ORIGINAL RESEARCH COMMUNICATION

Heme Attenuation Ameliorates Irritant Gas Inhalation-Induced Acute Lung Injury

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Abstract

Aims: Exposure to irritant gases, such as bromine (Br_2), poses an environmental and occupational hazard that results in severe lung and systemic injury. However, the mechanism(s) of Br_2 toxicity and the therapeutic responses required to mitigate lung damage are not known. Previously, it was demonstrated that Br_2 upregulates the heme degrading enzyme, heme oxygenase-1 (HO-1). Since heme is a major inducer of HO-1, we determined whether an increase in heme and heme-dependent oxidative injury underlies the pathogenesis of Br_2 toxicity. **Results:** C57BL/6 mice were exposed to Br_2 gas (600 ppm, 30 min) and returned to room air. Thirty minutes postexposure, mice were injected intraperitoneally with a single dose of the heme scavenging protein, hemo-pexin (Hx) (3 $\mu\text{g}/\text{gm}$ body weight), or saline. Twenty-four hours postexposure, saline-treated mice had elevated total heme in bronchoalveolar lavage fluid (BALF) and plasma and acute lung injury (ALI) culminating in 80% mortality after 10 days. Hx treatment significantly lowered heme, decreased evidence of ALI (lower protein and inflammatory cells in BALF, lower lung wet-to-dry weight ratios, and decreased airway hyperreactivity to methacholine), and reduced mortality. In addition, Br_2 caused more severe ALI and mortality in mice with HO-1 gene deletion ($\text{HO-1}^{-/-}$) compared to wild-type controls, while transgenic mice overexpressing the human HO-1 gene (hHO-1) showed significant protection. **Innovation:** This is the first study delineating the role of heme in ALI caused by Br_2 . **Conclusion:** The data suggest that attenuating heme may prove to be a useful adjuvant therapy to treat patients with ALI. *Antioxid. Redox Signal.* 24, 99–112.

Introduction

INHALED TOXIC IRRITANTS, such as bromine (Br_2), chlorine, ammonia, ozone, and sulfur mustard, dissolve in the aqueous environment of the respiratory tract mucosa and cause an inflammatory response typically due to the release of reactive intermediates. They predominantly damage the respiratory and alveolar epithelium, causing tracheitis, bronchitis, obliterative bronchiolitis, and acute respiratory distress syndrome (ARDS), which usually occurs within 24 h postexposure (19, 24). Rodents exposed to chlorine gas also develop systemic injury, including cardiac dysfunction (52), systemic hypocoagulation (53), and inactivation of pulmonary arterial endothelial nitric oxide synthase (18) leading to

hypertension, as well as increased susceptibility to fungal infections (12). Surviving animals develop subepithelial fibrosis and tracheal obstruction (30). Persons exposed to halogen gases may also develop bacterial infections and late pulmonary fibrosis (12, 31).

Innovation

This is the first study delineating the pathogenesis of the irritant gas, bromine, and induced respiratory insufficiency. The study has identified heme scavenging and heme oxygenase-1 (HO-1) induction as potential therapeutic interventions to mitigate initial lung insult upon catastrophic bromine exposure.

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Occupational accidents or deliberate use as pulmonary chemical warfare agents are the most frequent causes of exposure to these toxic gases. There is no specific antidote, and the current treatment for persons exposed to inhaled toxic gases is mainly palliative and includes the administration of supplemental oxygen, bronchodilators, and antibiotics in cases of infection. Therefore, there is a clear need for additional research to identify the pathophysiology of toxic gas-dependent morbidity and develop novel countermeasures to reduce morbidity and mortality.

In the present study, we investigated the mechanisms by which exposure of mice to Br₂ in concentrations likely to be encountered in the vicinity of industrial accidents, causes acute lung injury (ALI) and ARDS. Br₂ is a fuming liquid that targets the lungs, eyes, central nervous system, skin (32), and respiratory tract (51). Br₂ is used in the production of medicinal compounds, flame retardants, agricultural chemicals, gasoline additives, dyes, photographic chemicals, bleaching agents, and water disinfectants.

A recent study using the weanling swine burn model demonstrated that cutaneous exposure to Br₂ vapors increased the gene expression of heme oxygenase-1 (HO-1) (35). HO-1 catalyzes the first and rate-limiting step in heme degradation into equimolar amounts of iron, carbon monoxide (CO), and biliverdin (44). As heme is a major inducer of HO-1 expression, we hypothesized that heme levels are elevated upon exposure of mice to Br₂ and contribute to the

development of ALI and associated mortality. Furthermore, we tested the hypothesis that postexposure administration of hemopexin (Hx), a heme scavenging protein, will decrease lung injury and improve survival.

Heme is essential for biologic processes and serves as a functional group in proteins, such as hemoglobin, myoglobin, nitric oxide synthase, and cytochromes (37). However, excessive heme catalyzes the formation of free radicals, resulting in oxidative stress and cellular injury (16). Elevated heme levels have also been reported to underlie the endothelial injury after lipopolysaccharide exposure (26), lung injury after Libby amphibole asbestos exposure (40), as well as hyperoxia-induced lung injury (9), suggesting that the attenuation of heme may have broader implications in ameliorating lung oxidative damage.

Results

The literature on Br₂ inhalation-related lung toxicity is approximately four decades old. Therefore, we exposed C57BL/6 mice to various doses of Br₂ for different durations to determine the optimal dose and duration that would cause significant morbidity and mortality (Fig. 1A). More than 50% of mice exposed to 600 ppm Br₂ for 45 min died within 2 days postexposure. There was no significant difference in mortality among male and female mice. Thus, in all subsequent studies, we used male mice.

FIG. 1. Lung injury and mortality in mice exposed to Br₂ gas inhalation. Male and female C57BL/6 mice were exposed for different durations and various doses of Br₂ gas and assessed for mortality. Exposure to 600 ppm of Br₂ for 30 min caused mortality in 50% of mice within 6–7 days (A). There was no significant difference in the mortality rate between male and female mice ($n=12$ for male [400 ppm, 30 min], $n=8$ for male [600 ppm, 30 min], $n=4$ for male [600 ppm, 45 min], and $n=12$ for female [600 ppm, 45 min]) (A). Analysis of the BALF after 600 ppm Br₂ gas inhalation for 30 min showed a significant increase in BALF protein levels ($n=4-5$) (B) and total cell count ($n=4-5$) (C) within 24 h postexposure. Hematoxylin and eosin staining and the staining for myeloperoxidase ($n=3-4$) (E) of peripheral lung tissue 24 and 48 h post-Br₂ gas (600 ppm, 30 min) exposure demonstrated an increase in the lung injury score ($n=3-4$) (D), as depicted by the disruption of the airway parenchyma (black arrow), an increase in cellularity, proteinaceous debris accumulation, an increase in the alveolar and interstitial accumulation of neutrophils, and alveolar septal thickening. Values are mean $\pm$ SEM. All animals were males except where indicated. * $p < 0.05$ versus air exposed C57BL/6 mice. BALF, bronchoalveolar lavage fluid; Br₂, bromine.

