

HMGB1 release induced by liver ischemia involves Toll-like receptor 4–dependent reactive oxygen species production and calcium-mediated signaling

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Ischemic tissues require mechanisms to alert the immune system of impending cell damage. The nuclear protein high-mobility group box 1 (HMGB1) can activate inflammatory pathways when released from ischemic cells. We elucidate the mechanism by which HMGB1, one of the key alarm molecules released during liver ischemia/reperfusion (I/R), is mobilized in response to hypoxia. HMGB1 release from cultured hepatocytes was found to be an active process regulated by reactive oxygen species (ROS). Optimal production of ROS and subsequent HMGB1 release by hypoxic hepatocytes required intact Toll-like receptor (TLR) 4 signaling. To elucidate the downstream signaling pathways involved in hypoxia-induced HMGB1 release from hepatocytes, we examined the role of calcium signaling in this process. HMGB1 release induced by oxidative stress was markedly reduced by inhibition of calcium/calmodulin-dependent kinases (CaMKs), a family of proteins involved in a wide range of calcium-linked signaling events. In addition, CaMK inhibition substantially decreased liver damage after I/R and resulted in accumulation of HMGB1 in the cytoplasm of hepatocytes. Collectively, these results demonstrate that hypoxia-induced HMGB1 release by hepatocytes is an active, regulated process that occurs through a mechanism promoted by TLR4-dependent ROS production and downstream CaMK-mediated signaling.

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Abbreviations used: 4-HAE, 4-hydroxyalkenal; CaMK, calcium/calmodulin-dependent kinase; CaMKK, CaMK kinase; DAMP, damage-associated molecular pattern; DCF, 2',7'-dichlorofluorescein; HMGB1, high-mobility group box 1; I/R, ischemia/reperfusion; MDA, malondialdehyde; mRNA, messenger RNA; NAC, N-acetylcysteine; ROS, reactive oxygen species; sALT, serum alanine aminotransferase; siRNA, small interfering RNA; TLR, Toll-like receptor.

Activation of the innate immune system has long been recognized as a necessary step in mounting an antimicrobial response to pathogens. Sterile inflammatory insults initiated by trauma or ischemia activate many of the same innate immune signaling pathways. At the molecular level, these observations may be explained, at least in part, by the capacity of both pathogen-associated molecular pattern molecules and damage-associated molecular pattern (DAMP) molecules to activate cell signaling through common pattern recognition receptors of the innate immune system. DAMPs can be classified as normal cell constituents released by damaged or dying cells, or components of the extracellular matrix released by the action of proteases at the site of tissue damage. For example, Toll-like receptor (TLR) 4 signaling can be activated by cell constituents such as high-mobility group box 1 (HMGB1) (1, 2), heat

shock proteins (3), or uric acid crystals (4) or the matrix component heparin sulfate (5).

TLR proteins are pattern recognition receptors composed of 13 family members in mice and 10 in humans. Members of the TLR family have many structural similarities, both extracellularly and intracellularly, but they differ from each other in ligand specificity, expression patterns, and in some instances the signaling pathways they activate. TLR4 is one of a subset of pattern recognition receptors that recognize both DAMPs and pathogen-associated molecular patterns and, hence, is involved in immune response to both infection and injury. TLR4 was first characterized as part of the bacterial endotoxin recognition complex (6). More recently, the role of TLR4 in ischemia/reperfusion (I/R) injury has been revealed in studies in mice showing that the lack of TLR4 or impairment of TLR4 signaling results in less tissue damage and an attenuated inflammatory response in hepatic

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(2, 7), renal (8, 9), and cardiac (8) I/R, as well as hemorrhagic shock (10, 11). We have provided evidence that HMGB1 is essential to TLR4 signaling in hepatic I/R (2) and hemorrhagic shock (11, 12), whereas others have provided evidence for direct interaction of HMGB1 with the TLR4 receptor complex *in vitro* (1, 13).

HMGB1 is an evolutionarily conserved protein present in the nucleus of almost all eukaryotic cells, where it functions to stabilize nucleosomes and acts as a transcription factor (14, 15). Its proinflammatory properties were identified in experiments showing that HMGB1 is actively secreted by activated macrophages and that HMGB1 is a late mediator of lethality in sepsis models (16, 17). Although HMGB1 can also be released by cells undergoing necrosis (18, 19), the actions of HMGB1 in I/R occur before cell death, suggesting that HMGB1 is mobilized and released in response to hypoxia. In this paper, we show that hypoxia-induced HMGB1 release by hepatocytes occurs through a mechanism promoted by TLR4-dependent reactive oxygen species (ROS) production and downstream calcium/calmodulin-dependent kinase (CaMK)-mediated signaling. We also show that these signaling pathways are involved in I/R-induced injury and inflammation in the liver *in vivo*. Thus, TLR4 participates not only in the recognition of HMGB1 but also in its release through a mechanism involving ROS production and CaMK activation. These findings provide a mechanism by which ischemic tissues notify the immune system of the potential loss of tissue integrity.

RESULTS

Oxidative stress induces HMGB1 release from hepatocytes

We have previously shown that HMGB1 is a proximal mediator of inflammation and injury induced by I/R in the liver (20). Our observation that hypoxia leads to HMGB1 release by hepatocytes in the absence of cell death suggests that HMGB1 mobilization in these cells is a regulated process. To determine if ROS produced during hypoxia contributes to hypoxia-induced HMGB1 release, hepatocytes were cultured under normoxic (21% oxygen) or hypoxic (1% oxygen) conditions and in the presence or absence of the antioxidant *N*-acetylcysteine (NAC). Hypoxia led to a time-dependent increase in HMGB1 release into the media (Fig. 1 A). This release was prevented by NAC. To further establish the role of ROS in the mechanism of HMGB1 release, hepatocytes were treated with H₂O₂ in concentrations from 125 to 500 μ M. HMGB1 levels in the media were found to increase in a concentration-dependent manner at 8 h after H₂O₂ exposure (Fig. 1 B). Treatment with the antioxidants NAC or Trolox abrogated the H₂O₂-mediated HMGB1 release (Fig. 1 C).

