High-Mobility Group Box 1 Contributes to Lethality of Endotoxemia in Heme Oxygenase-1-Deficient Mice

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High-mobility group box 1 (HMGB1) is a nuclear protein that has been found to be a critical mediator of lethality in endotoxemia and sepsis. During the systemic inflammatory response, circulating levels of HMGB1 are increased, but in a delayed fashion compared with early inflammatory mediators. To counteract the inflammatory response of endotoxemia, a secondary anti-inflammatory response ensues in an attempt to prevent inflammation-induced tissue injury. One such cytoprotective gene that is induced during endotoxemia is heme oxygenase (HO)-1. HO-1, and its products of heme metabolism, possess anti-inflammatory and antioxidant properties to counter the damaging effects of endotoxemia. In the present study, we wanted to determine whether tissue and circulating levels of HMGB1 are increased further in the absence of HO-1 during endotoxemia, and whether this increase may contribute to the pathobiology of endotoxemia. Lung inflammation, HMGB1 protein levels, and expression of HMGB1 in inflammatory cells were increased in HO-1^{-/-} mice compared with HO-1^{+/+} mice. After the administration of LPS, tissue levels of HMGB1 were not increased further in HO-1^{-/-} mice; however, circulating levels of HMGB1 were higher when compared with HO-1^{+/+} mice. HO-1^{-/-} mice treated with a carbon monoxide-releasing molecule or biliverdin showed a reduction in plasma HMGB1, which was associated with a marked improvement in survival. HO-1^{-/-} mice given HMGB1-neutralizing antibody showed improvement in survival compared with control antibody. These data suggest that exaggerated circulating levels of HMGB1 contribute to endotoxin-induced mortality in the absence of HO-1.

Keywords: endotoxemia; heme oxygenase-1; inflammation; high-mobility group box 1; oxidative stress

Heme oxygenase (HO) enzymes are important cytoprotective enzymes that are critical for physiologic homeostasis (1–4). The inducible isoform, HO-1, responds rapidly to diverse physical and chemical stimuli by transcriptional activation and increased expression (5); however, HO-1 is also expressed under basal

CLINICAL RELEVANCE

These results may have implications for patients at increased risk for inflammation and oxidative stress. A deficiency of heme oxygenase (HO)-1 leads to increased lung inflammation and enhanced release of HMGB1 during endotoxemia. These data provide further insight into the pathophysiology of endotoxemia, and additional support for the therapeutic potential of HO-1-derived heme metabolites in inflammatory disease processes.

conditions. HO enzymes catalyze the first and rate-limiting step in the oxidative degradation of heme, resulting in the production of carbon monoxide (CO), biliverdin, and ferrous iron (2, 3, 6). Biliverdin is converted to bilirubin, a potent endogenous antioxidant (7, 8), and recently it has been recognized that biliverdin also has anti-inflammatory properties (9). CO also has numerous biological functions, with anti-inflammatory properties (1–4) being vital for its biological actions. Thus, these two products of heme catabolism have overlapping properties that may have benefit to counteract a systemic inflammatory stimulus. Using HO-1-deficient mice, it has been shown that the HO-1 enzyme is important for defending the body against inflammatory and oxidant-induced cellular and tissue injury (10–13). Previous work in our laboratory has shown, in a mouse model of endotoxemia, that an absence of HO-1 leads to increased oxidative stress, end-organ injury, and death (13).

The anti-inflammatory properties of HO-1, and the products of heme catabolism, have been shown to involve inhibition of early proinflammatory cytokines (e.g., TNF- α and IL-1 β) and induction of the anti-inflammatory cytokine, IL-10 (14). The balance of pro- and anti-inflammatory mediators characterizes the pathophysiologic response to endotoxemia, and, if an exaggerated proinflammatory response ensues, this will lead to enhanced oxidative stress and mortality. It has been suggested that the absence of HO-1 correlates with a shift toward a proinflammatory environment (15). Proinflammatory cytokines, such as TNF- α and IL-1 β , mediate the early inflammatory response to lethal endotoxemia and bacteremia (16). Recently, it has become apparent that another important mediator of endotoxemia and sepsis is high-mobility group box (HMGB) 1 (17–19). HMGB1 is a nuclear protein that is released during a systemic inflammatory response, resulting in increased circulating levels, but in a delayed fashion compared with TNF- α or IL-1 β (17–19). Cells critical for the production and active release of HMGB1 during an inflammatory response are monocytes and macrophages (17). Studies using neutralizing antibodies to HMGB1 have shown that increased circulating levels of HMGB1 contribute to the late lethality of endotoxemia and sepsis (17–20).

