Cutting Edge: High-Mobility Group Box 1 Preconditioning Protects against Liver Ischemia-Reperfusion Injury

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High mobility group box 1 (HMGB1) is a NF released extracellularly as a late mediator of lethality in sepsis and as an early mediator of inflammation following injury. Here we demonstrate that in contrast to the proinflammatory role of HMGB1, preconditioning with HMGB1 results in protection following hepatic ischemia/reperfusion (I/R). Pretreatment of mice with HMGB1 significantly decreased liver damage after I/R. The protection observed in mice pretreated with HMGB1 was associated with a higher expression of IL-1R-associated kinase-M, a negative regulator of TLR4 signaling, compared with controls. We thus explored the possibility that HMGB1 preconditioning was mediated through TLR4 activation. HMGB1 preconditioning failed to provide protection in TLR4 mutant (C3H/HeJ) mice, but successfully reduced damage in TLR4 wild-type (C3H/HeOuJ) mice. Our studies demonstrate that in contrast to the role of HMGB1 as an early mediator of inflammation and organ damage in hepatic I/R, HMGB1 preconditioning can be protective. The Journal of Immunology, 2006, 176: 7154–7158.

Ischemia/reperfusion (I/R) injury is a pathophysiologic process whereby hypoxic organ damage is accentuated following return of blood flow and oxygen delivery. Transient episodes of ischemia are encountered during solid organ transplantation, trauma, hypovolemic shock, and elective liver resection, when inflow occlusion or total vascular exclusion is used to minimize blood loss. The pathophysiology of liver I/R injury includes both direct cellular damage as the result of the ischemic insult as well as delayed dysfunc-

tion and damage resulting from activation of inflammatory pathways (1, 2). Our recent studies suggest that the initial ischemic injury activates inflammatory signaling through the release of high-mobility group box 1 (HMGB1) (3). HMGB1 is a nuclear protein that is involved in transcriptional activation and DNA folding (4). However, in addition to its nuclear role, extracellular HMGB1 has been shown to be a critical mediator of the innate immune response to infection and injury. HMGB1 is released from activated macrophages and immunostimulated gut epithelial cells in a delayed manner relative to the secretion of the classical early proinflammatory mediators TNF and IL-1 (5, 6). HMGB1 is also released from necrotic or damaged cells and serves as a signal for inflammation (7, 8). Our laboratory has recently shown that whereas HMGB1 is a late mediator of systemic inflammation, HMGB1 can also play a role as an early mediator following acute, local organ injury (3). HMGB1 levels are increased by ischemia/reperfusion in the liver and activation of the innate immune system by HMGB1 in this context requires TLR4-dependent signaling.

Like HMGB1, LPS is known to signal through TLR4 (4, 9). Because preexposure to low concentrations of LPS leads to tolerance to subsequent LPS challenges (10), we hypothesized that pretreatment with HMGB1 would precondition the liver against the damaging effects of I/R. Herein, we show that in contrast to the proinflammatory role of HMGB1 released posts insult, preconditioning with HMGB1 results in protection from inflammation and organ injury following hepatic I/R. In addition, we show that the protective effect of HMGB1 preconditioning in hepatic I/R injury is TLR4 dependent and is associated with IL-1R-associated kinase-M (IRAK-M) up-regulation, a negative regulator of TLR4 signaling.

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4 Abbreviations used in this paper: I/R, ischemia/reperfusion; HMGB1, high-mobility group box 1; IRAK-M, IL-1R-associated kinase-M; sALT, serum alanine aminotransferase; rHMGB1, recombinant HMGB1.
**Materials and Methods**

**Materials**

Recombinant HMGB1 (rHMGB1) derived from *Escherichia coli* strain BL21 (Novagen) was prepared as described previously (11). LPS from *E. coli* 0111:B4 was obtained from Sigma-Aldrich.

**Purification of HMGB1 from HeLa cells**

HeLa cells were lysed and centrifuged for 15 min at 16,000 × g to pellet nuclei and insoluble material. The supernatant was collected and filtered through a 0.45-μm filter. HMGB1 polyclonal Ab was obtained from New Zealand White rabbits immunized with the peptide sequence KSEAGKKGPGRTGTS corresponding to amino acids 166–181 of HMGB1. The affinity purification of the polyclonal anti-HMGB1 Ab was performed following standard procedures. To affinity purify HMGB1, 10 ml of freshly prepared and filtered HeLa lysate was loaded onto the rabbit polyclonal anti-HMGB1 affinity column and recirculated for 30 min. The column was then washed and the bound protein was eluted using 3 M potassium thiocyanate (pH 7.0). The eluted protein was dialyzed for 30 min.