Exposure to 600 ppm of Br₂ for 30 min caused 50% mortality within 6–7 days postexposure, which resembles data reported in the study by Bitron and Aharonson in 1978 (4). Exposure to 600 ppm Br₂ for 30 min also resulted in significant lung injury within 24 h postexposure, as demonstrated by an increase in bronchoalveolar lavage fluid (BALF) protein levels (Fig. 1B), total cell count (Fig. 1C), lung injury score (Fig. 1D), and the disruption of the airway parenchyma and increased lung neutrophils in lung sections stained with hematoxylin and eosin and myeloperoxidase (MPO) (Fig. 1E). Therefore, in our subsequent experiments, we subjected the mice to 600 ppm of Br₂ gas inhalation for 30 min and then returned them to room air. After 24 h of exposure, the effects of Br₂ on lung function and injury were investigated.

It has been previously reported that exposure to Br₂ vapors increased the gene expression of the heme degrading enzyme, HO-1 (35), suggesting that heme-dependent toxicity may play a significant role in Br₂-induced injury. There are two major sources of heme: intravascular hemolysis (red blood cell [RBC] destruction) and extravascular cell death. In fact, our data showed that the RBCs obtained from the Br₂ exposed mice were more susceptible to hemolysis following exposure to sodium dodecyl sulfate (SDS) (Fig. 2A).

In addition, significant higher levels of lactate dehydrogenase (LDH) (Fig. 2C) as well as higher fractions of late apoptotic and necrotic cells were seen among inflammatory cells in the BALF of Br₂ exposed mice (Fig. 2B). Our data also demonstrated that Br₂ induced the expression of the HO-1 enzyme in mouse peripheral lung tissue, as indicated by immunoblotting (Fig. 3A), and in mouse airway epithelia, as shown by immunohistochemistry analysis (Fig. 3B). Interestingly, an intraperitoneal injection of heme scavenging protein, Hx (3 µg/g body weight), 30 min post-Br₂ exposure

prevented the increase in HO-1 expression, suggesting that HO-1 induction is a compensatory response to an increase in heme.

In fact, Br₂ significantly increased total heme levels in the BALF (Fig. 4A), the plasma (Fig. 4B), and the peripheral lung tissue (Fig. 4C) of the exposed C57BL/6 mice at 24 h postexposure. However, treatment with Hx (3 µg/g body weight) decreased heme levels 24 h postexposure. The 3 µg/g body weight dose of Hx was selected after evaluating the efficacy of various concentrations of Hx in attenuating the Br₂-induced lung injury (data not shown). The fractionation of lung tissue indicated that Br₂ increased heme in the cytoplasm (Fig. 4D), whereas mitochondrial (Fig. 4E) and microsomal (Fig. 4F) heme did not change.

In our next series of experiments, we exposed mice over-expressing the human HO-1 enzyme (hHO-1), mice lacking endogenous HO-1 (HO-1^{-/-}) as well as their wild-type (WT) controls to Br₂ gas (600 ppm for 30 min) and returned them to room air (Fig. 5A). The BALF (Fig. 5B), plasma (Fig. 5C), and peripheral lung tissue (Fig. 5D) of total heme levels were significantly higher in the HO-1^{-/-} mice compared to the WT and the hHO-1 mice. Interestingly, the basal levels of heme (without Br₂ exposure) in the BALF, plasma, and peripheral lung tissue were not significantly different between the WT, HO-1^{-/-}, and the hHO-1 mice.

Elevated heme levels have been previously associated with an increase in oxidative stress and a proinflammatory response (38). In our study, heme scavenging by Hx prevented Br₂-dependent oxidation of lung proteins, as indicated by reduced levels of carbonyl (aldehydes and ketones) adducts in C57BL/6 mice (Fig. 6A). Similarly, lung protein carbonylation levels were higher in the HO-1^{-/-} mice compared to the WT or hHO-1 mice upon Br₂ gas exposure (Fig. 6B). In addition, Hx

FIG. 2. Br₂ gas inhalation increases the susceptibility to hemolysis and the apoptosis/necrosis of lung inflammatory cells. C57BL/6 mice were exposed to Br₂ gas (600 ppm) for 30 min and then brought to room air. The mice were harvested 24 h postexposure and their blood and BALF was obtained. An *ex vivo* hemolytic assay performed on the blood of these mice demonstrated an increased susceptibility to hemolysis at lower concentrations of the detergent, SDS, compared to the blood obtained from air exposed mice ($n = 10$) (A). Flow cytometry analysis of Annexin V FITC-stained cells collected from the BALF showed a significant increase in late apoptotic/necrotic (Quadrant [Q] 2) and necrotic (Quadrant [Q] 1) cells in the Br₂ exposed mice ($n = 3-6$) (B). Quadrants (Q) 3 and 4 show alive and early apoptotic cells, respectively. In addition, Br₂ inhalation also increased LDH levels in the BALF of exposed mice ($n = 8-10$) (C). Values are mean $\pm$ SEM. All animals were males. * $p < 0.05$ versus air exposed C57BL/6 mice. LDH, lactate dehydrogenase; SDS, sodium dodecyl sulfate.

FIG. 3. Br₂ gas inhalation increases lung HO-1 protein expression. C57BL/6 mice were exposed to Br₂ gas (600 ppm) for 30 min and then brought to room air. Mice were injected intraperitoneally with Hx (3 μ g/g body weight) 30 min after the exposure. After 24 h, peripheral lung tissue was harvested. Immunoblot analysis demonstrated that Br₂ inhalation significantly increased lung HO-1 protein levels ($n=6$) (A). Hx treatment prevented the increase in HO-1 protein levels. Similarly, immunohistological staining indicated higher HO-1 expression in the Br₂ exposed mice compared to the air exposed mice or Hx-treated mice ($n=4$) (B). Values are mean $\pm$ SEM. All animals were males. * $p < 0.05$ versus air exposed C57BL/6 mice, † $p < 0.05$ versus Br₂+Hx-treated C57BL/6 mice. HO-1, heme oxygenase-1; Hx, hemopexin.

FIG. 4. Heme is increased in C57BL/6 mice exposed to Br₂ gas inhalation. C57BL/6 mice were exposed to Br₂ gas (600 ppm) for 30 min and then brought to room air. Mice were given an intraperitoneal injection of Hx (3 μ g/g body weight) 30 min after the exposure. After 24 h, Br₂ inhalation significantly increased total heme levels in the BALF (A), the plasma (B), and the peripheral lung tissue (C) of the exposed C57BL/6 mice. Hx treatment reduced heme in these animals. Lung fractionation demonstrated that Br₂ mainly increased cytosolic heme (D), while the heme content in the mitochondria (E) and the microsomes (F) did not change after Br₂. Values are mean $\pm$ SEM ($n=5-7$). All animals were males. * $p < 0.05$ versus air exposed C57BL/6 mice, † $p < 0.05$ versus Br₂+Hx-treated C57BL/6 mice.