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Acute lung injury (ALI) is a consequence of a variety of insults, including inflammation, which may have subsequent systemic manifestations (21). When administered to the lungs, HMGB1 has been shown to be a mediator of ALI, resulting in inflammatory cell accumulation, pulmonary edema, and production of proinflammatory mediators (22). Moreover, the ability of HMGB1 to inhibit the phagocytosis of apoptotic neutrophils, and thus neutrophil clearance, may also contribute to its inflammatory response (23). Administration of neutralizing antibodies to HMGB1 results in decreased inflammatory cell migration and decreased edema in lungs after exposure to endotoxin (22). HMGB1 has also been shown to participate in the ALI associated with hemorrhage (24), another insult associated with a cascade of inflammatory mediators leading to injury. Thus, HMGB1 is a known mediator of lung injury associated with a systemic inflammatory response. Recent studies suggest that the inflammatory activity of HMGB1 is weak by itself (25); however, HMGB1 enhances this activity by binding with proinflammatory mediators, such as IL-1 β (26), and with bacteria-derived substances, such as lipids (23, 25), to increase its inflammatory response.

Because HO-1 is an endogenous cytoprotective enzyme that is involved in the pathophysiologic response to endotoxemia and ALI (13, 27, 28), we sought to determine whether tissue and circulating levels of HMGB1 are altered in the absence of HO-1 during endotoxemia *in vivo*, and whether an increase in HMGB1 may contribute to the lethality of endotoxemia in HO-1-deficient mice. We also sought to ascertain whether the downstream products of heme catabolism mediated by HO-1—namely, CO and biliverdin—could suppress the circulating levels of HMGB1 during endotoxemia and improve survival in HO-1-deficient mice.

MATERIALS AND METHODS

Reagents and Murine Endotoxemia Model

Pseudomonas aeruginosa LPS (serotype 10; Sigma, St. Louis, MO) was used for the endotoxemia studies. Wild-type (+/+) or homozygous-null (−/−) female mice for targeted disruption of HO-1 (12) on a BALB/c genetic background were studied at 8–10 weeks of age. Mice were treated with an intraperitoneal injection of LPS at the indicated doses and times. The vehicle used to dissolve the LPS, 0.9% NaCl, was used in the control mice. CO-releasing molecules (RM) are transition metal carbonyls, which liberate CO to elicit direct biological activities (29). The CO-RM used in our experiments was tricarbonyl-dichlororuthenium (II) dimer ([Ru(CO)₃Cl₂]₂; Sigma) dissolved in a 12.5% DMSO solution (which was used as the vehicle for control experiments). Biliverdin (Frontier Science, Logan, UT) was dissolved in 0.1 N NaOH and then adjusted to a pH of 7.4 with HCl (vehicle for control experiments was prepared exactly as the solution used to dissolve biliverdin). Both CO-RM and biliverdin were administered intraperitoneally at the indicated doses and times.

Immunohistochemical Analyses

Tissue was fixed in methyl Carnoy's solution and then in 70% ethanol before processing and embedding in paraffin. Immunohistochemistry was performed as previously described (30, 31), and sections were stained with antibodies against HMGB1 (1:500; Shino-Test Corp., Sagami-hara, Kanagawa, Japan) and CD45 (leukocyte common antigen, 1:1,000; BD Biosciences, San Jose, CA). To assess inflammatory cell accumulation and HMGB1 localization in the lung, the areas staining positive for CD45 and HMGB1 were measured by colorimetric analysis (30).