It is known that HMGB1 can be released from cells by either an active, regulated, process or passively released during cell necrosis. Previous work has shown that hypoxia alone leads to minimal hepatocyte death *in vitro* in the first 18–24 h (21, 22). Cell viability was not substantially different between hepatocytes exposed to hypoxia or normoxia or upon stimulation with H₂O₂ at concentrations up to 500 μ M with a crystal violet viability assay (Fig. 1 D). Lactate dehydrogenase levels were also

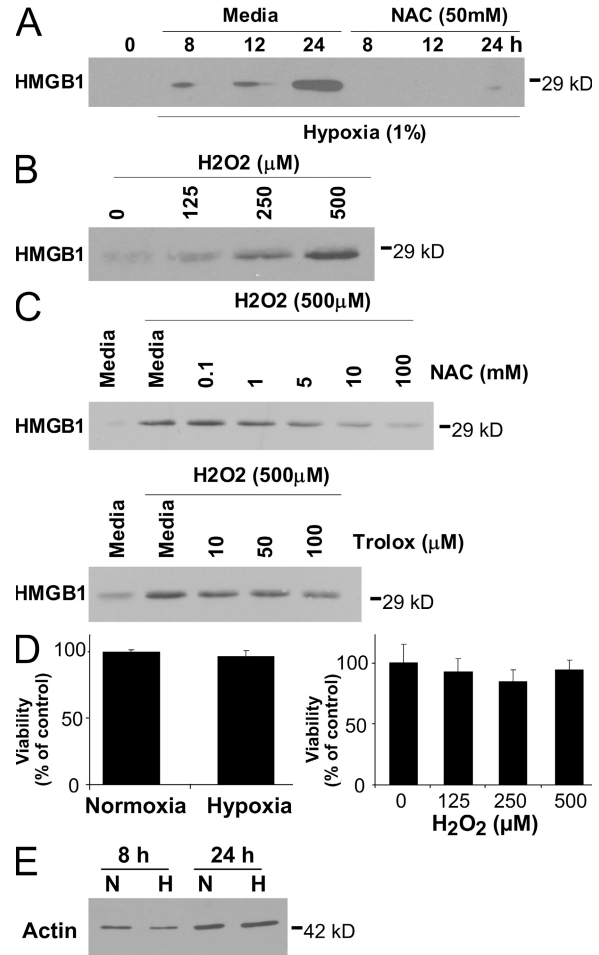


Figure 1. Oxidative stress induces HMGB1 release from hepatocytes. (A) Western blot analysis of supernatants from hepatocytes that underwent 8, 12, or 24 h of hypoxia (1% O₂). Cells were plated in Williams' medium E or Williams' medium E containing 50 mM NAC. The blot shown is representative of three different experiments with similar results. (B) Hepatocytes were stimulated with 125, 250, or 500 μ M H₂O₂ for 8 h, and supernatants were sampled and subjected to Western blot analysis for HMGB1. The blot shown is representative of three different experiments with similar results. (C) Hepatocytes were exposed to 500 μ M H₂O₂ for 30 min before treatment with NAC or Trolox in a dose-response fashion. Supernatants were sampled at 8 h and subjected to Western blot analysis for HMGB1. The blot shown is representative of three different experiments with similar results. (D) Viability was determined by crystal violet assay on hepatocytes subjected to either 24 h of hypoxia (1% O₂) or 8 h of oxidative stress from H₂O₂. Results are expressed as the mean \pm SEM. (E) Hepatocytes were exposed to normoxia (N) or hypoxia (H) for 8 and 24 h, and supernatants were sampled for Western blot analysis for HMGB1. The blot shown is representative of multiple experiments with similar results.

not substantially different between cells exposed to hypoxia or H₂O₂ compared with control cells (unpublished data). To further confirm whether differences found with HMGB1 release may reflect differences in cell death, actin levels in the supernatants of cells exposed to hypoxia or normoxia were also shown to be similar (Fig. 1 E). These results suggest that oxidant-induced

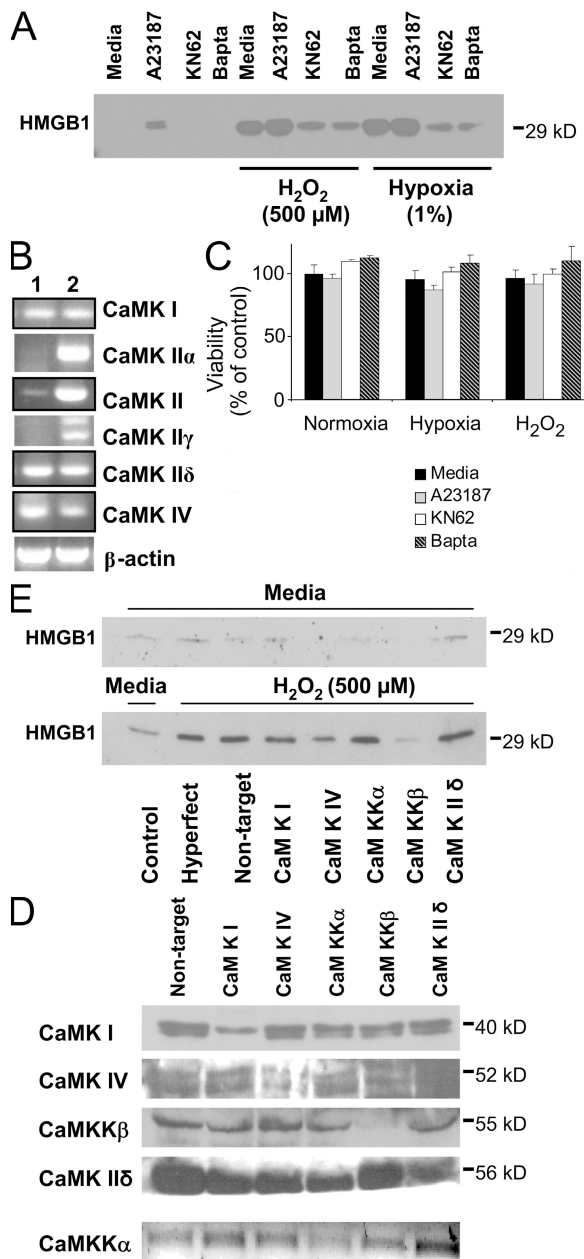


Figure 2. HMGB1 release from hepatocytes under oxidative stress is calcium dependent. (A) Hepatocytes were treated with A23187 (calcium ionophore), bapta (calcium chelator), or KN62 (CaMK inhibitor) for 30 min to alter intracellular calcium signaling. Cells were then exposed to 500 μ M H_2O_2 for 8 h or to hypoxia (1% O_2) for 24 h. Supernatants were sampled and subjected to Western blot analysis for HMGB1. The blot shown is representative of three different experiments with similar results. (B) CaMK mRNA expression levels in rat primary hepatocytes (lane 1) were detected by RT-PCR using specific primers for CaMK I, II α , II β , II γ , II δ , and IV, respectively. Rat cerebral cortex extract (lane 2) was used as a positive control. (C) Plated cells from the experiment in A underwent assessment of cell viability by crystal violet assay after supernatant samples had been taken. Results are expressed as the mean \pm SEM. The experiment shown is representative of three different experiments with similar results. (D) Hepatocytes were transfected with siRNA to each CaMK (I, II δ , and IV) and the upstream CaMKK (CaMKK α and CaMKK β). The specificity of each

HMGB1 release from hepatocytes is not mainly caused by cell death and is likely to be an active process regulated by ROS.