FIG. 5. HO-1 attenuates Br₂-induced heme. Immunoblot analysis demonstrated the expression of HO-1 protein in the peripheral lung tissue of the WT mice, the mice over-expressing the human heme oxygenase-1 enzyme (hHO-1), and the mice lacking endogenous HO-1 (HO-1^{-/-}) (*n*=5–6) (A). These mice were exposed to Br₂ gas (600 ppm) for 30 min and then brought to room air. After 24 h, Br₂ inhalation significantly increased total heme levels in the BALF of the WT and the HO-1^{-/-} mice compared to their air exposed controls (B). Br₂ exposure also increased total heme levels in the plasma (C) and the peripheral lung tissue (D) of the HO-1^{-/-} mice in comparison to the air exposed mice. In addition, the BALF, plasma, and whole lung heme levels were higher in the Br₂ exposed HO-1^{-/-} mice compared to the Br₂ exposed WT mice. Br₂ did not alter heme in the hHO-1 mice. Values are mean ± SEM (*n*=3 for air exposed mice and 5–6 for Br₂ exposed mice). All animals were males. **p* < 0.05 versus air exposed control mice, †*p* < 0.05 versus Br₂ exposed WT mice, and ‡*p* < 0.05 versus Br₂ exposed HO-1^{-/-} mice. WT, wild type.

reduced the Br₂-dependent increase in BALF protein levels (Fig. 7A) and BALF total cell count (Fig. 7B) and prevented the extravasation of neutrophils (Fig. 7C) in C57BL/6 mice. Likewise, BALF protein levels were elevated in the HO-1^{-/-} mice compared to the WT mice and mice overexpressing hHO-1 (Fig. 7D). Although Br₂ did not increase the total cell count in the BALF of HO-1^{-/-} mice versus WT or hHO-1 mice (Fig. 7E), the neutrophil count in the BALF was higher

in the HO-1^{-/-} mice compared to the WT mice and was significantly lower in the hHO-1 mice (Fig. 7F).

We also analyzed the BALF and the plasma for the presence of 25 cytokines/chemokines. However, we could only detect 10–11 cytokines in the BALF and 5 cytokines in the plasma. Of these cytokines, our results demonstrated that, in C57BL/6 mice, Br₂ significantly increased the levels of five cytokines/chemokines in the BALF and three cytokines/

FIG. 6. Heme induces lung oxidative stress in Br₂ gas exposed mice. C57BL/6 mice, WT mice, the mice over-expressing the human heme oxygenase 1 enzyme (hHO-1), and the mice lacking endogenous HO-1 (HO-1^{-/-}) were exposed to Br₂ gas (600 ppm) for 30 min and then brought to room air. C57BL/6 mice were given an intraperitoneal injection of Hx (3 μg/g body weight) 30 min after the exposure. After 24 h, carbonyl (aldehydes and ketones) adducts, a hallmark of the oxidation status of proteins, were measured by gel electrophoresis and Western blotting using the protein obtained from the whole lung tissue. Hx prevented the Br₂-dependent increase in protein carbonylation in C57BL/6 mice (A). Similarly, after Br₂ gas exposure, the lung protein carbonylation levels were significantly higher in the HO-1^{-/-} mice compared to the WT mice or the hHO-1 mice (B). Values are mean ± SEM (*n*=4). All animals were males. **p* < 0.05 versus air exposed C57BL/6 mice or Br₂ exposed WT mice, †*p* < 0.05 versus Br₂+Hx-treated C57BL/6 mice or Br₂ exposed HO-1^{-/-} mice.

FIG. 7. Br₂-induced lung injury is mediated by heme. C57BL/6 mice, WT mice, the mice overexpressing the human heme oxygenase 1 enzyme (hHO-1), and the mice lacking endogenous HO-1 (HO-1^{-/-}) were exposed to Br₂ gas (600 ppm) for 30 min and then brought to room air. C57BL/6 mice were given an intraperitoneal injection of Hx (3 μ g/g body weight) 30 min after the exposure. After 24 h, the BALF protein levels, total and differential cell counts, were measured. Hx significantly attenuated the Br₂-dependent increase in BALF protein levels ($n=6$) (A) and BALF total cell count ($n=5-6$) (B) and prevented the extravasation of neutrophils in C57BL/6 mice ($n=5-6$) (C). Furthermore, BALF protein levels ($n=5-6$) (D), but not the total number of cells ($n=5-6$) (E), were higher in the HO-1^{-/-} mice compared to the WT mice or the hHO-1 mice. Yet, the neutrophil count in the BALF was significantly higher in the HO-1^{-/-} mice compared to the WT mice and the hHO-1 mice ($n=5-6$) (F). Values are mean $\pm$ SEM. All animals were males except in (D-F), where two animals were females in each of the three groups: WT, HO-1^{-/-}, and hHO-1. * $p < 0.05$ versus air exposed C57BL/6 mice or Br₂ exposed WT mice, † $p < 0.05$ versus Br₂+Hx-treated C57BL/6 mice or Br₂ exposed HO-1^{-/-} mice.

chemokines in the plasma, including the neutrophil chemoattractant, keratinocyte-derived chemokine (KC), and the monocyte chemoattractant protein 1 (MCP-1) (Table 1). In addition, these cytokines/chemokines were also increased in the BALF and the plasma of HO-1^{-/-} mice after Br₂ exposure (Table 2). Both the treatment with Hx in C57BL/6 mice and the overexpression of hHO-1 prevented the increase in these cytokines/chemokines following exposure to Br₂.

Furthermore, Hx significantly reduced the Br₂-dependent increases in the lung wet-to-dry weight ratio (Fig. 8A), total lung resistance following challenge with methacholine (Fig. 8B), and Newtonian (central) airway resistance, a measure of resistance in central airways (Fig. 8C), as measured by flexiVent in C57BL/6 mice. Similarly, mice overexpressing the human HO-1 gene had a lower lung wet-to-dry weight ratio (Fig. 8D), total lung resistance (Fig. 8E), and Newtonian airway resistance (Fig. 8F) compared to the WT and the HO-1^{-/-} mice after Br₂ exposure.

The analysis of arterial blood gas (ABG) in C57BL/6 mice 24 h post-Br₂ gas exposure demonstrated that blood pH dropped to 7.1, which correlated with an increase in the partial pressure of carbon dioxide (pCO₂) to $\sim$ 60 mmHg (Table 3). In addition, the blood urea nitrogen (BUN) levels were significantly higher in Br₂ exposed mice (Table 3), suggesting kidney injury. Interestingly, Hx-treated mice had a blood pH of 7.3, a pCO₂ of 35 mmHg, and no significant increase in BUN.

Finally, postexposure administration of Hx reduced mortality within the first 2 days of exposure to 600 ppm Br₂ for 30 min, as indicated by a rightward shift of the Kaplan-Meier

curves (Fig. 9A). Incidentally, the overall mortality between both groups after 10 days was not different (Fig. 9A). This was most likely due to the fact that mice were injected with a single dose of Hx. In addition, the survival rate of hHO-1 mice after exposure to Br₂ gas was $\sim$ 80% compared to 35% for the WT mice and 0% for the HO-1^{-/-} mice after 10 days (Fig. 9B).