Preparation of Single-Cell Suspensions and Immunofluorescent Labeling for FACS Analyses

To isolate lung leukocytes, mice were killed and the lung vasculature was flushed with 20 ml of PBS by inserting a catheter into the right ventricle of

the heart. Whole mouse lungs were minced and digested in serum-free RPMI 1,640 medium supplemented with 1 mg/ml collagenase type IV (Sigma), 0.5 mg/ml DNase (DNase I from a bovine pancreas; Sigma), and 2 mg/ml collagenase/dispase (Roche, Indianapolis, IN) before filtering through a 70- μ m cell strainer (BD Biosciences) (32). The resulting cell suspension was centrifuged at 1,400 rpm for 5 minutes at 4°C, and leukocytes were isolated by separation on a 40–70% percoll (GE Lifesciences, Piscataway, NJ) gradient (33). Isolated cells were incubated with FITC-anti-CD11b (BD Biosciences), phycoerythrin (PE)-labeled F4/80 (Invitrogen, Carlsbad, CA), or PE-labeled Gr1 (Ly-6G; BD Pharmingen, San Diego, California) antibodies for 30 minutes on ice, and 10,000 cells in each group were analyzed by FACS on a FACScan Flow cytometer (BD Biosciences).

Primary Mouse Macrophages, Neutrophils, and Migration Assays

Primary macrophages were harvested from the peritoneum (34) and primary neutrophils from the bone marrow (35) of mice. For the macrophage studies, a migration assay was performed as described previously (35, 36) using Biocoat Matrigel Invasion Chambers (BD Biosciences), a modified Boyden chamber assay, with upper and lower chambers separated by an 8- μ m microporous, polyethylene terephthalate membrane coated with a uniform layer of Matrigel Matrix, a prototype basement membrane. The lower chambers were filled with medium alone (RPMI 1,640 containing 10 mM Hepes and 1% human serum albumin, pH 7.4) or medium containing mouse macrophage inflammatory protein (MIP)-1 α (10^{−9} M), whereas macrophages (3 $\times$ 10⁶ cells) were placed in the upper chambers. The chambers were placed overnight in a humidified incubator set at 37°C with 5% CO₂, and macrophages that migrated through the membrane were stained with Hema 3 (Fisher Scientific, Pittsburgh, PA) and counted in 10 random, high-magnification fields ($\times$ 1,000). For the neutrophil studies, a traditional Boyden chamber assay was performed, with upper and lower chambers separated by an 8- μ m microporous membrane. The neutrophils (4 $\times$ 10⁶ cells) were placed in the upper chamber, and the lower chamber contained medium alone (as above) or medium plus keratinocyte-derived chemokine (KC; 10^{−7} M), a neutrophil chemoattractant. The chambers were incubated for 4 hours at 37°C, and the neutrophils that migrated through the membrane were stained with Hema 3 and counted in 10 random, high-magnification fields ($\times$ 1,000).

Western Blot Analyses

Tissue was collected from mice receiving LPS or vehicle, and lysed in buffer containing 25 mM Tris (pH 7.5), 50 mM NaCl, 10 mM EDTA, and complete protease inhibitors (Roche). Protein extracts were subjected to 15% SDS-PAGE, transferred to nitrocellulose membranes (Whatman, Inc., Florham Park, NJ), and then probed with a chicken anti-HMGB1 antibody (Shino-Test Corp.). After incubation with a peroxidase-conjugated secondary antibody, immunoreactive bands were visualized using an enhanced chemiluminescence kit (GE Lifesciences).

Measurement of Plasma HMGB1 and HMGB1-Neutralizing Antibody Experiments

HMGB1 protein in plasma was quantified by ELISA (Shino-Test Corp.) following the manufacturer's instructions. In HO-1^{−/−} mice, chicken anti-HMGB1 or control chicken IgY antibody (200 μ g/mouse, intraperitoneally; Shino-Test Corp.) was administered 2 and 24 hours after the administration of LPS (10 mg/kg), and survival was then assessed.

Statistical Analysis

Data are expressed as mean ($\pm$ SEM). For comparisons between two groups, Student's two-tailed unpaired *t* test was used. For comparison between more than two groups, and multiple comparisons, an ANOVA test was used. Comparisons of mortality were made by analyzing Kaplan-Meier survival curves, and then log-rank test was performed to assess for differences in survival. The number of samples per group (*n*) is specified in the RESULTS section or in the figure legends. Statistical significance is accepted at *P* less than 0.05.

