Role of Heat Shock Protein 70 in Hepatic Ischemia/Reperfusion Injury in Mice

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Running Head: HSP70 and liver I/R injury

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Abstract

It is well established that liver ischemia/reperfusion induces the expression of HSP70. However, the biological function of HSP70 in this injury is unclear. In this study, we sought to determine the role of HSP70 in hepatic ischemia/reperfusion injury in mice. Male mice were subjected to 90 minutes of partial hepatic ischemia followed by up to 8 hours of reperfusion. HSP70 was rapidly upregulated after reperfusion. To explore the function of HSP70, sodium arsenite (8 mg/kg, i.v.) was injected prior to surgery. We found that this dose induced HSP70 expression within 6 hours of treatment. Induction of HSP70 with arsenite resulted in a >50% reduction in liver injury as determined by serum transaminases and histology. In addition, arsenite similarly reduced liver neutrophil recruitment, liver NF-κB activation, and attenuated serum levels of TNFα and MIP-2, but increased levels of IL-6. In HSP70-knockout mice, arsenite did not protect against liver injury but did reduce liver neutrophil accumulation. Arsenite-induced reductions in neutrophil accumulation in HSP70-knockout mice were found to be mediated by IL-6. To determine whether extracellular HSP70 contributed to the injury, recombinant HSP70 was injected prior to surgery. Intravenous injection of 10µg of recombinant HSP70 had no effect on liver injury after ischemia/reperfusion. The data suggest that intracellular HSP70 is directly hepatoprotective during ischemia/reperfusion injury and that extracellular HSP70 is not a significant contributor to the injury response in this model. Targeted induction of HSP70 may represent a potential therapeutic option for post-ischemic liver injury.

Key Words: liver, inflammation, neutrophils, cytokines
Introduction

Ischemia and reperfusion of the liver often occurs as a result of trauma, sepsis and liver surgery. This hepatocellular dysfunction/cell death caused by this insult results in severe intracellular oxidative stress as well as oxidant mediated killing by recruited neutrophils (16,17). Hepatocytes possess a number of intracellular cytoprotectants that help protect the cell from oxidative injuries. Notable amongst these are the antioxidants glutathione, superoxide dismutase, and heme oxygenase (10,12,35). These systems have been well studied and are known to counter oxidative damage to hepatocytes during ischemia/reperfusion injury. Another cytoprotectant protein, heat shock protein 70 (HSP70), is known to be expressed during hepatic ischemia/reperfusion (4), but whether it plays a significant role in protecting hepatocytes from injury during ischemia/reperfusion has not been elucidated.

HSP70 is a 70 kD protein originally described as a molecular chaperone and is intimately involved in trans-mitochondrial protein transport (18). In addition to its properties as a chaperone, HSP70 also functions as an important cytoprotectant. A number of studies have examined the protective nature of HSP70 to a variety of insults and have found that it is highly protective against oxidative stress and apoptosis. Overexpression of HSP70 in WEHI-S cells protected them from TNF-dependent and free radical-dependent killing by monocytes (15). In parallel, overexpression of HSP70 in the hepatocyte cell line, HepG2, protected against H$_2$O$_2$-induced killing in association with stabilization of the mitochondrial membrane potential (13). Likewise, induction of HSP70 by geranylgeranylacetone prevented hepatocyte apoptosis induced by H$_2$O$_2$ (14). The mechanism by which HSP70 inhibits apoptosis has been well studied. There is abundant evidence that HSP70 inhibits events upstream of caspase-3, and multiple laboratories
have reported that HSP70 binds to the apoptotic-protease-activating factor-1 (Apaf-1) and prevents the recruitment of procaspase-9 to the apoptosome (3,22,31).

HSP70 gene expression is regulated by the transcription factor, heat shock factor 1 (HSF1) (18). HSF1 exists in the cytoplasm of resting cells in monomeric form. With cellular stress, such as trauma/shock or ischemia/hypoxia, intracellular [Ca$^{2+}$] increases and PKC phosphorylates HSF1 monomers leading to their trimerization (8,26). HSF1 trimers then translocate into the nucleus, bind DNA and induce transcription of HSP70. Intracellular HSP70 has been shown to directly interact with the transcription factor, NF-κB, and prevent its activation (11). NF-κB is a transcription factor that is thought to have both proinflammatory and hepatoprotective roles in the hepatic response to ischemia/reperfusion (9,20,23,28). Thus, interference by intracellular HSP70 of NF-κB activation following hepatic ischemia/reperfusion may represent another protective mechanism.