Discussion

A previous study conducted on human volunteers demonstrated that a mere exposure to 0.9 ppm Br₂ for 5 min resulted in cough, headache, and irritation of the eyes, nose, and upper respiratory tract (36). Accidental exposure to higher doses of Br₂ and the ensuing respiratory complications can lead to severe morbidity and mortality, and yet, there is a lack of human and animal data available on Br₂ toxicity. This study is an attempt to underline the acute pathology (within 24 h of exposure) and potential therapeutic interventions that may be utilized to mitigate human morbidity and mortality associated with acute Br₂ exposure. The findings we present here emphasize the important role of heme in Br₂-induced airway and distal lung epithelial injury. Our data suggest that attenuating heme levels in BALF, peripheral lung tissue, and plasma reduces pulmonary oxidative stress, prevents lung inflammation, decreases protein extravasation and pulmonary edema, and improves airway function and survival.

There are three major known sources of heme: (i) hemoglobin in RBCs, (ii) heme-containing proteins in the live cell, and finally, (iii) heme released from dead cells into the

TABLE 1. HEMOPEXIN ATTENUATES BR₂-INDUCED CYTOKINE/CHEMOKINE GENERATION

Cytokines	Role in lung injury	Air (pg/ml) n=6	Br ₂ (pg/ml) n=6	Br ₂ +Hx (pg/ml) n=6
Bronchoalveolar lavage fluid				
IL-1 alpha	Proinflammatory interleukin	8.08 ± 1.79	20.32 ± 8.01	19.35 ± 4.10
IL-1 beta	Proinflammatory interleukin	2.22 ± 0.96	3.19 ± 1.19	2.22 ± 0.96
IL-2	Provides adaptive immunity	3.95 ± 0.62	4.41 ± 0.91	3.97 ± 0.83
IL-6	Both pro- and anti-inflammatory	2.03 ± 1.15	30.31 ± 9.21 ^a	8.65 ± 0.77 ^b
IL-9	Proproliferative and antiapoptotic	107.93 ± 29.57	79.74 ± 14.79	48.68 ± 7.79
IL-10	Anti-inflammatory	10.27 ± 2.44	17.75 ± 5.57	7.84 ± 1.63
IP-10	Angiogenic chemokine	12.65 ± 2.73	160.43 ± 59.44 ^a	47.96 ± 11.97 ^b
G-CSF	Stimulates granulocyte release	10.76 ± 5.46	284.01 ± 86.70 ^a	62.27 ± 12.72 ^b
KC	Chemoattractant for neutrophils	17.72 ± 3.28	48.20 ± 9.87 ^a	17.40 ± 1.66 ^b
MCP-1	Recruits monocytes	8.08 ± 3.24	22.47 ± 4.96 ^a	13.97 ± 1.44
Plasma				
IL-6	Both pro- and anti-inflammatory	3.73 ± 3.73	4797.0 ± 2723.7	209.45 ± 113.60
IP-10	Angiogenic chemokine	41.37 ± 10.88	342.75 ± 145.97 ^a	71.88 ± 15.03 ^b
G-CSF	Stimulates granulocyte release	106.13 ± 45.36	17111.8 ± 5097.4 ^a	9000.4 ± 4467.8
KC	Chemoattractant for neutrophils	32.47 ± 9.79	2050.9 ± 1475.5	421.43 ± 169.08
MCP-1	Recruits monocytes	22.37 ± 5.90	520.01 ± 241.13 ^a	37.84 ± 8.51 ^b

BALF and plasma cytokine/chemokine concentrations (pg/ml) in C57BL/6 mice are presented as mean ± SEM for groups (air: *n* = 5–6, Br₂: *n* = 5–6, Br₂+Hx: *n* = 5–6). All animals were males. Grayed rows indicate significant changes between Br₂ exposed mice and Br₂ exposed mice treated with Hx.

^a*p* < 0.05 versus Air.

^b*p* < 0.05 versus Br₂.

BALF, bronchoalveolar lavage fluid; Br₂, bromine; Hx, hemopexin; IP-10, IFN-γ-induced protein 10; G-CSF, granulocyte colony stimulating factor; KC, keratinocyte-derived chemokine; MCP-1, monocyte chemoattractant protein 1.

extracellular space. In our study, we found that heme levels were elevated in the plasma, BALF, and peripheral lung tissue of mice exposed to Br₂ gas. Our data demonstrated that the blood cells obtained from the mice after Br₂ exposure were more susceptible to hemolysis compared to the air exposed mice, probably due to the exposure to low pH and

higher oxidative stress (34) after Br₂ inhalation. Some clinical reports have also suggested a role of Br₂-related compounds, such as bromate, a bromine-based oxoanion, in the development of hemolytic anemia (14). In addition, chlorine-related compounds, which have very similar physical and chemical properties to Br₂, also cause anemia (7).

TABLE 2. HEME OXYGENASE-1 OVEREXPRESSION PREVENTS BR₂-DEPENDENT CYTOKINE/CHEMOKINE INDUCTION

Cytokines	Role in lung injury	WT (pg/ml) n=6	HO-1 ^{-/-} (pg/ml) n=6	hHO-1 (pg/ml) n=6
Bronchoalveolar lavage fluid				
IL-1 alpha	Proinflammatory interleukin	14.69 ± 6.09	21.54 ± 3.95	19.51 ± 7.48
IL-6	Both pro- and anti-inflammatory	65.04 ± 21.25	1214.7 ± 517.44 ^a	69.64 ± 15.31 ^b
IL-9	Proproliferative and antiapoptotic	57.38 ± 9.51	55.92 ± 21.86	73.53 ± 20.61
IL-10	Anti-inflammatory	8.14 ± 4.48	12.45 ± 4.66	9.18 ± 5.45
IL-13	Induces airway diseases	12.48 ± 9.13	39.14 ± 21.56	24.07 ± 11.38
IP-10	Angiogenic chemokine	13.93 ± 1.71	69.10 ± 23.19 ^a	6.56 ± 0.43 ^b
G-CSF	Stimulates granulocyte release	1698.5 ± 722.04	5833.5 ± 2005.4 ^a	59.47 ± 8.32 ^b
KC	Chemoattractant for neutrophils	45.87 ± 13.27	428.63 ± 168.61 ^a	22.57 ± 4.58 ^b
MCP-1	Recruits monocytes	20.52 ± 5.34	162.65 ± 69.38 ^a	8.43 ± 1.51 ^b
MIP-1 alpha	Recruits leukocytes	18.08 ± 3.94	26.97 ± 6.71	14.05 ± 1.92
MIP-1 beta	Recruits leukocytes	9.59 ± 4.98	12.32 ± 3.00	1.40 ± 1.13
Plasma				
IL-6	Both pro- and anti-inflammatory	1053.8 ± 519.41	4370.1 ± 1599.0 ^a	48.89 ± 21.10 ^b
IP-10	Angiogenic chemokine	89.59 ± 54.89	121.70 ± 45.32	25.56 ± 7.09
G-CSF	Stimulates granulocyte release	18369 ± 4598.8	16097 ± 4999.2	3199.2 ± 1454.9
KC	Chemoattractant for neutrophils	744.96 ± 221.15	1941.1 ± 674.08 ^a	217.55 ± 76.04 ^b
MCP-1	Recruits monocytes	67.00 ± 20.16	276.50 ± 104.39	21.73 ± 0.69

WT mice, mice with endogenous HO-1 deletion (HO-1^{-/-}), and mice overexpressing the human HO-1 gene (hHO-1) were exposed to Br₂ (600 ppm, 30 min), and then the cytokine/chemokine concentrations were measured in the BALF and the plasma 24 h postexposure. BALF and plasma cytokine/chemokine concentrations (pg/ml) are presented as mean ± SEM for groups (WT: *n* = 5–6, HO-1^{-/-}: *n* = 5–6, hHO-1: *n* = 5–6). All animals were males. Grayed rows indicate significant changes between HO-1^{-/-} mice and hHO-1 mice.