RESULTS

HO-1 and Lung Inflammation

To determine the role of HO-1 in lung inflammation, HO-1^{+/+} and HO-1^{-/-} mice were exposed to vehicle or LPS (5 mg/kg intraperitoneally). Lungs of the mice were harvested 24 hours later, and processed for immunohistochemical staining using an antibody against a common leukocyte antigen, CD45. In the present study, baseline inflammation in the lungs of HO-1^{-/-} mice appeared increased (Figure 1A, representative image). Quantitation of CD45 staining revealed a 2-fold increase ($P < 0.05$) at baseline in HO-1^{-/-} ($0.16 \pm 0.03\%$; $n = 8$) compared with HO-1^{+/+} ($0.08 \pm 0.02\%$; $n = 9$) lungs. We have shown previously that, after LPS administration in the trachea, distal airway inflammatory cell accumulation, pulmonary edema, and alveolar destruction were not different between HO-1^{+/+} and HO-1^{-/-} mice (13). In the present study, CD45 staining was also not different in the lungs of HO-1^{+/+} and HO-1^{-/-} mice 24 hours after systemic LPS administration ($0.12 \pm 0.08\%$ versus $0.11 \pm 0.04\%$, respectively). To further assess the composition of the inflammatory infiltrates in the interstitium of the lung (as airway inflammation is not prominent during endotoxemia), the lung tissue from HO-1^{+/+} and HO-1^{-/-} mice exposed to vehicle or LPS were minced, digested, and leukocytes isolated (as described in MATERIALS AND METHODS). The isolated cells were incubated with FITC-anti-CD11b antibody and then PE-labeled anti-F4/80 or anti-Gr1 antibodies to assess macrophages and neutrophils, respectively, by FACS analysis. Figure 1B shows a representative FACS analysis, and further quantitation was performed in three separate experiments. The fraction of macrophages (F4/80⁺ CD11b⁺) was increased in the lungs of HO-1^{-/-} compared with HO-1^{+/+} mice at baseline ($62 \pm 5\%$ versus $29 \pm 5\%$; $n = 3$), and neutrophil (Gr1⁺ CD11b⁺) fraction was 67% ($\pm 13\%$) versus 36% ($\pm 2\%$) ($n = 3$). The differences in cellular fractions in HO-1^{-/-} compared with HO-1^{+/+} mice were not as pronounced for macrophages ($76 \pm 3\%$ versus $52 \pm 6\%$; $n = 3$) and neutrophils ($78 \pm 4\%$ and $67 \pm 4\%$; $n = 3$) after LPS administration.

Migratory Potential of Primary Macrophages and Neutrophils in the Presence or Absence of HO-1

Because the cells that are critical for the production and release of HMGB1 during an inflammatory response are monocytes and macrophages (17), we initially focused on macrophages in these assays. Macrophages harvested from HO-1^{-/-} mice showed an increased ability to migrate in the absence of a chemokine gradient ($-MIP-1\alpha$) compared with HO-1^{+/+} macrophages (Figure 2A). These data suggest the HO-1-deficient macrophages have the potential to migrate even in the absence of a chemokine gradient, which may help explain the increased presence of inflammatory cells in the lungs of HO-1^{-/-} mice at baseline. In the presence of the chemokine, MIP-1 α (+), directed migration was increased in both groups of macrophages (Figure 2A). Although the overall number of migrated cells was higher in the absence of HO-1, the fold increase in migrated cells after MIP-1 α stimulation was greater in the HO-1^{+/+} cells (5.5-fold) compared with HO-1^{-/-} cells (2.3-fold). These data correlate with the inflammatory response *in vivo*, as there were increased numbers of macrophages in HO-1^{-/-} lungs at baseline, but the macrophage response to LPS stimulation was greater in the lungs of HO-1^{+/+} mice compared with HO-1^{-/-} mice. Analogous to the macrophage response, neutrophils harvested from HO-1^{-/-} mice showed an increased ability to