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**Results and Discussion**

**Pretreatment with HMGB1 protects against liver I/R injury**

To test our hypothesis that HMGB1 could precondition against organ injury following hepatic I/R, animals were given rHMGB1 at various concentrations 1 h before ischemia. Sixty minutes of warm hepatic ischemia followed by 6 h of reperfusion significantly increased sALT levels in control mice subjected to I/R. Pretreatment with 5 to 20 μg of rHMGB1 resulted in significant protection from hepatic injury in a dose-dependent manner (Fig. 1A). Liver histology confirmed the sALT estimation of liver damage (data not shown). Severe sinusoidal congestion and hepatocellular necrosis was present in liver tissue from control mice whereas minimal damage was noted in samples from HMGB1-treated mice.

Inflammatory cytokines, such as TNF and IL-6, have been shown to play key roles in the pathophysiology of hepatic I/R injury (12, 13). We measured serum levels of these cytokines after I/R. Compared with sham-treated animals, liver I/R in control animals resulted in increased levels of TNF and IL-6 (6 h after reperfusion; data not shown). However, animals pretreated with HMGB1 exhibited lower levels of serum TNF (Fig. 1B) and IL-6 (Fig. 1C) compared with control animals subjected to I/R.

**FIGURE 1.** Pretreatment with HMGB1 protects against liver I/R injury. *A,* Mice undergoing ischemia and 6 h of reperfusion were pretreated with rHMGB1 (2, 5, or 20 μg) or vehicle PBS i.v. 1 h before ischemia. Serum ALT levels were analyzed as a measure of hepatocellular injury. Data represent mean ± SE; *n = 6 mice per group, *p < 0.05 vs mice subjected to I/R given vehicle PBS. *(B)* Serum TNF and IL-6 (C) levels were measured following ischemia and 6 h of reperfusion in mice pretreated with 20 μg of HMGB1 or vehicle. Data represent mean ± SE; *n = 6 mice per group, *p < 0.05 vs mice subjected to I/R given vehicle PBS. *D,* NF-κB activation during hepatic I/R injury was assessed. Mice undergoing ischemia and 1 h of reperfusion were pretreated with HMGB1 Ab or vehicle PBS. Nuclear extracts were prepared from the ischemic livers and subjected to EMSA. Assay shown is representative of three experiments with similar results.

Cytoplasmic and nuclear proteins were extracted from frozen liver tissues as previously described (3). Protein concentration was quantitated with bicinchoninic acid protein assay reagent (Pierce).

**Liver ischemia**

A nonlethal model of segmental (70%) hepatic warm ischemia was used as previously described (3). Briefly, after induction of anesthesia, all structures in the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes were occluded with a microvascular clamp (Fine Science Tools). Heat-inactivated HMGB1 was used in certain experiments by boiling for 20 min at 100°C.

**Isolation of nuclear and cytoplasmic proteins**

Cytoplasmic and nuclear proteins were extracted from frozen liver tissues as previously described (3). Protein concentration was quantitated with bicinchoninic acid protein assay reagent (Pierce).

**Liver damage assessment**

To assess hepatic function and cellular injury following liver ischemia, serum alanine aminotransferase (sALT) levels were measured using the Opera Clinical Chemistry System (Bayer).

**Cytokine measurement**

Serum concentrations of immunoreactive TNF and IL-6 were determined using ELISA kits from Biosource International.

**SDS-PAGE and Western blotting**

Western blot analysis for HMGB1 (1:5000; BD Pharmingen), phosphorylated IRAK-1 (1:1000; Cell Signaling Technology), and IRAK-M (1:1000; Chemicon International) were performed as described (3). Membranes were developed with the SuperSignal West Pico chemiluminescent kit (Pierce) and exposed to film.

**EMSA**

NF-κB DNA binding activity was measured by EMSA using nuclear extracts from liver tissues as previously described (3).

**Statistical analysis**

Results are expressed as the mean ± SEM. Group comparisons were performed using Student’s *t* test or ANOVA. Differences were considered significant at *p < 0.05.*
NF-κB is a transcription factor involved in signal transduction of a variety of extracellular stress stimuli. It is activated in the setting of hepatic I/R (14) and regulates both proinflammatory and protective responses in the liver (15–17). Using EMSA, we found increases in NF-κB DNA binding in the ischemic liver 1 h after reperfusion in control mice when compared with sham-treated animals (Fig. 1D). Mice pretreated with HMGB1 exhibited less NF-κB DNA binding activity. The specificity of the NF-κB bands were confirmed by cold competition in the presence of excess unabeled NF-κB consensus motif. We previously performed supershift studies to determine that the NF-κB complex was a heterodimer composed of both p65 and p50 subunits (17).

These results demonstrate that HMGB1 preconditioning can protect against warm hepatic I/R injury and that the protection is associated with a decrease in NF-κB activation and serum proinflammatory cytokine levels.

Protection with HMGB1 preconditioning in liver I/R associated with increase in IRAK-M expression

Recent in vitro and in vivo evidence suggest that TLR4 acts as a receptor to HMGB1 (3, 18, 19). The TLRs are one of the components by which the innate immune system senses the invasion of pathogenic microorganisms or tissue damage by recognizing specific molecular patterns that are present in microbial products (pathogen-associated molecular pattern molecules or PAMPs) or endogenous molecules released by damaged tissues (DAMPs) (20). Perhaps more than any of the other TLR family members, TLR4 sits at the interface of microbial and sterile inflammation by responding to both bacterial endotoxin and multiple other endogenous ligands, including hyaluronic acid (21), heparin sulfate (22), fibrinogen (23), HMGB1 (3, 18), and heat shock proteins (24).