^a*p* < 0.05 versus WT.

^b*p* < 0.05 versus HO-1^{-/-}.

HO-1, heme oxygenase-1; MIP, macrophage inflammatory protein; WT, wild type.

FIG. 8. Heme reduction improves airway function post-Br₂ gas exposure. Lung wet-to-dry ratio, methacholine-dependent peripheral lung resistance (R), and central (Newtonian) airway resistance (Rn) were measured in mice exposed to Br₂ gas (600 ppm) for 30 min. Br₂ increased the lung wet-to-dry weight ratio in C57BL/6 mice, which was attenuated by treatment (30 min postexposure) with Hx (3 μ g/g body weight) ($n=6$) (A). The methacholine-dependent increase in R ($n=4-10$) (B) and Rn ($n=6$) (C) was also higher in the Br₂ exposed C57BL/6 mice compared to the air exposed mice. Hx prevented the increase in both R and Rn (B, C). Similarly, the mice lacking the endogenous heme oxygenase-1 gene (HO-1^{-/-}) had a higher lung wet-to-dry ratio ($n=5-7$) (D) and an elevated methacholine-dependent increase in R ($n=3-7$) (E) and Rn ($n=4-7$) (F) compared to the WT mice and the mice overexpressing the human HO-1 gene (hHO-1). Values are mean $\pm$ SEM. All animals were males. * $p < 0.05$ versus air exposed C57BL/6 mice or Br₂ exposed WT mice, † $p < 0.05$ versus Br₂+Hx-treated mice or Br₂ exposed HO-1^{-/-} mice.

It should be noted that the plasma heme levels in our study are total heme levels that include free heme and also heme bound to hemoglobin and albumin, as previously reported in humans (54). Furthermore, the increase in BALF heme was possibly derived from the death of inflammatory cells or the sloughing of epithelial cells lining the airways, as evident by the increased number of apoptotic and necrotic cells and elevated LDH levels in the BALF of exposed mice. Similar sloughing of airway epithelial cells has been previously shown after chlorine inhalation in rats (8), pigs (15), and mice (45).

Heme (iron protoporphyrin IX) is an essential functional group of various proteins. However, these heme moieties are readily oxidized and cause oxidative damage, impair cellular integrity (38), and contribute to inflammatory injury (50). Br₂ gas inhalation increased lung protein oxidation, lung inflammation, resistance, and lung wet-to-dry weight ratio.

A similar increase in pulmonary edema was reported in a patient requiring endotracheal intubation, artificial ventila-

tion for 5 days, and hospitalization for 41 days after inadvertently inhaling Br₂ vapors (20). Yet, in another patient who was admitted to an emergency ward after accidental exposure to Br₂ gas, irritation of the airways by Br₂ resulted in coughing and transitory respiratory obstruction, which led to the rupture of the alveoli and mediastinal emphysema (27). Br₂ exposure has also been linked to the acute development of pneumonitis and also delayed respiratory complications, such as pulmonary fibrosis in a nonsmoking patient who accidentally inhaled Br₂ vapors for 5–10 min (22).

In addition, the ABG analysis demonstrated that the blood pH in Br₂ exposed mice dropped to 7.1, which corresponded to an increase in the pCO₂ to ~ 60 mmHg. These results are consistent with the available data on patients showing respiratory acidosis with a drop in blood pH and a rise in the pCO₂ to 52 mmHg immediately after Br₂ exposure (27). However, it seems that this change in blood gas may be transitory. In a similar patient 3 days after Br₂ gas exposure,

TABLE 3. MOUSE ARTERIAL BLOOD GAS ANALYSIS

	pH	pCO ₂ (mmHg)	HCO ₃ (mM)	Na ⁺ (mM)	K ⁺ (mM)	tHb (g/dl)	SO ₂ (%)	Hct	BUN (mg/dl)
Air	7.4	27.5 $\pm$ 2.3	14.5 $\pm$ 0.6	151.7 $\pm$ 1.2	2.7 $\pm$ 0.1	12.5 $\pm$ 0.4	96.1 $\pm$ 1.7	37.7 $\pm$ 1.1	17.5 $\pm$ 0.7
Br ₂	7.1 ^a	59.4 $\pm$ 4.5 ^a	18.5 $\pm$ 1.3	154.8 $\pm$ 2.7	3.4 $\pm$ 0.3	12.9 $\pm$ 0.8	93.1 $\pm$ 3.1	38.9 $\pm$ 2.4	39.4 $\pm$ 8.2 ^a
Br ₂ +Hx	7.3 ^b	34.8 $\pm$ 5.3 ^b	20.5 $\pm$ 4.1	157.3 $\pm$ 3.2	3.4 $\pm$ 0.4	12.8 $\pm$ 0.8	91.0 $\pm$ 2.3	38.3 $\pm$ 2.3	21.2 $\pm$ 4.0 ^b

C57BL/6 mice were exposed to Br₂ gas (600 ppm, 30 min) and then kept in room air for 30 min. These mice were then treated with Hx (3 μ g/g body weight) through an intraperitoneal injection. After 24 h of exposure, the ABG was measured from the arterial blood drawn from the abdominal aorta using OPTI[®] CCA-TS Blood Gas and Electrolyte Analyzer with E-BUN cassettes. Heme scavenging by Hx prevented the decline in arterial pH and a subsequent increase in pCO₂. Hx also attenuated Br₂-dependent increase in BUN levels. Values are mean $\pm$ SEM ($n=7-10$). All animals were males.

^a $p < 0.05$ versus air exposed C57BL/6 mice.

^b $p < 0.05$ versus Br₂+Hx-treated C57BL/6 mice.

ABG, arterial blood gas; BUN, blood urea nitrogen; pCO₂, partial pressure of carbon dioxide.