In contrast, release of HSP70 into the extracellular compartment (i.e. serum) has also been documented. Extracellular HSP70 has been proposed to function as a proinflammatory cytokine, signaling through toll-like receptors 2 and 4 (2). Serum levels of HSP70 in trauma patients correlates with survival, whereas in patients undergoing liver resection serum HSP70 was associated with liver dysfunction (19,29). We have previously shown that serum levels of HSP70 are elevated following hepatic ischemia/reperfusion (27), however the significance of this finding has not been established. In the current studies, we sought to examine the function of HSP70 during hepatic ischemia/reperfusion injury. To do this, we utilized HSP70-knockout mice as well as sodium arsenite (as an inducer of HSP70 expression).
Materials and Methods

Hepatic Ischemia/Reperfusion Injury Model. Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME), and wild-type (on a B6129F1/Tac background) or HSP70-knockout mice (Taconic, Germantown, NY) were used in these experiments. All mice weighed 25-30g. This project was approved by the University of Cincinnati Animal Care and Use Committee and was in compliance with the National Institutes of Health guidelines. The animals underwent either sham surgery, or non-lethal ischemia/reperfusion. Partial hepatic ischemia was induced as described previously (21). Briefly, mice were anaesthetized with sodium pentobarbital (60mg/kg, i.p.). A midline laparotomy was performed and an atraumatic clip was used to interrupt blood supply to the left lateral and median lobes of the liver. After 90 minutes of partial hepatic ischemia, the clip was removed to initiate hepatic reperfusion. Sham control mice underwent the same protocol without vascular occlusion. Some mice were pretreated with normal saline or 8 mg/kg sodium arsenite intravenously prior to induction of ischemia. Additionally, some mice were injected intravenously with 3 mg/kg non-specific IgG or rabbit polyclonal antibodies to interleukin-6 (IL-6) 30 minutes prior to ischemia. Other mice were injected intravenously with 10µg recombinant human HSP70 (rHSP70) 2 hours prior to ischemia. rHSP70 was generated as previously described (37). There was a low level of contamination by lipopolysaccharide (9.8 ng/µg) in these preparations, therefore 98 ng of lipopolysaccharide (E. coli, serotype 0111:B4, Sigma Chemical Co. St. Louis, MO) was added to the vehicle (phosphate buffered saline). Mice were sacrificed after the indicated periods of reperfusion and blood and samples of the left lateral lobe were taken for analysis. Liver samples were not pooled for analysis.
**Western Blot Analyses.** Liver samples were homogenized in lysis buffer (10 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.6% NP-40, 0.5 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotonin, 10 µg/ml soybean trypsin inhibitor, 1 µg/ml pepstatin) on ice. Homogenates were sonicated and centrifuged at 5,000 rpm to remove cellular debris. Protein concentrations were determined as described for nuclear extracts. Samples containing equal amounts of protein in equal volumes of sample buffer were separated in a denaturing 10% polyacrylamide gel and transferred to a 0.1 µm pore nitrocellulose membrane. Nonspecific binding sites were blocked with TBS (40 mM Tris, pH 7.6, 300 mM NaCl) containing 5% non-fat dry milk for 12 hours at 4°C. Membranes were then incubated with antibodies to HSP70 or IκBα (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS with 0.1% Tween 20 (TBST). Membranes were washed and incubated with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive proteins were detected by enhanced chemiluminescence.