HMGB1 release from hepatocytes under oxidative stress is calcium dependent

To elucidate the downstream signaling pathways involved in hypoxia-induced HMGB1 release from hepatocytes, we examined the role of calcium signaling in this process. It is known that intracellular calcium is released after oxidative stress, leading to the activation of calcium-dependent signaling pathways (23, 24). Increasing intracellular levels of calcium with the calcium ionophore A23187 resulted in the release of HMGB1 from resting hepatocytes (Fig. 2 A). HMGB1 release induced by either H_2O_2 exposure or hypoxia was markedly reduced by treatment with the calcium chelator bapta (Fig. 2 A). Because a wide range of calcium-linked signaling events are mediated by a family of CaMKs, we asked if these enzymes were involved in HMGB1 release. The CaMK inhibitor KN62 markedly inhibited both hypoxia- and H_2O_2 -induced HMGB1 release (Fig. 2 A). We confirmed that hepatocytes express CaMK using RT-PCR (Fig. 2 B). Isoforms I, II δ , and IV were detected at the messenger RNA (mRNA) level. The effect of calcium modulation on HMGB1 release was not caused by cell death because cell viability was not substantially different among the different treatment groups (Fig. 2 C). These results suggest that HMGB1 release by ischemic hepatocytes is regulated by calcium-mediated signaling involving the CaMK cascade. To further elucidate the specific CaMK isotypes involved in oxidant-mediated HMGB1 release, small interfering RNA (siRNA) technology was used to complement the pharmacologic inhibitor experiments. Hepatocytes were transfected with siRNA to the various isotypes of CaMKs (I, II δ , and IV) and their upstream activators, CaMK kinases (CaMKKs) α and β (25). The specificity of the various CaMK siRNAs were determined by Western blotting (knockdown of CaMKK α was determined by RT-PCR, as there is no commercially available antibody to this protein; Fig. 2 D). The cells were stimulated with H_2O_2 , and their release of HMGB1 was examined (Fig. 2 E). We found that CaMK IV and the CaMKK β appear to be involved in oxidant stress-mediated HMGB1 release in hepatocytes.

CaMK inhibition protects against liver I/R injury

Our laboratory has recently shown that HMGB1 is an early mediator of injury and inflammation in liver I/R (2). Because HMGB1 release from hepatocytes under oxidative stress was mediated in part by CaMKs, we asked if CaMK-dependent signaling played a role *in vivo* during liver I/R. Animals were given KN93, an inhibitor of CaMK activity, or KN92, an inactive analogue of KN93, 12 and 1 h before ischemia.

siRNA was confirmed by Western blot (CaMK I, CaMK IV, CaMKK β , and CaMK II δ) or RT-PCR (CaMKK α). (E) HMGB1 release was examined in the transfected cells stimulated with or without H_2O_2 . The experiment shown is representative of three different experiments with similar results.

Serum alanine aminotransferase (sALT) levels were used to estimate the magnitude of liver damage. We found that KN92 did not alter the level of injury in mice subjected to I/R. Treatment with KN93 resulted in significant protection from hepatic injury (Fig. 3 A). Histologic analysis confirmed the sALT estimation of liver damage. Severe sinusoidal congestion and hepatocellular necrosis were present in liver tissue

from mice treated with control KN92, whereas minimal damage was noted in samples from KN93-treated mice (not depicted). To determine if CaMK inhibition affected HMGB1 release during liver I/R, serum HMGB1 levels of mice treated with KN93 and control KN92 were examined using Western blotting. Circulating HMGB1 was increased in control KN92-treated mice compared with sham-treated animals at 6 h after

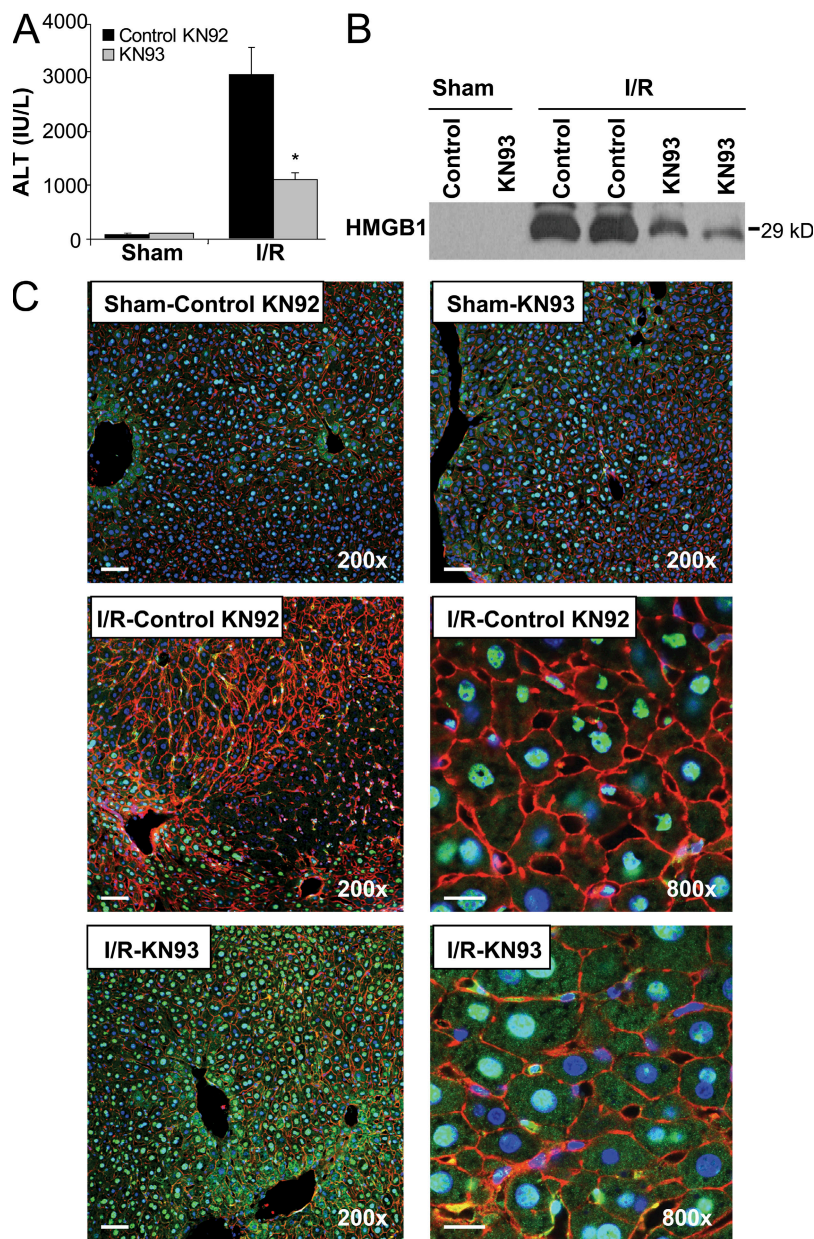


Figure 3. CaMK inhibition protects against liver I/R injury. (A) Mice were given 16-mg/kg intraperitoneal injections of KN93, a CaMK inhibitor, or KN92, an inactive analogue, 12 and 1 h before ischemia. Mice underwent 1 h of ischemia and 6 h of reperfusion before serum samples were taken and tested for ALT levels. Sham animals that were injected with the same solutions were subjected only to laparotomy without ischemia. Data represent means \pm SE ($n = 6-10$ animals). * $P < 0.05$ versus mice subjected to I/R and treated with KN92 control. (B) Western blot analysis for HMGB1 was also performed on serum samples from animals. Each lane represents a different animal. The blot shown is representative of two experiments with similar results. (C) Immunofluorescent stain of HMGB1 from sections of livers subjected to sham operation or 60 min of ischemia and 6 h of reperfusion. Mice were given intraperitoneal injections of KN93, a CaMK inhibitor, or KN92, an inactive analogue, 12 and 1 h before ischemia. Images are representative liver sections from six mice per group (green, HMGB1; blue, nuclei; red, F-actin). Bars: ($\times 200$) 50 μm ; ($\times 800$) 10 μm .