FIG. 9. Heme attenuation improves survival after Br₂ gas exposure. Mice were exposed to Br₂ gas (600 ppm) for 30 min and then immediately brought to room air. After 30 min of exposure, C57BL/6 mice were given an intraperitoneal injection of Hx (3 μ g/g body weight). Heme scavenging by Hx reduced the early increase in mortality within the first 2 days from 60% in the vehicle-treated to 25% in the Hx-treated mice after Br₂ exposure. However, the overall survival according to the log-rank test was not significant between the two groups in the C57BL/6 mice ($n = 20$) (A). The Kaplan-Meier curve also demonstrated that the mice overexpressing the human heme oxygenase 1 enzyme (hHO-1) had lower mortality in comparison to the WT mice and the mice lacking endogenous HO-1 (HO-1^{-/-}) postexposure to Br₂ gas ($n = 7$ for HO-1^{-/-}, 14 for WT, and 17 for hHO-1) (B). All animals were males. * $p < 0.05$ versus Br₂ exposed WT mice, † $p < 0.05$ versus Br₂ exposed HO-1^{-/-} mice.

arterial blood pH was 7.45 and the pCO₂ was 28 mmHg possibly due to renal compensation, although the indices of respiratory function, such as total lung capacity and minute forced expiratory volume (FEV₁), were abnormal (22).

Furthermore, the mortality after Br₂ inhalation seemed biphasic (early vs. late). There was an immediate decline in survival within the first 2 days followed by a period of 4–5 days with no deaths and a subsequent increase in mortality thereafter. The delayed pattern of mortality after Br₂ exposure is associated with higher tissue solubility of Br₂ and therefore its higher potency (41). Br₂ is able to penetrate deeper into the lung tissue and cause peribronchiolar abscess, persistent bronchial spasm, and a delay in lung healing (39).

To determine if attenuating heme levels can have potential therapeutic effects on Br₂-induced toxicity, we utilized two experimental approaches. In the first approach, we scavenged heme by treating the C57BL/6 mice with the heme scavenging protein, Hx, 30 min after Br₂ exposure. In the second approach, we exposed mice overexpressing or lacking the heme degrading enzyme, HO-1. Hx, an intravascular protein, has superior heme binding affinity than albumin or haptoglobin and transports intravascular heme to the liver (49).

Heme-stimulated lipid peroxidation cannot be inhibited by the iron chelator, desferrioxamine, or by apotransferrin, but is strongly attenuated by Hx and, to a lesser extent, by albumin (16). Moreover, Hx is not oxidized by heme, presumably because H₂O₂ cannot interact with the bis-histidyl heme-Hx complex, whereas albumin and glutathione *S*-transferases, which also decrease heme-catalyzed lipid peroxidation, are subjected to oxidation (48). At higher concentrations (2–10 μ M), heme-Hx exerts pleiotropic protective effects by increasing cell survival (10). Although the Hx-deficient mice are normal under physiological conditions, when subjected to acute hemolysis, these mice have high lipid peroxidation and recover more slowly (46). Brass *et al.* demonstrated that the administration of 5 μ M of Hx attenuated free radical production in a rat model of reperfusion injury (5). Similarly, in our study, we have also shown that a single injection of Hx (3 μ M) is able to decrease lung oxidative stress and ameliorate the associated tissue damage and decline in respiratory function after Br₂ inhalation.

Heme induces a set of proteins, such as HO-1, metallothionein-1 (MT-1), and ferritin, which are able to protect the cell against oxidative stress (1, 2). The HO-1 enzyme catalyzes the oxidant, heme, into the catabolites, CO, biliverdin, and bilirubin, which have antioxidant properties. Although HO-1 protein levels were increased in Br₂ exposed mice, it seems this increase is not enough to overcome the initial insult. In fact, some studies have also shown that HO-1 induction is transitory after stress (13). Therefore, we used mice constitutively overexpressing human HO-1 and mice lacking endogenous HO-1. Our results suggested that, while HO-1^{-/-} mice are more susceptible to Br₂ toxicity, hHO-1 mice are largely protected.

Lung injury results in an early influx of neutrophils followed by a late increase in macrophages. KC, a major chemoattractant for neutrophils, was elevated in the BALF of Br₂ exposed mice. Heme attenuation either by Hx or hHO-1 overexpression attenuated the levels of KC, suggesting that heme is responsible for the early phase of inflammation. Surprisingly, we found that, the overexpression of hHO-1 has superior protection against Br₂-induced lung toxicity in terms of neutrophil suppression and overall survival than Hx treatment probably due to additional anti-inflammatory effects of CO, which are generated by the action of HO-1 during the degradation of heme (33).

Both HO-1 induction and CO administration have been previously shown to be protective against airway pathologies, such as asthma (6), bleomycin-induced pulmonary fibrosis (47), and hypoxia-induced inflammation and hypertension (29). However, it should be noted that some studies have reported that excessive production of HO-1 may be detrimental due to the generation of reactive iron during heme metabolism (23, 43).

In conclusion, our study has demonstrated that heme-dependent airway injury underlies the pathogenesis of Br₂ toxicity. Thus, therapeutic approaches that reduce heme levels such as Hx, and to some extent albumin and haptoglobin, in addition to pharmacological induction of HO-1 may prove to be beneficial countermeasures that can be exploited by emergency room physicians to treat patients in the immediate aftermath of Br₂ gas exposure to reduce morbidity and mortality. It should be noted that our data also demonstrated that Br₂ inhalation increased BUN, suggesting possible renal damage in the exposed mice. Therefore,

further studies would be required to fully elucidate the complex pathology of Br₂ toxicity.

Materials and Methods

Animals

Adult male and female C57BL/6 mice (20–25 g) were bought from Charles River (Wilmington, MA), non-Frederick/NCL. All the C57BL/6 mice used in the study were males except in Figure 1A where the survival between male and female mice was compared.

Mice overexpressing the human HO-1 gene, hHO-1, were generated as described earlier (21). Briefly, a 87 Kb bacterial artificial chromosome (BAC) clone (GenBank Accession No. Z82244) containing the entire human HO-1 gene was purified, and sets of 200 C57BL/6J oocytes were microinjected with 30 ng of the purified BAC DNA. Fertilized ova were subsequently implanted into pseudopregnant females, and the offspring were analyzed for the insertion of the HO-1 BAC. Two independent founder lines were produced that were confirmed to transmit the transgene. The inserted BAC DNA contained the entire human HO-1 gene along with all of its regulatory regions. These HO-1 BAC mice were then bred with HO-1^{-/-} mice to generate hHO-1 mice, as described previously (21).

The HO-1^{-/-} mice, hHO-1 mice, and the WT littermate mice also had a mixed background of C57BL/6 and FVB. All the WT, hHO-1, and HO-1^{-/-} mice used in the study were male except in Figure 7D–F, where two animals in each of the three groups were females due to the unavailability of sufficient male mice. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Alabama in Birmingham.

Exposure to Br₂

Mice were exposed to Br₂ gas (400 or 600 ppm) in a cylindrical glass chamber for 30 or 45 min, as previously described for chlorine gas (25, 42). Control mice were exposed to room air in the same experimental conditions as Br₂ exposed mice. Exposures were performed with two mice in the same chamber at any one time, and all exposures were performed between 6:00 AM and 12:00 PM. Tanks were replaced when the pressure in the tanks reached 500 psi. In each case, immediately following exposure, mice were returned to room air. All experiments involving animals were conducted according to protocols approved by the UAB IACUC.