**Electrophoretic Mobility Shift Assay.** Nuclear extracts of liver tissue were prepared by the method of Deryckere and Gannon (7), and analyzed by electrophoretic mobility shift assay (EMSA). Briefly, double-stranded consensus oligonucleotides to NF-κB (Promega, Madison, WI) or HSF1 (University of Cincinnati DNA Core) were end-labeled with γ[^32P] ATP(3,000 Ci/mmol at 10mCi/ml, Amersham, Arlington Heights, IL). Binding reactions (total volume 15 µl) containing equal amounts of nuclear protein extract (20 µg) and 35 fmols (~50,000 cpm, Cherenkov counting) of oligonucleotide and were incubated at room temperature for 30 minutes. Binding reaction products were separated in a 4% polyacrylamide gel and analyzed by autoradiography.
Liver Neutrophil Accumulation. Liver myeloperoxidase (MPO) content was assessed by methods described elsewhere (33). Briefly, liver tissue (100 mg) was homogenized in 2ml of buffer A (3.4 mmol/L KH₂HPO₄, 16 mmol/L Na₂HPO₄, pH 7.4). After centrifugation for 20 minutes at 10,000 × g, the pellet was resuspended in 10 volumes of buffer B (43.2 mmol/L KH₂HPO₄, 6.5mmol/L Na₂HPO₄, 10mmol/L EDTA, 0.5% hexadecyltrimethylammonium, pH 6.0) and sonicated for 10 seconds. After heating for 2 hours at 60°C, the supernatant was reacted with 3,3’,3,5’-tetramethylbenzidine, and the optical density was read at 655nm.

Blood and Tissue Analysis. Blood was obtained by cardiac puncture for analysis of serum alanine amino transferase (ALT) as an index of hepatocellular injury. Measurements of serum ALT were made using a diagnostic kit (Wiener Laboratories, Rosario, Argentina). Serum levels of TNFα, macrophage inflammatory protein-2 (MIP-2), and IL-6 were measured by sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer’s instructions (R&D Systems, Minneapolis, MN). Liver tissues were fixed in 10% formalin and then embedded in paraffin for light microscopy. Sections were stained with hematoxylin and eosin (H&E) for histological examination.

Statistical analysis. All data are expressed as mean ± SEM. Data were analyzed with a one-way analysis of variance with subsequent Student-Newman-Keuls test. Differences were considered significant when \( P<0.05 \).
Results

Induction of HSP70 during Ischemia/Reperfusion and After Treatment with Sodium Arsenite. HSP70 has been shown to be expressed in other models of liver ischemia/reperfusion (4). We have previously documented that HSP70 is induced after ischemia and 8 hours of reperfusion (27). To determine the kinetics of HSP70 protein expression in the liver after ischemia/reperfusion, we subjected liver protein extracts to Western blot analyses. Liver HSP70 was not induced by ischemia alone (Figure 1). However, within 1 hour of reperfusion, HSP70 expression was maximal and remained elevated for up to 8 hours after reperfusion. In order to determine if HSP70 could be induced in the liver, we injected 8 mg/kg sodium arsenite intravenously and examined HSP70 expression by Western blot. Hepatic HSP70 expression peaked 6 hours after injection in otherwise unmanipulated mice (Figure 2A). In all subsequent experiments with sodium arsenite, mice were injected 6 hours prior to ischemia or sham surgery. We next assessed whether hepatic ischemia/reperfusion induced DNA-binding of HSF1 with or without arsenite treatment. In mice undergoing sham surgery, there was no HSF1 activation detected in mice pretreated with saline (Figure 2B). However, pretreatment with arsenite caused activation of HSF1. Hepatic ischemia and 1 hour of reperfusion resulted in a similar increase in HSF1 activation. When mice were pretreated with arsenite and then subjected to hepatic ischemia/reperfusion, HSF1 activation was much greater than that in mice pretreated with saline (Figure 2B). The degree of HSF1 activation was directly related to the amount of HSP70 protein expressed after ischemia and 1 hour of reperfusion; mice pretreated with arsenite had higher HSP70 expression than mice pretreated with saline (Figure 2C).
Effects of Arsenite Pretreatment and Associated HSP70 Induction on Liver Ischemia/Reperfusion Injury. We next assessed if pretreatment with sodium arsenite had any effect on ischemia/reperfusion injury. Mice pretreated with saline and subjected to ischemia and 8 hours of reperfusion had the expected level of liver injury and inflammation (Figure 3). In these mice, serum ALT was significantly elevated above sham-operated mice (Figure 3A), as was the amount of neutrophil accumulation, as determined by liver MPO (Figure 3B). Histopathology showed that mice pretreated with saline followed by sham surgery had normal hepatic architecture (Figure 3C). However, mice pretreated with saline followed by ischemia/reperfusion had substantial hepatocellular necrosis and neutrophilic infiltrates (Figure 3D). Pretreatment with sodium arsenite reduced liver injury and inflammation as evidenced by reduced serum levels of ALT and liver MPO content (Figure 3A and B, respectively). Furthermore, mice pretreated with arsenite undergoing sham surgery had normal hepatic architecture (Figure 3E), while those undergoing ischemia/reperfusion (Figure 3F) displayed far less hepatocellular necrosis and neutrophil recruitment than those pretreated with saline (Figure 3D).