Figure 1. Increased inflammatory cells in the lungs of heme oxygenase (HO)-1^{-/-} compared with HO-1^{+/+} mice. (A) Lung tissue from HO-1^{+/+} and HO-1^{-/-} mice were immunostained with CD45 antibody. (B) After HO-1^{+/+} and HO-1^{-/-} mice were injected intraperitoneally with 5 mg/kg LPS for 24 hours, lung tissues were harvested, digested, and cells were isolated. FACS was performed by staining for markers of macrophages (F4/80, orange, upper panels) and neutrophils (Gr1, blue, lower panels) on CD11b-positive inflammatory cells. All experiments were performed a minimum of three independent times.

migrate in the absence of a chemokine gradient ($-KC$; Figure 2B). In the presence of the chemokine, KC (+), directed migration was increased in both groups, but the fold increase was more robust in the HO-1^{+/+} cells (2.1-fold) compared with the HO-1^{-/-} cells (1.5-fold). HO-1^{+/+} cells were used in the neutrophil migration assay, but we have shown previously that the response to an inflammatory stimulus is similar in HO-1^{+/+} and HO-1^{-/-} mice (13).

Figure 2. Increased migratory potential of HO-1^{-/-} inflammatory cells. (A) HO-1^{+/+} (closed bars) and HO-1^{-/-} (open bars) mouse macrophages were added to the upper compartments of Matrigel chambers, and the lower chambers contained vehicle (-; $n = 12$ in each group) or macrophage inflammatory protein (MIP)-1 α (+; 10^{-9} M; $n = 7$ in each group). At 12 hours later, the cells in the upper chamber were removed by swabbing, the bottom of the membrane was stained, and cells that had migrated were counted in 10 random, high-power fields (HPF, $\times 1,000$). * $P < 0.05$ versus vehicle-treated HO-1^{+/+} (5.5-fold increase) and HO-1^{-/-} (2.3-fold increase) cells, respectively; † $P < 0.05$ versus vehicle-treated HO-1^{+/+} cells. (B) HO-1^{+/+} (gray bars) and HO-1^{-/-} (open bars) mouse neutrophils were added to the upper compartments of Boyden chambers; the lower chambers contained vehicle (-; $n = 5$ in each group) or KC (+; 10^{-7} M; $n = 5$ in each group). At 4 hours later, the bottom of the membrane was stained, and cells that had migrated were counted in 10 random, high-power fields (HPF; $\times 1,000$). * $P < 0.05$ versus vehicle-treated HO-1^{+/+} (2.1-fold increase) and HO-1^{-/-} (1.5-fold increase) cells, respectively; † $P < 0.05$ versus vehicle-treated HO-1^{+/+} cells. Data are expressed as mean ($\pm$ SEM).

Expression of HMGB1 in Tissue and Plasma of HO-1^{-/-} Mice

Similar to the inflammatory response at baseline, there was evidence of increased HMGB1 immunostaining in the lungs of HO-1^{-/-} mice (Figure 3A, representative images), and the immunostaining appeared to be predominantly in inflammatory cells. Quantitation of HMGB1 revealed an increased level of immunostaining ($P < 0.05$) at baseline in HO-1^{-/-} ($1.10 \pm 0.004\%$; $n = 6$) compared with HO-1^{+/+} ($0.26 \pm 0.09\%$; $n = 8$) lungs. Interestingly, in the absence of HO-1, immunostaining for HMGB1 did not increase after the administration of LPS (Figure 3A) in HO-1^{-/-} (0.81 ± 0.20 ; $n = 7$) or HO-1^{+/+} (0.013 ± 0.003 ; $n = 5$) lungs. If anything, HMGB1 staining tended to decrease. This HMGB1 response was confirmed by Western blot analysis of lung tissue, which showed a higher level of HMGB1 at baseline in HO-1^{-/-} compared with HO-1^{+/+} tissue (Figure 3B). Moreover, in HO-1^{-/-} lungs, LPS administration did not cause an increase in HMGB1 protein expression. This absence of an increase in HMGB1 protein in HO-1^{-/-} mice after LPS was also evident in kidney tissue (Figure E1 in the online supplement), in which protein levels for HMGB1 decreased. Figure 3C emphasizes the point that expression of HMGB1 in primary macrophages is increased in HO-1^{-/-} cells compared with HO-1^{+/+} cells.