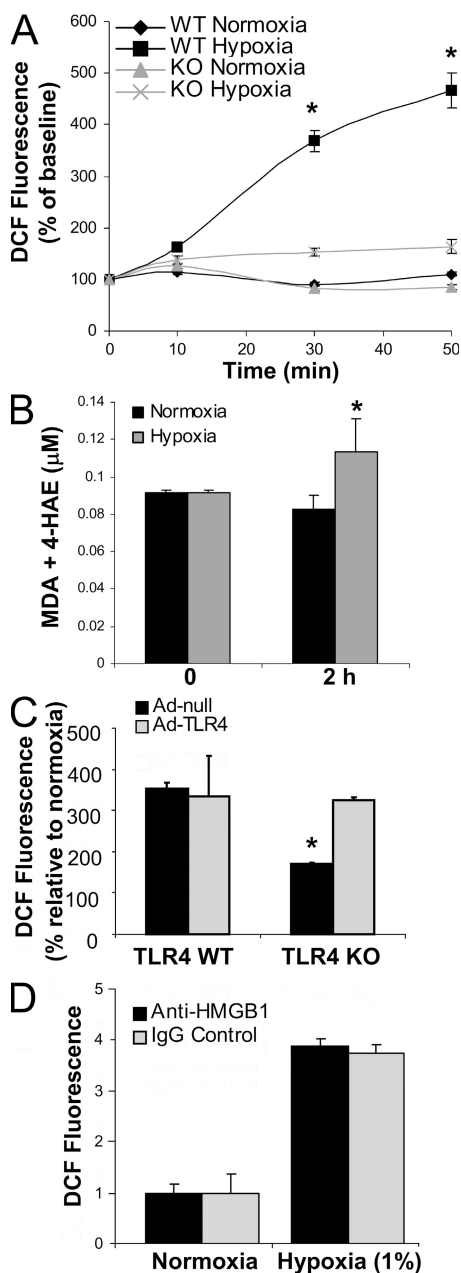


Figure 4. Functional TLR4 signaling required for optimal ROS production. (A) Hepatocytes from TLR4 wild-type and KO mice were plated and incubated with 10 μ M DCF-DA for 30 min before treatment. Cells were subjected to normoxia or hypoxia (1% O_2) for times up to 50 min, and ROS production was measured by fluorescence spectrophotometry. Data represent means \pm SE. *, $P < 0.05$ versus TLR4 KO hepatocytes subjected to hypoxia. (B) Hepatocytes plated in 10-cm plates were exposed to either normoxia or hypoxia for 2 h. Baseline samples, in addition to 2-h samples, were assayed for byproducts of lipid peroxidation, MDA and 4-HAE. *, $P < 0.05$ versus 2-h normoxic controls. (C) Hepatocytes from TLR4 wild-type and KO mice were transfected with adenoviral vector encoding either murine TLR4 (Ad-TLR4) or empty adenoviral vector control (Ad-null). Cells were subjected to 30 min of hypoxia. ROS production measured by DCF assay shown is the percent increase relative to respective normoxic controls. *, $P < 0.05$ versus TLR4 wild-type controls treated with Ad-null virus. (D) Hepatocytes were treated with either anti-HMGB1 neutralizing

antibody or IgG control and exposed to normoxia or hypoxia (1% O_2) for 30 min. ROS production was measured by DCF assay, and results are reported as the percentage relative to normoxic controls. Results in B–D are expressed as the mean \pm SEM.

reperfusion (Fig. 3 B). Serum HMGB1 levels were markedly lower after I/R in the KN93-pretreated mice. To determine the effect of CaMK inhibition on the cellular localization of HMGB1 in the liver, immunofluorescence staining was performed. Consistent with our previous observations (2), HMGB1 was almost exclusively localized in the nucleus of hepatocytes in the livers of sham-treated mice (Fig. 3 C). There was no considerable difference in HMGB1 staining between sham animals treated with KN93 or the control compound KN92. After I/R, the intensity of HMGB1 staining increased in some hepatocytes, whereas others were depleted of HMGB1 in the control KN92-treated group. In livers of mice pretreated with KN93 before I/R, there was a clear accumulation of HMGB1 in the cytoplasm of hepatocytes compared with the livers of mice pretreated with control KN92 before I/R. These findings suggest that the protective effect of CaMK inhibition against liver I/R may be in part caused by the prevention of HMGB1 release from hepatocytes.

Functional TLR4 signaling required for optimal ROS production and HMGB1 release from hepatocytes during oxidative stress

TLR4 signaling is involved in I/R-induced inflammation and injury. We have provided evidence that HMGB1 stimulates TLR4 signaling in I/R in the liver (2, 20); however, it is unknown whether TLR4 participates in hypoxia-induced ROS production and HMGB1 release. We initially assessed this in vitro using cultured hepatocytes. Wild-type hepatocytes exposed to hypoxia produced ROS over time, as measured by 2',7'-dichlorofluorescein (DCF) fluorescence compared with normoxia (Fig. 4 A). An assay to measure two markers of lipid peroxidation, malondialdehyde (MDA) and 4-hydroxyalkenal (4-HAE), was also used as an estimate of oxidant production to demonstrate that hypoxia resulted in ROS generation (Fig. 4 B). In contrast to wild-type hepatocytes, TLR4 KO hepatocytes generated significantly lower levels of ROS compared with the wild-type counterparts (Fig. 4 A). To confirm that functional TLR4 signaling was involved in ROS production in hepatocytes during hypoxia, TLR4 KO hepatocytes were transfected with empty adenoviral vector control (Ad-null) or recombinant adenovirus encoding TLR4 (Ad-TLR4). Transfection of TLR4 KO hepatocytes with Ad-null resulted in lower ROS production during hypoxia (30 min) compared with wild-type levels (Fig. 4 C). However, transfection of TLR4 KO hepatocytes with Ad-TLR4 increased ROS production during hypoxia to levels comparable to TLR4 wild type. Our previous experiments have suggested that HMGB1 may be a ligand for TLR4 (2). The mechanism of TLR4-dependent ROS production did not involve an HMGB1–TLR4 interaction because blockade of HMGB1 did not affect hypoxia-induced ROS production (Fig. 4 D).

Furthermore, a supernatant from hypoxic cells transferred to normoxic cells did not trigger HMGB1 release (unpublished data), suggesting that a soluble factor is not involved.