We next determined whether pretreatment with arsenite had any effects on NF-κB, an inflammation-associated transcription factor known to be involved in this injury. Pretreatment with arsenite in mice undergoing sham surgery resulted in modest activation of liver NF-κB compared to mice pretreated with saline (Figure 4A). Conversely, mice pretreated with saline and undergoing hepatic ischemia/reperfusion had very high levels of NF-κB activation. Pretreatment with arsenite markedly reduced NF-κB activation induced by ischemia/reperfusion (Figure 4A). This was associated with decreased degradation of IκBα (Figure 4B). In mice
undergoing ischemia/reperfusion, IκBα was almost fully degraded in mice receiving saline pretreatment, whereas mice receiving arsenite had far less degradation of IκBα (Figure 4B).

NF-κB is known to regulate the expression of a number of inflammatory cytokines, including TNFα and MIP-2, and HSF1 is known to regulate IL-6 (30). Because each of these cytokines is known to contribute to (TNFα and MIP-2) or protect against (IL-6) hepatic ischemia/reperfusion injury (5,6,21), we assessed the levels of these mediators. In saline-pretreated mice, all three cytokines were elevated after ischemia and 1 or 8 hours of reperfusion (Figure 5). Pretreatment with arsenite resulted in significantly decreased TNFα and MIP-2 expression. Interestingly, arsenite pretreatment itself resulted in elevated IL-6 expression in sham-operated mice (Figure 5). After 1 hour of reperfusion, arsenite-treated mice had much higher levels of serum IL-6 than saline-treated mice. However, after 8 hours of reperfusion, IL-6 levels in arsenite-treated mice were lower than those in saline-treated mice (Figure 5).

**Extracellular HSP70 Does Not Affect Hepatic Ischemia/Reperfusion Injury.** Because extracellular HSP70 has been implicated in recovery from traumatic injury (28), as well as organ dysfunction after liver resection (19), it is unclear as to whether circulating HSP70 plays a positive or negative role in organ injury. In order to determine if hepatic ischemia/reperfusion injury could be modulated by circulating HSP70, we evaluated the effects of recombinant human HSP70 in our model of injury. Mice underwent hepatic ischemia and were injected intravenously with either vehicle or 10 µg of rHSP70 2 hours prior to ischemia. The 10 µg dose has been shown to result in substantial hepatic uptake of rHSP70 (34). Injection of rHSP70 had no effects on liver injury (serum ALT) or neutrophil accumulation (MPO content) after 8 hours of reperfusion (Figure 6).
HSP70 Does Not Influence Hepatic Inflammation. To more directly examine the function of HSP70 during hepatic ischemia/reperfusion injury, we employed HSP70-knockout mice. Unexpectedly, HSP70-knockout mice did not differ from wild-type mice in their response to hepatic ischemia/reperfusion (Figure 7). HSP70-knockout mice had similar serum ALT and liver MPO content as wild-type mice (Figure 7A and B, respectively). In addition, no differences were observed in liver histopathology. Both wild-type and HSP70-knockout mice undergoing sham surgery had normal liver architecture (Figure 7C and F, respectively). After ischemia and 8 hours of reperfusion, hepatocellular necrosis and neutrophilic infiltration were similar in wild-type and HSP70-knockout mice (Figure 7D and G, respectively). We next evaluated whether the effects observed with arsenite treatment were due to HSP70. In these experiments, wild-type and HSP70-knockout mice were pretreated with arsenite and evaluated for liver injury after ischemia/reperfusion. Unlike its effects in wild-type mice, arsenite showed no benefit on liver injury in HSP70-knockout mice (Figure 7A). However, arsenite treatment reduced liver MPO content in both wild-type and HSP70-knockout mice (Figure 7B). These results were supported by histology, with wild-type mice that were pretreated with arsenite showing reduced amounts of necrosis and neutrophil accumulation (Figure 7E) and HSP70-knockout mice treated with arsenite showing high levels of necrosis but reduced neutrophil infiltration (Figure 7H).