Circulating levels of HMGB1 were also assessed in plasma of HO-1^{+/+} and HO-1^{-/-} mice in the presence or absence of endotoxemia. Administration of a sublethal dose of *P. aeruginosa* LPS (5 mg/kg for 24 h) led to no increase in HMGB1 plasma levels in HO-1^{+/+} mice, but a marked increase in HMGB1 in HO-1^{-/-} mice (Figure 4). Next, CO-RM (20 μ M/kg

Figure 3. Tissue and cellular expression of high-mobility group box (HMGB)-1 are increased in the lungs of HO-1^{-/-} mice. After HO-1^{+/+} and HO-1^{-/-} mice were injected with 5 mg/kg LPS for 24 hours, lung tissues were harvested. (A) Immunostaining for HMGB1 in the lungs of HO-1^{+/+} and HO-1^{-/-} mice in the presence of vehicle or LPS. (B) Western blot analysis of HMGB1 protein expression in the lungs of HO-1^{+/+} and HO-1^{-/-} mice after the intraperitoneal injection of vehicle (-) or LPS (+; 5 mg/kg). (C) Western blot analysis of HMGB1 in primary macrophages harvested from HO-1^{+/+} and HO-1^{-/-} mice. In (B) and (C), the same membranes were blotted against tubulin to assess protein loading. All experiments were performed at a minimum of two independent times.

intraperitoneally) or biliverdin (50 μ M/kg intraperitoneally) was injected into HO-1^{-/-} mice at 24 hours and at 30 minutes before LPS administration (5 mg/kg intraperitoneally), and plasma HMGB1 levels were assessed. Administration of CO-RM or biliverdin to HO-1^{-/-} mice was able to decrease the circulating levels of HMGB1 (Figure 4). Taken together, these data suggest that endogenous HMGB1 protein expression in tissue is not up-regulated by LPS, but that the increased numbers of inflammatory cells expressing greater amounts of HMGB1 at baseline release the protein during LPS exposure, leading to increased circulating levels in HO-1-deficient mice. The enhanced circulating levels of HMGB1 in HO-1^{-/-} mice can be blunted by treatment with CO-RM or biliverdin.

Effects of CO-RM or Biliverdin, and Neutralizing Antibody to HMGB1, on the Outcome of HO-1^{-/-} Mice Exposed to Endotoxin

We demonstrated that CO-RM and biliverdin were able to decrease the circulating levels of HMGB1 in HO-1^{-/-} mice during endotoxemia. Next, to determine whether CO-RM or biliverdin was able to rescue HO-1^{-/-} mice from the detrimental consequences of endotoxemia, mice were administered

Figure 4. Reduction of endotoxin-induced circulating levels of HMGB1 by administration of carbon monoxide (CO)-releasing molecule (RM) or biliverdin to HO-1^{-/-} mice. Vehicle ($n = 7$) or LPS (5 mg/kg intraperitoneally; $n = 7$) was administered to HO-1^{+/+} and HO-1^{-/-} mice. In a separate group of mice, CO-RM (20 μ M/kg intraperitoneally; $n = 3$) or biliverdin (50 μ M/kg intraperitoneally; $n = 3$) were injected at 24 hours and at 30 minutes before LPS administration (5 mg/kg intraperitoneally). At 24 hours after LPS exposure in each group, plasma levels of HMGB1 protein were quantified by ELISA. * $P < 0.05$ versus vehicle- (-) and LPS (+)-treated HO-1^{+/+} and HO-1^{-/-} mice. Data are expressed as mean ($\pm$ SEM).

a higher dose of LPS (10 mg/kg intraperitoneally), and lethality was assessed (Figure 5). CO-RM or biliverdin was also injected twice before LPS administration, with the dose of CO-RM and biliverdin being 20 μ M/kg and 50 μ M/kg, respectively. Both CO-RM (Figure 5A, $P = 0.008$) and biliverdin (Figure 5B, $P = 0.015$) were able to dramatically improve survival of HO-1^{-/-} mice (by 57 and 50%, respectively) after administration of LPS.