To determine the role of TLR4 signaling in HMGB1 release in response to oxidant stress, TLR4 wild-type and KO hepatocytes were exposed to hypoxia. Both TLR4 wild-type and TLR4 KO hepatocytes exhibited a time-dependent increase in HMGB1 release during hypoxia (Fig. 5 A). However, the release of HMGB1 from TLR4 KO hepatocytes was markedly less. TLR4 viral transfection enhanced hypoxia-induced HMGB1 release in TLR4 KO and, to a lesser extent, in TLR4 wild-type cells (Fig. 5 B). The overexpression of TLR4 by transfection in both KO and wild-type hepatocytes was confirmed by Western blotting for TLR4 (not depicted). Although the hypoxic-induced HMGB1 release in the TLR4 KO hepatocytes was less than in wild-type cells, the release observed in these cells was still dependent on calcium, CamK, and ROS (Fig. 5, C and D). The differences in HMGB1 release in TLR4 KO hepatocytes was not caused by baseline differences in HMGB1 levels between hepatocytes from the two strains of mice because both wild-type and KO hepatocytes contained similar levels of HMGB1 (Fig. 5 E). Additionally, treatment with H₂O₂ resulted in similar levels of HMGB1 release in both TLR4 wild-type and KO hepatocytes (Fig. 5 F). These observations indicate that intact TLR4 signaling promotes ROS production and HMGB1 release by hypoxic hepatocytes.

Antioxidant-mediated hepatic protection during I/R is TLR4 dependent

We next sought to determine if functional TLR4 was required for ROS-mediated I/R injury in the liver. TLR4 mutant (C3H/HeJ) and wild-type control (C3H/HeOuJ) mice were subjected to liver I/R mice and were treated with NAC. As we (2) and others (7) have previously shown, TLR4 mutant mice exhibit less hepatic injury after I/R compared with the wild-type mice. NAC reduced the injury in wild-type mice to the level observed in TLR4 mutant mice but failed to reduce the injury in the TLR4 mutant mice (Fig. 6 A). Histologic evaluation demonstrated severe sinusoidal congestion and hepatocellular necrosis in livers from wild-type mice treated with saline, whereas minimal damage was noted in liver sections from NAC-treated mice or the treated or control TLR4 mutant groups (not depicted). Similar findings were observed when the antioxidant superoxide dismutase was used (unpublished data). As an estimate of oxidant production, we measured the formation of MDA and 4-HAE, markers of ROS-mediated lipid peroxidation, in wild-type and TLR4 mutant mice at 1 h after reperfusion. Changes in lipid peroxidation were found to parallel hepatocellular injury. Although treatment of wild-type mice with NAC resulted in decreased hepatic lipid peroxidation, antioxidant treatment did not further reduce the already lower levels of lipid peroxidation in the TLR4 mutant mice (Fig. 6 B). Inflammatory cytokines, such as TNF and IL-6, have been shown to play key roles in the pathophysiology of hepatic I/R injury (26, 27). Using real-time RT-PCR, we measured steady-state mRNA levels for

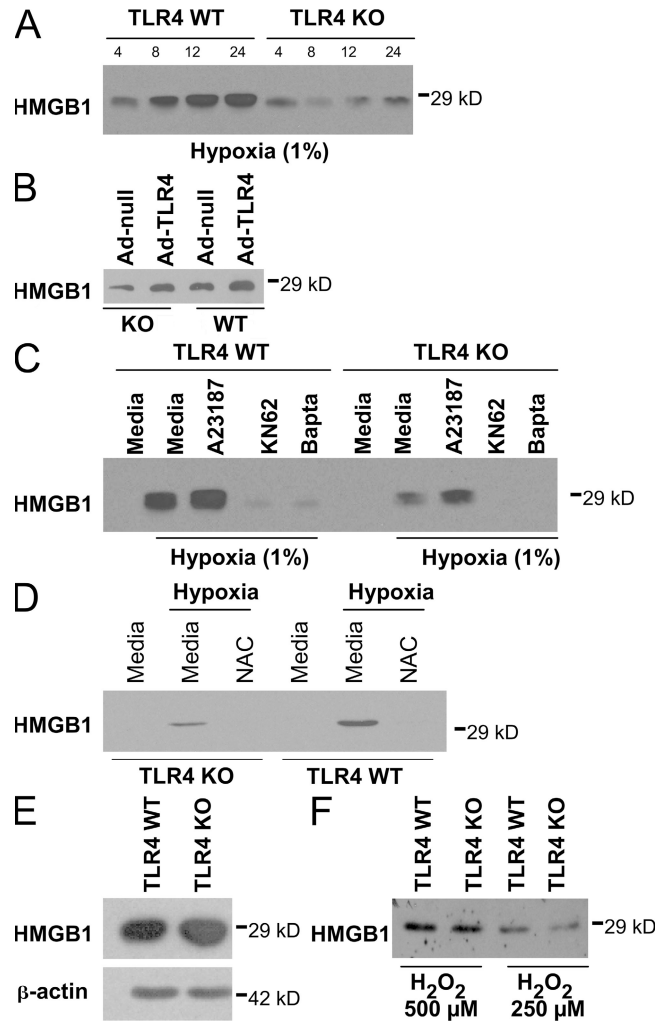


Figure 5. Functional TLR4 signaling required for HMGB1 release from hepatocytes during oxidative stress. (A) TLR4 wild-type and KO hepatocytes were plated and subjected to hypoxic (1% O₂) conditions for various times up to 24 h. The supernatants were sampled at predetermined time points and subjected to Western blot analysis for HMGB1. The blot shown is representative of three different experiments with similar results. (B) Supernatants from Fig. 4 C were sampled at 24 h and subjected to Western blot analysis for HMGB1. The blot shown is a representative blot from multiple experiments. (C) TLR4 wild-type and KO hepatocytes were treated with A23187, KN62, or bapta and subjected to normoxia or hypoxia (1% O₂). Supernatants were sampled at 24 h and subjected to Western blot analysis for HMGB1. The blot shown is representative of three different experiments with similar results. (D) TLR4 wild-type and KO hepatocytes were subjected to hypoxia (1% O₂) with or without 50-mM NAC treatment. Supernatant was sampled at 24 h and subjected to Western blot analysis for HMGB1. The blot shown is representative of three different experiments with similar results. (E) Baseline intracellular HMGB1 levels were determined in TLR4 wild-type and KO hepatocytes. β -actin was used as a loading control. (F) H₂O₂ was used to stimulate TLR4 wild-type and KO hepatocytes to release HMGB1. Cells were subjected to 250 or 500 μ M H₂O₂ for 8 h, and supernatants were subjected to Western blot analysis for HMGB1. The blot shown is representative of three different experiments with similar results.