Finally, because our earlier results showed that arsenite pretreatment induced IL-6 production (Figure 5), and that knockout of HSP70 had no effect on neutrophil recruitment, we next examined whether increased IL-6 production may be responsible for the reduced neutrophil accumulation. HSP70-knockout mice were pretreated with saline or arsenite and subsequently treated with anti-IL-6 antibodies 30 minutes prior to induction of ischemia. HSP70-knockout
mice pretreated with saline and receiving anti-IL-6 had similar liver MPO content after ischemia and 8 hours of reperfusion as mice receiving only saline pretreatment (Figure 8). However, blockade of IL-6 partially reversed the arsenite-induced reduction in liver MPO content (Figure 8), suggesting that arsenite-induced IL-6 was at least partially responsible for the reduction in neutrophil accumulation.
Discussion

The present study suggests that HSP70 may be hepatoprotective during ischemia/reperfusion injury. While previous studies, including our own work, have shown that HSP70 is upregulated after liver reperfusion (4,27), the manner in which HSP70 protects against liver injury has not been previously studied. Here we show that HSP70 is induced early after reperfusion and its expression remains elevated for at least 8 hours after reperfusion. This increased expression coincided with increased activation of the transcription factor, HSF1. Interestingly, direct application of HSP70-knockout mice in these studies revealed no phenotype, and analysis of these studies alone would suggest that HSP70 is not an important protein in this response. However, the lack of phenotypic differences in knockout versus wild-type mice may be due to genetic compensation. While our data do not confirm this possibility, we show that treatment with sodium arsenite induces HSP70 expression in the liver and that this is accompanied by marked decreases in liver injury after ischemia/reperfusion. In HSP70-knockout mice, treatment with arsenite did not reduce liver injury, suggesting that HSP70 is required for the hepatoprotective effects of arsenite. Supporting this concept are previous in vitro studies that demonstrated that overexpression of HSP70 in HepG2 cells limited H₂O₂-induced cell death (13).

Interestingly, the reduction in neutrophil accumulation by arsenite treatment was also observed in HSP70-knockout mice. Thus, it appears that the protective effects of HSP70 are independent of the inflammatory process, or at least independent of the hepatic recruitment of neutrophils. This leaves open the manner in which sodium arsenite attenuates this response. Treatment with arsenite increased HSF1 activity and HSF1 activation has been linked to inhibition of NF-κB activation and repression of proinflammatory cytokine expression in a
number of other experimental systems (1, 25, 39). NF-κB is a well-known transcriptional regulator of proinflammatory cytokines as well as cytoprotective proteins (9, 28). The role of NF-κB in hepatic ischemia/reperfusion injury has not been fully elucidated. Activation of NF-κB in whole liver during ischemia/reperfusion injury is an indicator of the extent of injury. The precise function of NF-κB activation in hepatocytes during this response is controversial and there is evidence to support both protective (20) and injurious (23) actions. Increased activation of HSF1, as observed after arsenite treatment, would be expected to reduce both NF-κB activation as well as proinflammatory cytokine expression, which is precisely the profile we observed (Figure 9). Secondly, it is known that HSF1 regulates IL-6 expression (30), and that IL-6 reduces hepatic ischemia/reperfusion-induced inflammation (5). In our studies, we found that neutralization of IL-6 in HSP70-knockout mice partially reversed arsenite-mediated reductions in neutrophil accumulation. These data suggest that HSF1 activated following ischemia/reperfusion and augmented by arsenite treatment, results in induction of both HSP70 and IL-6. HSP70 appears to have direct protective effects on hepatocytes and IL-6 appears to function in part by attenuating the recruitment of neutrophils to the injured liver (Figure 9).