Finally, to determine whether HMGB1 may play a role in the detrimental outcome of endotoxemia in the absence of HO-1, we administered an HMGB1 neutralizing antibody to HO-1^{-/-} mice (200 μ g intraperitoneally) 2 and 24 hours after the onset of endotoxemia (10 mg/kg LPS). Lethality was subsequently monitored over the next several days. Figure 6 reveals that administration of HMGB1-neutralizing antibody improved survival compared with control IgY antibody. Although the improvement in survival was not as dramatic as CO-RM or biliverdin replacement in HO-1^{-/-} mice (given before LPS), the ability of HMGB1-neutralizing antibody to improve outcome after the onset of endotoxemia demonstrates that increased circulating HMGB1 contributes, in part, to the lethality of endotoxemia in the absence of endogenous HO-1.

DISCUSSION

HO-1 is an inducible, cytoprotective enzyme that breaks down the pro-oxidant heme to generate products, including CO and biliverdin/bilirubin (1–4). These products of heme catabolism have important endogenous anti-inflammatory and antioxidant properties, which help to maintain physiologic homeostasis. CO has been shown to inhibit LPS-induced production of proinflammatory cytokines (TNF- α , IL-1 β , MIP-1 β , and IL-6), and increase production of the anti-inflammatory cytokine, IL-10 (4, 6, 14). CO also has an inhibitory effect on granulocyte-macrophage colony-stimulating factor, which is known to promote the secretion of proinflammatory mediators (4, 37) and the differentiation of hematopoietic progenitor cells into macrophages and neutrophils (4). Administration of bilirubin was recently shown to blunt the inflammatory cascade in a model of rodent sepsis by decreasing IL-6 and monocyte chemoattractant protein-1, and increasing IL-10 (38). It has also been demonstrated that HO-1 and its products are able to modulate the expression of adhesion molecules, and leukocyte-endothelial interactions (39–42). In total, these studies imply that an absence of HO-1 promotes an inflammatory milieu. In the current study, we assessed the inflammatory response both at baseline and after LPS adminis-

Figure 5. CO-RM and biliverdin rescue HO-1^{-/-} mice from the mortality of endotoxemia. HO-1^{-/-} mice were treated with either (A) CO-RM ($n = 7$) or an equal volume of vehicle ($n = 6$), or (B) biliverdin ($n = 6$) or an equal volume of vehicle ($n = 4$). CO-RM and biliverdin were also injected intraperitoneally twice before LPS administration (10 mg/kg intraperitoneally), in a manner analogous to that in Figure 4, with the total dose of CO-RM and biliverdin being 20 and 50 μ M/kg, respectively. Survival was assessed for 5 days after the LPS administration. $P = 0.008$ (A) and $P = 0.015$ (B).

tration in HO-1^{+/+} and HO-1^{-/-} mice. Interestingly, the predominant difference in inflammation between these groups of mice (more inflammatory cells in HO-1^{-/-} lungs) was evident at baseline, even more so than after LPS administration (Figure 1). In addition, total leukocytes and the percentage of macrophages and neutrophils at baseline were increased in lungs of HO-1^{-/-} mice. This suggests that HO-1 functions in the basal regulation of physiologic inflammation in the lung.

Although the mediation of cytokine and chemokine profiles by HO-1 and its metabolites has been studied extensively, to further explain the increased presence of inflammatory cells in the lungs of HO-1^{-/-} mice, we also sought to assess the migratory potential of macrophages and neutrophils in the presence and absence of HO-1. Migration of macrophages, in the absence of a chemokine gradient, was increased in HO-1^{-/-} compared with HO-1^{+/+} cells (Figure 2A). Chemokinesis was also increased in neutrophils of HO-1^{-/-} cells (Figure 2B). Other investigators have shown that adenoviral expression of HO-1 was critical for resolution of neutrophil migration into the lung after LPS (43). These data suggest that modulation of chemokinesis, in addition to chemotaxis, may contribute to the inflammatory response in HO-1-deficient mice.