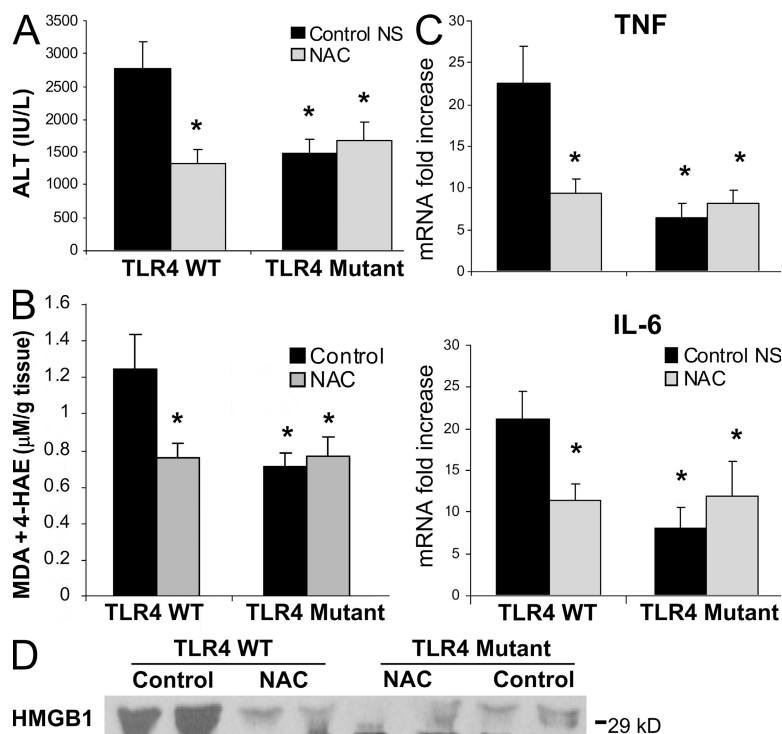


Figure 6. Antioxidant-mediated hepatic protection during I/R is TLR4 dependent. (A) TLR4 wild-type and mutant mice were used for in vivo study of antioxidant effect. Mice were given 300-mg/kg intravenous injections of NAC or control normal saline immediately before ischemia and immediately after reperfusion. Mice underwent 1 h of ischemia and 6 h of reperfusion before serum samples were taken and tested for ALT levels. Data represent means \pm SE ($n = 4-6$ mice per group). *, $P < 0.05$ versus TLR4 wild-type mice that were subjected to I/R and given control normal saline. (B) TLR4 wild-type and mutant mice were treated as described for ischemia and were given 1 h of reperfusion. Hepatic tissue was taken and used for approximating the oxidant stress state by measuring levels of MDA and 4-HAE, lipid peroxidation by-products produced from oxidant stress. Data represent means \pm SE ($n = 4-6$ mice per group). *, $P < 0.05$ versus TLR4 wild-type mice that were subjected to I/R and given control normal saline. (C) Hepatic TNF and IL-6 mRNA expression were obtained in TLR4 wild-type and mutant mice after ischemia and 6 h of reperfusion. Mice were treated as described with NAC or control normal saline. Results are expressed as the relative increase of mRNA expression compared with sham animals. Data represent means \pm SE ($n = 4-6$ mice per group). *, $P < 0.05$ versus TLR4 wild-type mice that were subjected to I/R and given control normal saline. (D) Serum samples from mice subjected to ischemia and 6 h of reperfusion underwent Western blot analysis for HMGB1. Each lane represents a different animal. The blot shown is representative of two experiments with similar results.

these cytokines in the liver after I/R. NAC treatment inhibited the expression of TNF and IL-6 mRNA to the levels seen in TLR4 mutant mice after I/R (Fig. 6 C). Again, NAC had no effect on cytokine mRNA levels in the TLR4 mutant mice subjected to I/R.

To determine if antioxidant treatment affected HMGB1 release during liver I/R in TLR wild-type or mutant mice, serum HMGB1 levels of animals treated with NAC or control saline were examined at 6 h after reperfusion using Western blotting. Circulating HMGB1 levels were increased in the control saline-treated mice over sham animals (Fig. 6 D). NAC treatment prevented this increase. HMGB1 levels increased minimally in TLR4 mutant mice subjected to I/R. These results provide in vivo evidence that HMGB1 release during I/R is mediated, in part, by TLR4 signaling and parallels the extent of oxidant production.

DISCUSSION

Ischemic tissues require mechanisms to notify the immune system of the impending cell damage and possible breach of

tissue integrity. Cells of the innate immune system are activated after ischemic injury to initiate tissue repair processes and to provide defense against microbial invasion. Excessive activation can lead to exaggerated local and systemic inflammation that may extend the tissue damage. In this study, we sought to determine how one of the key alarm molecules released during liver I/R, HMGB1, was mobilized in response to hypoxia. We show that hypoxic hepatocytes release HMGB1 through an active process facilitated by TLR4-dependent ROS production. ROS, in turn, induce HMGB1 release through a CaMK-dependent mechanism. That these events may be important in vivo was shown by experiments in a mouse liver I/R model in which both CaMK inhibitors and antioxidants reduce HMGB1 release and liver damage to the level seen in mice deficient in TLR4 signaling.

HMGB1 is becoming increasingly recognized as the prototypic alarmin (28). Initially identified as a chromatin-binding protein in the nucleus, its cytokine-like properties were revealed in experiments showing that HMGB1 is released by activated macrophages and that it acts as a late mediator of

lethality in sepsis models (16, 17, 29). Ample evidence now exists to indicate the HMGB1 also acts as an early inflammatory mediator in ischemia (2, 7–9), hemorrhagic shock (10), non-infectious hepatitis (19), and peripheral tissue trauma (unpublished data). Both passive (19) and active (30, 31) pathways for HMGB1 release have been described. The passive pathway requires the loss of cell membrane integrity, as seen in necrosis. The active pathway, best described in LPS- or TNF-stimulated macrophage cell lines (30) and cytokine-treated enterocyte cell lines (32), appears to involve HMGB1 hyperacetylation and packaging into secretory vesicles, at least for enterocytes. Recent evidence suggests that phosphorylation on serine residues within the two nuclear localization sequences prevents return of the mobilized HMGB1 to the nucleus (31). We postulated that the initial HMGB1 release in ischemia would be an active process triggered by redox signaling. Indeed, antioxidants prevented HMGB1 release in hypoxic hepatocytes *in vitro* and systemic release of HMGB1 in liver I/R. 250–500 μM H_2O_2 also induced hepatocyte HMGB1 release without causing measurable cell death. Interestingly, exogenous H_2O_2 was also recently shown to induce HMGB1 release from a macrophage cell line in the absence of cell death (33). Not previously shown was the requirement for intact TLR4 signaling for hypoxia-induced ROS production and HMGB1 release, as well as the involvement of CaMK signaling in HMGB1 release.

TLR4 has been shown to be involved in I/R-induced inflammation in several organs in addition to the liver (8, 9). The only molecule known to stimulate TLR4 signaling shown to be involved in I/R-induced injury is HMGB1 (2). We previously reported that hepatocytes express functional TLR4 (34). Furthermore, others have shown that oxidant stress up-regulates TLR4 expression (35). However, the requirement for intact TLR4 signaling for hypoxia-induced ROS production is novel. Whether a TLR4-activating ligand is required for this response is unclear. A neutralizing anti-HMGB1 antibody had no effect on the production of ROS by hypoxic hepatocytes. Therefore, at least the initial signaling is not dependent on the release of HMGB1 into the extracellular space. In HEK294 T cells, TLR4 has been linked to the activation of the NADPH oxidase (36). However, the source of the hypoxia-induced ROS production is unknown. Some HMGB1 release was observed in the absence of TLR4 signaling. This may be caused by production of ROS through other pathways or by the involvement of other signaling events. The suppression of even this lower level of HMGB1 release by CaMK inhibitors suggests that TLR4 serves primarily to amplify ROS production. This reduction but not inhibition of HMGB1 in the absence of hepatocyte TLR4 signaling is consistent with our observations in TLR4 chimeric mice where the absence of TLR4 on only parenchymal cells caused only a minor reduction in I/R-induced inflammatory signaling compared with the reduction seen in an absence of TLR4 signaling on nonparenchymal cells (20). The possibility that even very low levels (<1%) of contaminating nonparenchymal cells in our hepatocyte preparation could contribute to the ROS generation and HMGB1 release remains a possibility. However, hepatocyte purity exceeded

99%, and we have previously shown that hepatocytes express a functional TLR4 (34).