Hemopexin administration

After 30 min of Br₂ exposure, C57BL/6 mice were given a single intraperitoneal injection of Hx (Product No. 16-16-080513; Athens Research and Technology, Athens, GA) in 1× phosphate-buffered saline (PBS) (0.1–6 µg/kg final concentration). Hx stocks were prepared daily in sterile PBS with injection volumes of 75 µl.

Histological analysis and HO-1 staining of the mouse lung

Lung tissues were removed and fixed in 10% formalin for 24 h and dehydrated in 70% ethanol before embedding in paraffin. Paraffin-embedded tissues were cut into 4 µm sections, deparaffinized, and rehydrated using CitriSolv

(d-limonene-based solvent) and isopropanol, respectively. The sections were stained with hematoxylin and eosin.

In addition, sections were deparaffinized in xylenes and rehydrated in a series of ethanol washes (100%/95%/70%). Retrieval of antigen for anti-HO-1 was performed by steaming in the Trilogy solution. Sections were blocked with the PBS buffer containing 1% BSA/0.2% nonfat dry milk/0.3% Triton X-100 for 1 h and then incubated for 16 h at 4°C with the polyclonal rabbit anti-HO-1 antibody (Product No. SPA-895; Enzo Life Sciences, Farmingdale, NY) diluted 1:1000 in a blocking buffer. The peroxidase-conjugated goat anti-rabbit antibody was applied for 1 h at room temperature followed by PBS washes. Vector VIP substrate was added as per the manufacturer's instructions. The sections were dehydrated and mounted in the xylene mounting medium. Images were captured using a Leica DMI 6000 B microscope (Leica Microsystems, Inc., Bannockburn, IL) and Leica Application Suite V4.2 software.

MPO staining

Sections (5 µm) were cut from paraffin blocks and mounted on treated slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA). The slides were air-dried overnight, placed in a 60°C oven for 30 min, deparaffinized in xylene, and run through graded alcohol to distilled water. Endogenous peroxidases were quenched with 0.3% H₂O₂ for 5 min followed by two rinses with distilled water. The slides were then stained with the anti-MPO antibody (Product No. ab9535; Abcam, Cambridge, MA), using the HRP-DAB Cell and Tissue Staining Kit (Product No. CTS005; R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The MPO-stained slides were then evaluated by scoring for the presence of neutrophils within the alveolar and interstitial spaces.

Lung injury scoring

Lung injury was scored using five parameters: (i) neutrophils in the alveolar space; (ii) neutrophils in the interstitial space; (iii) development of hyaline membranes; (iv) proteinaceous debris filling the airspaces; and (v) alveolar septal thickening. All the guidelines laid down by the official American Thoracic Society workshop report (28) on features and measurements of experimental ALI in animals were followed. The slides were analyzed by a double-blinded study.

ALI assessment

Mice were euthanized with an intraperitoneal injection of ketamine and xylazine (100 and 10 mg/kg body weight, respectively). An incision was made at the neck to expose the trachea, and a 3-mm endotracheal cannula was inserted. The lungs were lavaged with 1 ml of PBS; ~0.9–0.7 ml was recovered in all groups. Mice were then exsanguinated by cardiac puncture for the collection of blood.

Recovered aliquots of lavage fluid were kept on ice and centrifuged immediately at 3000 g for 10 min to pellet the cells. Supernatants were removed and stored on ice for protein analysis using the BCA Protein Assay Kit (Product No. 23225; Thermo Fisher Scientific, Rockford, IL). Cells were resuspended in 100 µl of PBS and counted using a Neubauer hemocytometer. Cells were then placed on slides using a Cellspin (Tharmac, Drosselweg, Germany) and stained using

a two-stain set consisting of Eosin Y and a solution of thiazine dyes (Quik-Stain; Siemens, Washington, DC). Differential counts (specifically monocytes, neutrophils, and lymphocytes) were then performed on slides *via* light microscopy.

Staining for apoptotic/necrotic cells

Cells from the BALF were collected from air or Br₂ (600 ppm, 30 min) exposed mice 24 h after exposure, as mentioned above, and stained with Annexin V FITC Assay Kit (Product No. 600300; Cayman Chemicals Company, Ann Arbor, MI), as per the manufacturer's instructions. The stained cells were then analyzed for the presence of early apoptotic, late apoptotic/necrotic, and necrotic cells using flow cytometry.

LDH assay

The release of LDH, a sign of cell death, was measured in the BALF of air or Br₂ (600 ppm, 30 min) exposed mice 24 h after exposure. The LDH Cytotoxicity Assay Kit (Product No. 88953; Thermo Fisher Scientific) was used for this purpose, and all the instructions were followed, as per the manufacturer's protocol.

Ex vivo hemolysis assay

Ex vivo hemolysis was measured using a modified version of a previously published protocol (11). Briefly, blood from mice was collected, as described above. From each mouse, blood (500 μ l) was obtained, split (100 μ l each) into five microcentrifuge tubes (labeled 0–4), and centrifuged at 500 g for 5 min. Plasma (yellowish, upper layer) was aspirated. PBS (pH 7.4), equivalent to the amount of aspirated plasma, was added to the hematocrit (red, lower layer), gently mixed, and centrifuged again at 500 g for 5 min. This washing of cells was repeated thrice to completely rid the cells of plasma.

Finally, the supernatant was aspirated and replaced by increasing concentrations of SDS solution in PBS (0%, 0.02%, 0.04%, 0.08%, and 0.1%), mixed gently, and incubated at 37°C for 1 h. Subsequently, the mixture was centrifuged for 5 min at 500 g, the supernatant was carefully transferred to a 96-well plate, and the absorbance was read at 540 nm. Our repeated experiments showed that at higher concentrations of SDS, such as 1% and 12%, the maximum hemolysis was equivalent to the hemolysis caused by 0.1% SDS. Therefore, the results were depicted as a percentage of hemolysis by using 0.1% SDS as the benchmark for 100% hemolysis.

BALF and plasma heme assay

Heme levels were measured in mouse BALF and plasma using the QuantiChrom heme assay kit (Product No. DIHM-250; BioAssay Systems, Hayward, CA), according to the manufacturer's instructions.

Lung fractionation and heme assay

Lungs were perfused with PBS *in situ*. About a third of each lung was taken and 5 $\times$ (w/v) a sucrose buffer (0.25 M sucrose with 0.01 M Tris-HCl, pH 7.4) was added. Then, the lungs were homogenized and sonicated on ice. The lysate was centrifuged at 3000 rpm at 4°C for 20 min. The pellet was discarded, and a small fraction of the supernatant was used to

measure total lung heme levels. The remaining supernatant was further centrifuged at 12,000 g at 4°C for 20 min. The pellet was resuspended in phosphate buffer (400 μ l) and used to determine mitochondrial heme levels. The supernatant was then used for further fractionation. Using an ultracentrifuge, the supernatant was centrifuged at 105,000 g (37,000 rpm) for 1 h. The resultant pellet from this step was resuspended in 1 ml phosphate buffer and utilized to analyze the microsomal heme fraction, while the supernatant provided the cytosolic heme levels.