A limitation of our studies is the effects of arsenite other than induction of HSP70. Arsenite is a pharmacological inducer of the heat shock response, of which HSP70 induction is but one component. It is known that components of the heat shock response other than HSP70 may interfere with NF-κB signaling and resultant cytokine expression (24). It is therefore possible that some of the arsenite effects observed in our studies were unrelated to its induction of HSP70. However, our data with HSP70-knockout mice do demonstrate a requirement for HSP70 for the hepatoprotection afforded by arsenite.
We have previously reported that serum levels of HSP70 are elevated following hepatic ischemia/reperfusion (27). Elevated circulating HSP70 has also been demonstrated during trauma, septic shock, myocardial infarction and liver resection (19,29,32,38). In liver resection and myocardial infarction, circulating HSP70 was correlated with increased infection or systemic inflammation and poor outcome, whereas in trauma, circulating levels correlate with survival (19,29,32,38). Extracellular HSP70 has been shown to signal via toll-like receptors 2 and 4 (TLR2 and TLR4) (2). Interestingly, TLR4 expressed on Kupffer cells has been shown to contribute to the development of hepatic ischemia/reperfusion injury (36). In our studies, administration of rHSP70 did not alter hepatic ischemia/reperfusion injury, suggesting that the infused HSP70 does not have any protective or injurious function in this setting.

In summary, induction of HSP70 with sodium arsenite resulted in significant protection from liver injury and inflammation. The hepatoprotective effects of arsenite were due specifically to HSP70 as HSP70-knockout mice treated with arsenite showed no protection against injury. However, the reduction in liver inflammation observed with arsenite treatment was not related to HSP70 but appeared to be, at least partly, dependent upon arsenite-induced IL-6. Intravenous injection of rHSP70 did not change the degree of inflammatory injury after ischemia/reperfusion. These data suggest that intracellular HSP70 is hepatoprotective during ischemia/reperfusion injury and that extracellular HSP70 has no impact on the injury response in this model. Targeted induction of HSP70 may be a potentially important therapeutic option for post-ischemic liver injury.
Grants

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References


Figure Legends

**Figure 1.** Hepatic expression of HSP70 during ischemia/reperfusion injury. HSP70 protein was assessed by Western blot. std, molecular weight standards; PC, positive control (activated macrophage cell lysates, Santa Cruz Biotechnology, sc-24767). Chemiluminescence films were quantitated by image analysis. Results are representative of duplicate experiments.

**Figure 2.** Effects of sodium arsenite on HSP70 expression and activation of HSF1. (A) Unmanipulated mice were injected intravenously with 8 mg/kg sodium arsenite and HSP70 protein expression in the liver was monitored for up to 48 hours thereafter by Western blot. Chemiluminescence films were quantitated by image analysis. Data are mean ± SEM with n=3-4 per group with $P<0.05$ compared to: *0, 3, 24, and 48 hour groups, †0 and 3 hour groups. (B) Mice were injected intravenously with saline or 8 mg/kg sodium arsenite 6 hours prior surgery. After ischemia and 1 hour of reperfusion, liver nuclear extracts were assessed by electrophoretic mobility shift assay for HSF1 activation. Results were quantitated by image analysis of autoradiograms. Data are mean ± SEM with n=3-4 per group with *$P<0.05$ compared to saline-treated groups. (C) Hepatic HSP70 protein expression in mice pretreated with saline or sodium arsenite prior to ischemia and 1 hour of reperfusion. std, molecular weight standards; PC, positive control (activated macrophage cell lysates, Santa Cruz Biotechnology, sc-24767). Chemiluminescence films were quantitated by image analysis. Data are mean ± SEM with n=5 per group with *$P<0.05$ compared to the saline-treated group.

**Figure 3.** Effects of sodium arsenite on hepatic ischemia/reperfusion injury. Mice were injected intravenously with saline or 8 mg/kg sodium arsenite 6 hours prior to surgery. Samples were
analyzed after ischemia and 8 hours of reperfusion. (A) Liver injury was measured by serum levels of alanine aminotransferase (ALT). Data are mean ± SEM with n=6-9 per group. *P<0.05 compared to saline-treated mice. (B) Neutrophil accumulation was determined by liver content of myeloperoxidase (MPO). Data are mean ± SEM with n=6-9 per group. *P<0.05 compared to saline-treated mice. Liver histology in sham-operated mice pretreated with saline (C) or arsenite (E) showed normal hepatic architecture. After ischemia/reperfusion, livers from saline-pretreated mice had large areas of necrosis with marked neutrophilic infiltrates (D), whereas mice pretreated with arsenite had far less evidence of necrosis or neutrophil accumulation (F). For C-F, original magnification was 50X.