The capacity of redox stress to mobilize calcium in hepatocytes was recognized more than two decades ago (37); however, the involvement of CaMKs in I/R injury has not been previously shown. CaMKs are a family of proteins composed of CaMK I–IV, myosin light chain kinase, and phosphorylase kinase (38, 39). Activation typically requires Ca–calmodulin binding and can be augmented or sustained by phosphorylation. H_2O_2 treatment has been shown to activate CaMKs II and IV in Jurkat T cells (40), and redox activation of CaMKs may occur through both calcium-dependent and -independent pathways (41). Our observation that calcium chelation abrogates hypoxia-induced HMGB1 release suggests that calcium-dependent CaMK activity is required. The specific CaMK family members involved in hypoxia-induced HMGB1 release is uncertain, and we show that hepatocytes express CaMKs I, II, and IV. Based on experiments in other cells, it is possible that more than one isoform could be involved.

The downstream targets for CaMK in triggering HMGB1 release are not known. The greater accumulation of HMGB1 in the cytosolic compartment in the presence of CaMK inhibitors during I/R suggests that HMGB1 secretion and not nuclear mobilization is the process regulated by CaMK activity. Whether CaMKs directly modify HMGB1 is uncertain.

Models of organ I/R have revealed unique insights into how tissues respond to ischemic stress to activate the resident cells of the innate immune system. That the pattern recognition receptors involved in the recognition of pathogens are also used to activate innate immune pathways in response to ischemia or tissue damage is now clear. From this and previous studies, it is important to appreciate that TLR4-dependent signaling is among the most proximal events in I/R-induced inflammation and may occur in response to ischemia alone to trigger downstream signaling events. CaMKs now enter the equation as a potential therapeutic target in acute ischemic events, along with TLR4 and HMGB1. A recent study also points to the importance of resident NKT cells in the early inflammatory response after I/R (42). Whether NKT cell involvement is dependent on TLR4 signaling is not yet known; however, the central role of nonparenchymal cells and, more specifically, immature dendritic cells (43) in the TLR4-dependent responses seems more certain. A scheme whereby I/R induces inflammation through the activation of TLR4 signaling by an endogenously released cell constituent supports tenants of the “Danger Theory” initially proposed by Matzinger (44, 45).

MATERIALS AND METHODS

Materials. HMGB1 polyclonal antibody was obtained from New Zealand white rabbits immunized with the peptide sequence KSEAGKKGPRPTGS, corresponding to amino acids 166–181 of HMGB1. The affinity purification of the polyclonal anti-HMGB1 antibody was performed according to standard procedures (antibody provided by M.T. Lotze, University of Pittsburgh Medical Center, Pittsburgh, PA). KN62, KN92, KN93, A23187, and Bapta were obtained from EMD. All other chemicals and reagents were obtained from Sigma-Aldrich, except where noted.

Animals. Male wild-type (C57BL/6, C57BL/10SnJ, and C3H/HeOuj) and TLR4-defective (KO, C57BL/10ScCr; mutant, C3H/HeJ) mice (8–12 wk old) were purchased from the Jackson Laboratory. Male Sprague-Dawley rats, also pathogen free, weighing 350–400 g were obtained from Charles River Laboratories. All animals were maintained in a laminar-flow, specific pathogen-free atmosphere at the University of Pittsburgh. Animal protocols were approved by the Animal Care and Use Committee of the University of Pittsburgh, and the experiments were performed according to the National Institutes of Health guidelines for the use of laboratory animals.

Liver ischemia. A previously described I/R protocol involving a nonlethal model of segmental (70%) hepatic warm ischemia was used (2). Sham animals underwent anesthesia, laparotomy, and exposure of the portal triad without hepatic ischemia.

Experimental design. For CaMK inhibition studies, mice received 16-mg/kg doses of either KN93 (water-soluble inhibitor of CaMK activity) or KN92 (inactive analogue of KN93) by intraperitoneal injection 12 and 1 h before ischemia. Studies involving antioxidants consisted of mice receiving a 300-mg/kg dose of NAC by intravenous injection immediately before ischemia and immediately after reperfusion. Animals were killed at predetermined time points after reperfusion for serum and liver samples.

Hepatocyte isolation. Hepatocytes were isolated from rats and mice by an in situ collagenase (type IV; Sigma-Aldrich) perfusion technique, which was modified as previously described (46). Hepatocytes were separated from the nonparenchymal cells by two cycles of differential centrifugation (400 rpm [50 g] for 2 min) and further purified over a 30% Percoll gradient. Hepatocyte purity was >98%, as assessed by light microscopy, and viability was typically >95%, as determined by a Trypan blue exclusion assay.

Cell culture and treatment. 3×10^6 hepatocytes were plated onto 6-cm gelatin-coated plastic tissue culture dishes. The initial culture medium was Williams' medium E containing 10% calf serum, 15 mM HEPES, 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin. Hepatocytes were allowed to attach to plates overnight. For experiments involving hypoxia, the medium was replaced with hypoxic medium (equilibrated with 1% O₂, 5% CO₂, and 94% N₂) and placed into a modular incubator chamber (Billups-Rothenberg), which was flushed with the same hypoxic gas mixture. The cells were exposed to hypoxia for various time points for determination of ROS production or HMGB1 release in the supernatants. In TLR4 transfection experiments, hepatocytes were transfected with recombinant adenovirus encoding mouse TLR4 (Ad-TLR4) or empty adenoviral vector control (Ad-null), prepared as previously described (34), and subjected to hypoxia for ROS analysis. For H₂O₂ studies, 4×10^5 hepatocytes per well were plated on sixwell plates and allowed to attach to the plates overnight. The media was replaced before stimulation of the cells with the appropriate amount of H₂O₂ and incubated at 37°C for the times indicated in the figures. Where applicable, hepatocytes treated with NAC or trolox were allowed to incubate for 30 min with H₂O₂ before antioxidant treatment.