The protein content of each sample was analyzed by the BCA assay to normalize the heme levels. The following steps to determine heme levels were performed in a fume hood. The samples were brought up to 800 μ l in a phosphate buffer; 400 μ l of pyridine (Product No. P368-1, ACS stock; Fisher Scientific) was added and mixed. To the mixture, 400 μ l of 1 N NaOH was added and again mixed. The mixture was then split into two equal volumes of 790 μ l each in a cuvette. One half was oxidized and the other reduced. To oxidize, 3 μ l of 0.1 M K₃Fe(CN)₆ solution was added to one volume of the sample, while 1–2 mg of dithionite was added to the other half to reduce the sample. Each sample was mixed and covered with parafilm. Within 5 min of oxidation/reduction, the reduced samples were scanned from 570 to 520 nm. The oxidized samples were used to blank the spectrophotometer.

Western blot analysis

Mice were anesthetized and euthanized. Their lungs were perfused with PBS until they were clear of blood, removed, and homogenized in a radioimmunoprecipitation assay lysis buffer (Product No. 89901; Thermo Fisher Scientific) containing protease inhibitors. The samples were then sonicated for 10 s thrice on ice in 1.5-ml Eppendorf tubes using an ultrasonic liquid processor and centrifuged at 14,000 g for 20 min at 4°C.

Cleared supernatants were used to measure the protein concentration by the BCA assay. Equal amounts of protein (150–200 μ g) were loaded in 10% Tris-HCl Criterion precast gels (Product No. 567-1093; Bio-Rad Laboratories, Hercules, CA); proteins were transferred to polyvinylidene difluoride membranes (Product No. 162-0177; Bio-Rad Laboratories) and immunostained with an anti-HO-1 antibody (1:1000 dilution; Enzo Life Sciences). Bands were detected by the Protein Detector LumiGLO Western Blot Kit (Product No. 95059-302; VWR, Radnor, PA). Protein loading was normalized by reprobing the membranes with an antibody specific to β -actin.

Measurement of carbonyl adducts in lung protein

The presence of protein carbonyl groups was assessed using the OxyBlot Protein Oxidation Detection Kit (Product No. S7150; EMD Millipore, Billerica, MA), according to the manufacturer's protocol. Briefly, the carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone by reacting with 2,4-dinitrophenylhydrazine. Precisely, 10 μ g of protein was used for each sample, and the 2,4-dinitrophenol-derivatized protein samples were separated by polyacrylamide gel electrophoresis, as described previously. Polyvinylidene fluoride membranes were incubated for 1 h in the stock primary antibody (1:150 in 1% PBS/TBST buffer) and after washing for 1 h in the stock secondary antibody (1:300 in %

PBS/TBST buffer). Membranes were washed 3× in TBST and visualized, as described previously.

The abundance of protein carbonylation was assessed by densitometry of each lane. Normalization for lane protein loading was performed by probing for β -actin levels.

BALF cytokine measurements

A panel of 25 cytokines (G-CSF, GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 [p40], IL-12 [p70], IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1 α , MIP-1 β , MIP-2, RANTES, TNF- α) were assessed in duplicate in 75 μ l of BALF and plasma from mice using a cytokine magnetic bead assay (MILLIPLEX Mouse Cytokine Magnetic Bead Panel-Premixed 25 Plex-Immunology Multiplex Assay, Product No. MXMCY70KPMX25MAG; EMD Millipore). The cytokine concentrations were measured using the Bio-Plex multiplex suspension cytokine array (Bio-Rad, Philadelphia, PA).

Lung leak assessment *in vivo*

Lung leak assessment *in vivo* was performed by measuring the lung wet-to-dry weight ratio. Briefly, the lungs were excised, and the tissue wet weight was measured immediately followed by drying at 80°C for 36 h to obtain dry weight measurements.

Assessment of respiratory mechanics

Mice were mechanically ventilated and challenged with increasing concentrations of methacholine, as described previously (3, 12). Briefly, 24 h postair or Br₂ exposure, mice were anesthetized with pentobarbital (50 mg/kg i.p.; Vortech Pharmaceuticals, Dearborn, MI), paralyzed with pancuronium (4 mg/kg i.p.; Gensia Sicor Pharmaceuticals, Irvine, CA), intubated, connected to an FX-1 module of the FlexiVent® (SCIREQ, Montreal, PQ, Canada), and ventilated at a rate of 160 breaths per minute at a tidal volume of 0.2 ml with a positive end-expiratory pressure of 3 cm H₂O.

Total, as well as Newtonian, respiratory system resistance (R and R_N) and elastance (E and E_N) were recorded continuously, as previously described (17). Baseline was set *via* deep inhalation. Increasing concentrations of methacholine chloride (0–40 mg/ml; Sigma-Aldrich, St. Louis, MO) were administered *via* aerosolization within an administration time of 10 s. Airway responsiveness was recorded every 15 s for 3 min after each aerosol challenge. Broadband perturbation was used, and impedance was analyzed *via* the constant phase model. Assessments of Newtonian *versus* total respiratory resistances allow us to differentiate the contribution of the central *versus* peripheral airways in the observed increase in total lung resistance.

ABG analysis

Blood pH, PCO₂, PO₂, Na⁺, K⁺, BUN, tHb, SO₂, HCO₃⁻, and Hct were assessed using OPTI® CCA-TS Blood Gas and Electrolyte Analyzer (OPTI Medical, Atlanta, GA) with E-BUN cassettes (Product No. BP7558; OPTI Medical). For blood collection, mice were anesthetized with ketamine/xylazine (40 μ l ketamine/20 μ l xylazine). The abdominal cavity was exposed, and abdominal aorta was isolated from connected viscera and injected with 25 μ l heparin.

Immediately following, 200 μ l arterial blood was collected *via* a 25-gauge needle and used for analysis.

Statistical analyses

Statistical analysis was performed using GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA). The mean $\pm$ SEM was calculated in all experiments, and statistical significance was determined by either the one-way or the two-way ANOVA. For one-way ANOVA analyses, Newman–Keuls *post hoc* testing was employed, while for two-way ANOVA analyses, Bonferroni posttests were used. Overall survival was analyzed by the Kaplan–Meier method. Differences in survival were tested for statistical significance by the log-rank test. A value of $p < 0.05$ was considered significant.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ABG = arterial blood gas
 ALI = acute lung injury
 ARDS = acute respiratory distress syndrome
 BALF = bronchoalveolar lavage fluid
 Br₂ = bromine
 BSA = bovine serum albumin
 BUN = blood urea nitrogen
 CO = carbon monoxide
 FEV₁ = minute forced expiratory volume
 HO-1 = heme oxygenase-1
 Hx = hemopexin
 KC = keratinocyte-derived chemokine
 LDH = lactate dehydrogenase
 MCP-1 = monocyte chemoattractant protein 1
 MIP = macrophage inflammatory protein
 MPO = myeloperoxidase
 MT-1 = metallothionein-1
 PBS = phosphate-buffered saline
 pCO₂ = partial pressure of carbon dioxide
 RBC = red blood cell
 SDS = sodium dodecyl sulfate
 SEM = standard error of the mean
 TBST = Tris-buffered saline and Tween 20
 WT = wild type.