**Figure 4.** Effects of sodium arsenite on hepatic NF-κB activation and IκBα protein degradation during ischemia/reperfusion injury. Mice were injected intravenously with saline or 8 mg/kg sodium arsenite 6 hours prior to surgery. Samples were analyzed after ischemia and 1 hour of reperfusion. (A) Liver nuclear extracts were analyzed by electrophoretic mobility shift assay for NF-κB activation. Results were quantitated by image analysis of autoradiograms. Data are mean ± SEM with n=3-4 per group with *P<0.05 compared to saline-treated groups. (B) Liver lysates were assessed by Western blot for protein expression of IκBα. Chemiluminescence films were quantitated by image analysis. Data are mean ± SEM with n=3-4 per group with *P<0.05 compared to the saline-treated group.

**Figure 5.** Effects of sodium arsenite on serum levels of TNFα, MIP-2 and IL-6 during hepatic ischemia/reperfusion injury. Mice were injected intravenously with saline or 8 mg/kg sodium arsenite 6 hours prior to surgery. Serum samples were analyzed by enzyme-linked
immunosorbent assay for TNFα, MIP-2 and IL-6 after ischemia and 1 or 8 hours of reperfusion. Data are mean ± SEM with n=5-9 per group. *P<0.05 compared to saline-treated mice.

**Figure 6.** Effects of recombinant HSP70 on hepatic ischemia/reperfusion injury. Ten micrograms of rHSP70 was injected intravenously 2 hours prior to surgery. This dose and time schedule was chosen based on a previous report demonstrating significant hepatic uptake of rHSP70 at this time point (26). Liver injury was measured by serum levels of alanine aminotransferase (ALT) and neutrophil accumulation was determined by liver content of myeloperoxidase (MPO) after 8 hours of reperfusion. Data are mean ± SEM with n=6 per group.

**Figure 7.** Response of HSP70-knockout mice to hepatic ischemia/reperfusion injury. Wild-type or HSP70-knockout mice were injected intravenously with saline or 8 mg/kg sodium arsenite 6 hours prior to surgery. Samples were analyzed after ischemia and 8 hours of reperfusion. (A) Liver injury was measured by serum levels of alanine aminotransferase (ALT). Data are mean ± SEM with n=4-7 per group. *P<0.05 compared to saline-treated mice. (B) Neutrophil accumulation was determined by liver content of myeloperoxidase (MPO). Data are mean ± SEM with n=4-6 per group. *P<0.05 compared to saline-treated mice. Liver histology in sham-operated wild-type (C) or HSP70-knockout (F) mice pretreated with saline showed normal hepatic architecture. After ischemia/reperfusion, livers from saline-pretreated wild-type (D) and HSP70-knockout (G) mice had large areas of necrosis with marked neutrophilic infiltrates. In contrast, livers from arsenite-pretreated wild-type mice, had far less liver injury and neutrophil accumulation (E), whereas HSP70-knockout mice had marked necrosis with reduced neutrophilic infiltrates (H). For C-H, original magnification was 50X.
**Figure 8.** Effects of anti-IL-6 on hepatic neutrophil recruitment in HSP70-knockout mice after ischemia and 8 hours of reperfusion. HSP70-knockout mice were injected intravenously with saline or 8 mg/kg sodium arsenite 6 hours prior to surgery. In addition, mice were injected intravenously with non-specific (ns) IgG or anti-IL-6 30 minutes prior to surgery. Neutrophil accumulation was determined by liver content of myeloperoxidase (MPO). Data are mean ± SEM with n=4-6 per group. *P<0.05, *compared to saline-treated mice; †compared to arsenite + ns IgG.

**Figure 9.** Proposed pathways by which arsenite treatment reduces hepatic ischemia/reperfusion injury. ↓ indicates an activating pathway; ⊥ indicates an inhibitory pathway.
### HSP70 Protein Expression (RIU)

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**Figure:**
- **Top:** Western blot analysis showing HSP70 protein expression at different time points after reperfusion.
- **Bottom:** Bar graph depicting HSP70 protein expression levels in sham and after various hours of reperfusion.
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NF-κB

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IκB

NF-κB Activation (RIU)

IκB Protein Expression (RIU)
MPO (U/g)

- Saline + ns IgG
- Saline + arsenite + ns IgG
- Saline + anti-IL-6
- Saline + arsenite + anti-IL-6

* and † indicate significant differences.