Transfection of fluorescein-labeled cyclophilin, CaMKK α , CaMKK β , CaMK I, CaMK IV, and CaMK II δ siRNA. Murine hepatocytes at a concentration of 1.5×10^5 were seeded in each well of a 24-well plate in 1 ml of culture medium to yield a confluence of ~80%. Hepatocytes were allowed to adhere for 4 h. Before transfection, the medium was exchanged for 500 μ l per well of culture medium without antibiotics. Fluorescein-labeled cyclophilin, CaMK I, CaMK IV, CaMKK α , CaMKK β , and CaMK II δ siRNA were obtained (Dharmacon) that incorporate four siRNAs into one siRNA pool. The sequences used for each target were as follows: CaMK I sequence 1, (sense) GAACGAGAUUGCCGUCUUAUU and (antisense) UAAGACGGCAAUCUCGUUCUU; CaMK I sequence 2, (sense) CGGAAGACAUUAGGGAUUAUU and (antisense) AUAUCCCUAAUGUCUCCGUU; CaMK I sequence 3, (sense) GGAGAGCUGUUUGACCAGGUU and (antisense) UUCGGUCAACAGCUCUCCUU; CaMK I sequence 4, (sense)

AUACAGCUCUGGAUAAGAAUU and (antisense) UUCUUAUC-CAGAGCUGUAUUU; CaMK IV sequence 1, (sense) GAGAUCCUCUG-GCGGAUUUUU and (antisense) AAAUCGCCAGAGGAUCUCUU; CaMK IV sequence 2, (sense) UCAAGGAAAUAUUCGAAACUU and (antisense) GUUUCGAAUUAUUCCUUGAUU; CaMK IV sequence 3, (sense) GGUGCUACAUCAUUGUGUUU and (antisense) ACACAAUGGAUGUAGCACC UU; CaMK IV sequence 4, (sense) GGGGAUGAGU-GUCUUUAAAUU and (antisense) UUUAAAGACACUUCUACUCCUU; CaMKK α sequence 1, (sense) GGGCUCAAGUUGGGCUUAAU and (antisense) UAAGGCCCAACUUGAGCCUU; CaMKK α sequence 2, (sense) GCGUGUGUAUCAUGACAUUUU and (antisense) AAUGUCAUGAU-ACACAGC UU; CaMKK α sequence 3, (sense) GGAAGUGCCCUGCGA-CAAGUU and (antisense) CUUGUCGCGAGGGCACUUCUU; CaMKK α sequence 4, (sense) GGAAGUGCCCUGUUCUUGAUU and (antisense) UCAAUGAACGGGCACUUCUU; CaMKK β sequence 1, (sense) GGCUGAGAAUUCAGUCAAAUU and (antisense) UUUGACUGAAU-UCUCGACC UU; CaMKK β sequence 2, (sense) GGACUCUCAUC-CUUAAGUUAUU and (antisense) UACUUAAGGAUGAGAGUCCUU; CaMKK β sequence 3, (sense) GAACGAAUCAUGUGUUUGCUU and (antisense) GCAAACACAUGAUUCGUUCUU; and CaMKK β sequence 4, (sense) UCACACCAGUCUCCAUUAAUU and (antisense) UAAUGGAGACGUGGUGUGAUU. 2.5 pmol siRNA was diluted in 100 μ l of culture medium without serum. 23 μ l of Hiperfect transfection reagent (QIAGEN) was added to the diluted siRNA and mixed. The transfection mixture was incubated for 10 min at room temperature, then applied to each well and incubated for 72 h. Transfection efficiency was determined at 48 h by fluorescence microscopy of cells subjected to RNA interference (RNAi) with fluorescent cyclophilin B siRNA. For each experiment, at least three microscopic visual fields (200-fold magnification) were counted, thereby calculating the ratio of fluorescent cyclophilin-expressing cells to nonfluorescent cells. The specificity of RNAi was determined by Western blot or RT-PCR (RT-PCR was performed to confirm knockdown of CaMK α because there are no commercially available antibodies recognizing this protein). All experiments and cell number determinations were performed in triplicate.

Determination of ROS. 3×10^6 hepatocytes were plated onto 6-cm gelatin-coated plastic tissue culture dishes in Williams' medium E as described in Cell culture and treatment. 2 h before the experiment, cells were washed with cold KHB (pH 7.4) and incubated in warm KHB (pH 7.4). 30 min before the start of the experiment, cells were again washed with KHB (pH 7.4) and incubated with KHB containing 1 μ l/ml of a 10-mM DCF diacetate (DCF-DA) in DMSO stock solution for a final concentration of 10 μ M (Invitrogen). After 30 min of incubation, the media was changed to fresh KHB (pH 7.4) for normoxia cells or hypoxic KHB (pH 6.2, equilibrated with 1% O₂, 5% CO₂, and 94% N₂) and placed into the appropriate incubator. After treatment, cells were scraped on ice and lysed with CellLytic M reagent (Sigma-Aldrich) followed by centrifugation at 10,000 rpm for 10 min. Supernatants were read in a fluorescence spectrophotometer (SpectraMAX Gemini XS; MDS Analytical Technologies) with 485-nm excitation and 530-nm emission wavelengths.

Liver damage assessment. To assess hepatic function and cellular injury after liver ischemia, sALT levels were measured using a clinical chemistry system (opeRA; Bayer Co.).

Measurement of lipid peroxidation. Assessment of the level of oxidative stress in the hepatocytes taken from the in vivo experiments was assessed by measuring by-products of lipid peroxidation, specifically MDA and 4-HAE. This was done according to the protocol described by the Lipid Peroxidation Assay Kit (EMD).

Confocal immunofluorescence imaging. A segment of the left lobe of the liver was fixed in 2% paraformaldehyde in PBS overnight and cryoprotected in 30% sucrose in PBS for another 24 h. Livers were frozen in liquid nitrogen-cooled isopentane. These samples were stored at -80°C until sectioned. 6- μ m

sections were washed three times with PBS and blocked with 2% BSA in PBS for 45 min, followed by three washes with 0.5% BSA in PBS. This was followed by anti-HMGB1 antibody (1:1,000 in 0.5% BSA) for 60 min at room temperature. The slides were washed with 0.5% BSA, and a secondary antibody (goat anti-rabbit Alexa Fluor 488, 1:500 in 0.5% BSA; Invitrogen) was applied with F-actin counterstain (rhodamine phalloidin, 1:250 in PBS; Invitrogen) for another 60 min. The slides were rinsed and HOECHST dye (1 mg/100 ml bisbenzimidazole) was applied for 30 s. The slides were rinsed with PBS and covered with gelvatol, a water-soluble mounting media (21 g polyvinylalcohol, 52 ml water, sodium azide, 106 ml 0.2-M Tris buffer). Slides were visualized with a confocal microscope (Fluoview 1000; Olympus).

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis and Western blotting. Western blot analysis for HMGB1 was as follows. Primary polyclonal rabbit antibody to HMGB1 (1:1,000) was used for Western blotting. Membranes were developed with the Super Signal West Pico chemiluminescent kit (Thermo Fisher Scientific) and exposed to film.

SYBR green real-time RT-PCR. Total RNA was extracted from the liver using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. mRNA for TNF- α , IL-6, and GAPDH was quantified in duplicate by SYBR green two-step, real-time RT-PCR. After removal of potentially contaminating DNA with DNase I (Invitrogen), 1 μ g of total RNA from each sample was used for reverse transcription with oligo dT (Invitrogen) and Superscript II (Invitrogen) to generate first-strand cDNA. The PCR reaction mixture was prepared using SYBR green PCR Master Mix (Applied Biosystems) using previously described primers (47, 48). Thermal cycling conditions were 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min on a sequence detection system (ABI PRISM 7000; Applied Biosystems). Each gene expression was normalized with GAPDH mRNA content.

Statistical analysis. Results are expressed as the mean \pm SEM. Group comparisons were performed using the Student's *t* test or analysis of variance. Differences were considered significant at $P < 0.05$.

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