Our recent studies suggest a central role for HMGB1 in the TLR4-dependent component associated with hepatocyte damage, and the resultant enhanced inflammation following hepatic I/R injury (3). We thus asked whether the mechanism of HMGB1 preconditioning involved down-regulation of TLR4 signaling. Upon TLR stimulation, multiple adaptor molecules are recruited to the TLR signaling complex. One molecule, IRAK-M, has been shown to be a negative regulator of TLR signaling (25). Thus, we examined the role of IRAK-M in HMGB1 preconditioning. Following 60 min of warm ischemia, IRAK-M protein expression was up-regulated in the liver of control mice (Fig. 2). However, mice pretreated with HMGB1 exhibited higher hepatic IRAK-M levels after reperfusion compared with control mice. Because IRAK-M expression was increased in preconditioned mice, we sought to determine whether TLR4 signaling was down-regulated in these protected mice. One of earliest events of TLR signaling involves the phosphorylation of the adaptor molecule IRAK-1 (26). Levels of phosphorylated IRAK-1 were increased in control mice after reperfusion (Fig. 2). In mice pretreated with HMGB1, phosphorylated IRAK-1 expressions were lower compared with control mice. Thus, the lower IRAK-1 phosphorylation in the livers of mice treated with HMGB1 was associated with increased hepatic IRAK-M expression after I/R.

HMGB1 preconditioning involves TLR4

Because the protection seen with HMGB1 preconditioning appeared to involve down-regulation of TLR4 signaling, we sought to determine whether TLR4 was required for the HMGB1-mediated preconditioning. TLR4 mutant (C3H/HeJ) mice and wild-type control (C3H/HeOuJ) mice were subjected to liver I/R with or without HMGB1 pretreatment. In agreement with previous reports, TLR4 mutant mice were protected from hepatic I/R injury compared with wild-type mice (27) (Fig. 3A). Similar to that seen with C57BL/6 mice, HMGB1 treatment before the ischemic insult in TLR4 wild-type animals lead to protection from hepatic I/R damage (Fig. 3A). However, this protection was not seen in TLR4 mutant mice undergoing hepatic I/R. Whereas the liver damage in these mice is 50% less than wild-type mice, it is still much greater than sham animals. HMGB1 pretreatment failed to reduce the damage in TLR4 mutant animals. We also examined the expression of serum TNF (Fig. 3B) and IL-6 (Fig. 3C) in the wild-type and mutant animals. Although TLR4 wild-type mice...
treated with HMGB1 exhibited decreased circulating TNF and IL-6 levels compared with control I/R animals, there was no difference in levels of these cytokines after I/R in TLR4 mutant mice treated with HMGB1 compared with mutant mice subjected to I/R but not receiving HMGB1.

Hepatic IRAK-M expression was also examined in both TLR4 mutant and wild-type mice (Fig. 4). Following 60 min of warm ischemia, wild-type mice pretreated with HMGB1 had higher expressions of hepatic IRAK-M after reperfusion compared with control mice. However, there was no significant difference in IRAK-M expression between TLR4 mutant animals treated with or without HMGB1. Of note, IRAK-M levels dropped to baseline levels by 6 h in the TLR4 mutant animals but remained elevated in the wild-type mice subjected to I/R. The up-regulation of IRAK-M in the TLR4 mutant animals indicates an early pathway for IRAK-M independent of TLR4, whereas the drop at 6 h indicates that the persistence is due to TLR4 activation.

**FIGURE 4.** HMGB1 preconditioning in TLR4-mutant animals does not change IRAK-M expression. TLR4 mutant and wild-type mice were subjected to liver ischemia and varying times of reperfusion. Animals were pretreated with 20 µg of HMGB1 or vehicle PBS 1 h before ischemia. Western blot analysis for IRAK-M was performed for hepatic protein lysates of the ischemic lobes at the time points shown, with each lane representing a separate animal. Blot shown is representative of three experiments with similar results.

**FIGURE 5.** HMGB1 preconditioning is not mediated by LPS. A, Mice undergoing ischemia and 6 h of reperfusion were pretreated with heat-inactivated HMGB1 (20 µg) or vehicle PBS i.v. 1 h before ischemia. Data represent mean ± SE; n = 4 mice per group. B, Mice undergoing I/R were pretreated with HMGB1 derived from HeLa cells (20 µg) or vehicle PBS i.v. 1 h before ischemia. Data represent mean ± SE; n = 4–6 mice per group. *p < 0.05 vs mice subjected to I/R given vehicle PBS.

**Disclosures**

The authors have no financial conflict of interest.

**References**