Beyond the inflammatory response, oxidative stress is a critical component of the pathophysiologic response to systemic inflammatory diseases, such as endotoxemia and sepsis (44). In fact, we have shown previously that absence of HO-1 leads to increased oxidative stress, which contributes to end-organ injury and death in mice (13). Because it has recently been shown that release of HMGB1 is increased by oxidative stress in macro-

Figure 6. Neutralizing antibody to HMGB1 improves survival of HO-1^{-/-} mice during endotoxemia. Control antibody (IgY; 200 μ g; $n = 5$) or anti-HMGB1 (200 μ g; $n = 6$) were administered intraperitoneally to HO-1^{-/-} mice 2 and 24 hours after the onset of endotoxemia (10 mg/kg LPS, intraperitoneally). Survival was assessed after the administration of LPS. $P = 0.002$.

phages and monocytes (45), we sought to determine whether absence of a molecule with antioxidant properties, HO-1, plays a role in the modulation of HMGB1 during systemic endotoxemia *in vivo*. When evaluating lung expression of HMGB1, levels were higher in HO-1^{-/-} mice compared with HO-1^{+/+} mice at baseline, and expression was localized to inflammatory cells, as confirmed in HO-1^{-/-} macrophages (Figure 3). Investigators have shown previously that remarkably increased levels of HMGB1 occur in the serum of mice 16 hours after LPS administration, and levels remain elevated for more than 36 hours (17, 18). Whereas Hagiwara and colleagues (46) showed increased expression of HMGB1 in rat lungs 12 hours after intraperitoneal LPS administration, we did not find increased tissue expression of HMGB1 at a later time point (24 h) in either HO-1^{+/+} or HO-1^{-/-} mice. Despite the lack of increased tissue expression by LPS in the present study (and a trend toward a decrease in lung and kidney expression [Figure 3B and Figure E1, respectively]), circulating levels of HMGB1 were higher in HO-1-null mice (Figure 4). This suggests a release of HMGB1 from inflammatory cells in HO-1^{-/-} mice, which occurred at a low dose of LPS administration (5 mg/kg)—a dose at which wild-type mice do not exhibit increased circulating levels of HMGB1 (Figure 4). Although this dose of LPS is not typically sufficient to cause death in wild-type mice, increased mortality occurs in HO-1-deficient mice (13).

A previous study investigated the effect of an inducer of HO-1, cobalt protoporphyrin (CoPP) IX, on expression of TNF- α and HMGB1 during LPS-induced lung injury. This investigation showed less inflammation and tissue injury in mice receiving LPS pretreated with CoPPIX, in association with less HMGB1 mRNA and less protein in bronchoalveolar lavage fluid (47). However, the definitive role of HMGB1 in this CoPPIX response was not proven. In the present study, treatment with CO-RM or biliverdin was able to suppress the increased circulating levels of HMGB1 in HO-1^{-/-} mice (Figure 4), and even when a higher dose of LPS (10 mg/kg) was administered, replacement of CO-RM or biliverdin was able to significantly rescue HO-1^{-/-} mice from the lethality of endotoxemia (Figure 5). To confirm that HMGB1 contributes to the pathobiology of endotoxemia in the absence of HO-1, a neutralizing antibody to HMGB1 was given to HO-1^{-/-} mice after the onset of endotoxemia. Treatment with anti-HMGB1

antibody compared with a control antibody was able to significantly improve survival in HO-1^{-/-} mice (Figure 6). Thus, the present study suggests that endogenous HO-1 is an important mediator of HMGB1 release in the setting of endotoxemia, and that the increased circulating levels of HMGB1 contribute to the enhanced lethality of endotoxemia in the absence of HO-1. These results may have interesting implications, particularly for patients with increased risk for inflammation and oxidative stress. Although the incidence of HO-1 deficiency in humans is rare, with one reported case (48), there has been increased appreciation of patients with polymorphisms of the HO-1 promoter leading to reduced expression of this cytoprotective enzyme (28, 49). The present study provides further insight into potential pathophysiologic mechanisms of disease when HO-1 is deficient. Moreover, the present study provides additional supportive data in regard to the therapeutic potential of HO-1-derived heme metabolites in inflammatory disease processes.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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