wang X, Wang Z, Sun L, Zhang R, Du H, Zhu Z: Interferon regulatory factor-1 activates autophagy to aggravate hepatic ischemia-reperfusion injury via the P38/P62 pathway in mice. *Sci Rep* 2017;7:43684.
- 18 Yang M, Cao L, Xie M, Yu Y, Kang R, Yang L, Zhao M, Tang D: Chloroquine inhibits HMGB1 inflammatory signaling and protects mice from lethal sepsis. *Biochem Pharmacol* 2013;86:410-418.
- 19 Li P, Du Q, Cao Z, Guo Z, Evankovich J, Yan W, Chang Y, Shao L, Stolz DB, Tsung A, Geller DA: Interferon-gamma induces autophagy with growth inhibition and cell death in human hepatocellular carcinoma (HCC) cells through interferon-regulatory factor-1 (IRF-1). *Cancer Lett* 2012;314:213-222.
- 20 Tang D, Kang R, Zeh HJ, 3rd, Lotze MT: High-mobility group box 1, oxidative stress, and disease. *Antioxid Redox Signal* 2011;14:1315-1335.
- 21 Huang Q, Yang Z, Zhou JP, Luo Y: HMGB1 induces endothelial progenitor cells apoptosis via RAGE-dependent PERK/eIF2alpha pathway. *Mol Cell Biochem* 2017;431:67-74.
- 22 Zhao P, Ye T, Yan X, Hu X, Liu P, Wang X: HMGB1 release by H2O2-induced hepatocytes is regulated through calcium overload and 58-F interference. *Cell Death Discov* 2017;3:17008.
- 23 Kang R, Livesey KM, Zeh HJ, 3rd, Lotze MT, Tang D: HMGB1 as an autophagy sensor in oxidative stress. *Autophagy* 2011;7:904-906.
- 24 Liu L, Yang M, Kang R, Wang Z, Zhao Y, Yu Y, Xie M, Yin X, Livesey KM, Lotze MT, Tang D, Cao L: HMGB1-induced autophagy promotes chemotherapy resistance in leukemia cells. *Leukemia* 2011;25:23-31.
- 25 Pan PH, Cardinal J, Li ML, Hu CP, Tsung A: Interferon regulatory factor-1 mediates the release of high mobility group box-1 in endotoxemia in mice. *Chin Med J (Engl)* 2013;126:918-924.
- 26 Wu D, Pan P, Su X, Zhang L, Qin Q, Tan H, Huang L, Li Y: Interferon Regulatory Factor-1 Mediates Alveolar Macrophage Pyroptosis During LPS-Induced Acute Lung Injury in Mice. *Shock* 2016;46:329-338.
- 27 Manne N, Arvapalli R, Graffeo VA, Bandrupalli VVK, Shokuhfar T, Patel S, Rice KM, Gijupalli GK, Blough ER: Prophylactic Treatment with Cerium Oxide Nanoparticles Attenuate Hepatic Ischemia Reperfusion Injury in Sprague Dawley Rats. *Cell Physiol Biochem* 2017;42:1837-1846.
- 28 Gao XJ, Qu YY, Liu XW, Zhu M, Ma CY, Jiao YL, Cui B, Chen ZJ, Zhao YR: Immune complexes induce TNF-alpha and BAFF production from U937 cells by HMGB1 and RAGE. *Eur Rev Med Pharmacol Sci* 2017;21:1810-1819.
- 29 Xu Z, Yu J, Wu J, Qi F, Wang H, Wang Z, Wang Z: The Effects of Two Anesthetics, Propofol and Sevoflurane, on Liver Ischemia/Reperfusion Injury. *Cell Physiol Biochem* 2016;38:1631-1642.
- 30 Seidu RA, Wu M, Su Z, Xu H: Paradoxical Role of High Mobility Group Box 1 in Glioma: A Suppressor or a Promoter? *Oncol Rev* 2017;11:325.
- 31 Wang D, Liu K, Wake H, Teshigawara K, Mori S, Nishibori M: Anti-high mobility group box-1 (HMGB1) antibody inhibits hemorrhage-induced brain injury and improved neurological deficits in rats. *Sci Rep* 2017;7:46243.