Heme Oxygenase-1/Carbon Monoxide-regulated Mitochondrial Dynamic Equilibrium Contributes to the Attenuation of Endotoxin-induced Acute Lung Injury in Rats and in Lipopolysaccharide-activated Macrophages

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ABSTRACT

Background: Sepsis-associated acute lung injury remains the major cause of mortality in critically ill patients and is characterized by marked oxidative stress and mitochondrial dysfunction. Mitochondrial dynamics are indispensable for functional integrity. Additionally, heme oxygenase (HO)-1/carbon monoxide conferred cytoprotection against end-organ damage during endotoxic shock. Herein, we tested the hypothesis that HO-1/carbon monoxide played a critical role in maintaining the dynamic process of mitochondrial fusion/fission to mitigate lung injury in Sprague-Dawley rats or RAW 264.7 macrophages exposed to endotoxin.

Methods: The production of reactive oxygen species, the respiratory control ratio (RCR), and the expressions of HO-1 and mitochondrial dynamic markers were determined in macrophages. Concurrently, alterations in the pathology of lung tissue, lipid peroxidation, and the expressions of the crucial dynamic proteins were detected in rats.

Results: Endotoxin caused a 31% increase in reactive oxygen species and a 41% decrease in RCR levels (n = 5 per group). In parallel, the increased expression of HO-1 was observed in lipopolysaccharide-stimulated macrophages, concomitantly with excessive mitochondrial fission. Furthermore, carbon monoxide-releasing molecule-2 or hemin normalized mitochondrial dynamics, which were abrogated by zinc protoporphyrin IX. Additionally, impaired mitochondrial dynamic balance was shown in Sprague-Dawley rats that received lipopolysaccharide, accompanied by pathologic injury, elevated malondialdehyde contents, decreased manganese superoxide dismutase activities, and lowered RCR levels in rat lung mitochondria. However, the above parameters were augmented by zinc protoporphyrin IX and were in turn reversed by hemin.

Conclusions: The HO-1/carbon monoxide system modulated the imbalance of the dynamic mitochondrial fusion/fission process evoked by lipopolysaccharide and efficiently ameliorated endotoxin-induced lung injury *in vivo* and *in vitro*.
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SEPSIS-DEPENDENT multiple organ failure, which presents a high mortality rate, is a compelling clinical problem in the field of medicine.[1,2] The lung is one of the most vulnerable organs when subjected to sepsis. The exogenous administration of endotoxin causes acute lung injury (ALI), called mild acute respiratory distress syndrome by the Berlin definition, which has come to be an accepted model to analyzing the complex pathophysiologic responses to endotoxemia in humans.[3,4] Many studies have focused on oxidative stress, with aberrantly activated reactive oxygen species (ROS) involved in multiple organ failure during severe sepsis.[5] Mitochondria, an abundant source of intracellular ROS, have been implicated as the major target for oxidative damage.[6] As reported by Mannam *et al.*,[7] mitochondrial dysfunction is a paramount

What We Already Know about This Topic

- Heme oxygenase-1 is an important element in protection against oxidant-induced injury, and mitochondrial dysfunction is important in such injury

What This Article Tells Us That Is New

- Using isolated cell and *in vivo* models, it was found that heme oxygenase-1 regulates mitochondrial dynamic equilibrium in the setting of lipopolysaccharide injury, and this contributes to a negative feedback loop that dampens endotoxin-induced acute lung injury

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mediator of sepsis-induced lung injury. However, the ultimate mechanism of the modulation of mitochondrial processes is poorly understood.

Mitochondria, the principal source of cellular adenosine triphosphate, are dynamic organelles that undergo delicate fusion and fission cycles to maintain their functions when cells experience metabolic or environmental stress.[8] Notably, their dynamics are mediated by several prime guanosine-5′-triphosphatases in the dynamin family. In mammals, fusion is regulated by mitofusins 1 and 2 (Mfn1/2), situated in mitochondrial outer membranes, whereas optic atrophy 1 (OPA1) is situated in mitochondrial inner membranes. Concurrently, dynamin-related protein 1 (Drp1) is recruited from the cytosol to mitochondria to initiate the fission process.[8,9] Intriguingly, the balance of mitochondrial dynamic behavior is a prerequisite for functional integrity and, if impaired, triggers various pathologic processes, such as cellular senescence, ischemia–reperfusion, and neuronal injury.[10,11] Moreover, the progression of organ failure during sepsis is due at least partially to mitochondrial dysfunction initiated by oxidative stress, along with a decreased mitochondrial respiratory control ratio (RCR).[12,13] Because a properly regulated fusion/fission balance is essential, exploiting a potential antioxidant to preserve mitochondrial dynamics may be a vital determinant in protecting mitochondria from oxidative damage in animal models of sepsis-induced organ failure.

Heme oxygenase-1 (HO-1), namely, heat shock protein 32, is potentially a stress-inducible protein induced by endotoxin, cytokines, and ROS and tends to impede oxidative cellular injury.[14] As the initial and rate-limiting enzyme, HO-1 is ubiquitous in mammalian tissues and catalyzes the oxidation of heme to equal moles of free iron, biliverdin, and carbon monoxide. Under stressful conditions, the endogenous production of carbon monoxide or the administration of carbon monoxide-releasing molecules has antiinflammatory, antiapoptotic, and cytoprotective properties.[15,16] Our previous study described the defensive role of HO-1, followed by endogenous carbon monoxide, against oxidative stress and end-organ damage during endotoxic shock.[17] To date, most experimental evidence indicates that the induction and mitochondrial localization of HO-1 are novel cytoprotective mechanisms for resisting mitochondrial oxidative stress–mediated apoptotic tissue injury.[18,19] Of note, whether the HO-1/carbon monoxide system exerts antioxidant effects to sustain the integrity of mitochondrial function *via* the modulation of mitochondrial dynamics to suppress endotoxin-induced ALI has yet to be examined.

Macrophages serve as the first cell line to confront invading pathogens through the clearance of cellular debris, antigen presentation, and immunomodulation.[20] Consequently, the current study was designed to clarify whether HO-1/carbon monoxide preserves the balance of mitochondrial fusion and fission *via* the modulation of dynamic-related proteins to attenuate ALI induced by endotoxin in rats and in lipopolysaccharide-stimulated macrophages.

Materials and Methods

Reagents

Dulbecco's modified Eagle medium, fetal bovine serum, and penicillin–streptomycin were purchased from Invitrogen (Grand Island, USA). Antibodies against HO-1 (SC-10789), Mfn1 (SC-50330), Mfn2 (SC-50331), OPA1 (SC-367890), Drp1 (SC-32898), and β-actin were supplied by Santa Cruz Biotechnology (USA). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) for ROS was from commercial assay kits supplied by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The mitochondrial reductase function test, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was from Roche Applied Science (USA). All other chemicals, including lipopolysaccharide (L2630), zinc protoporphyrin IX (ZnPP), carbon monoxide–releasing molecule-2 (CORM2), and hemin, were obtained from Sigma-Aldrich (USA).

Cell Culture

Murine RAW 264.7 macrophage cell lines were purchased from the American Type Culture Collection (USA) and cultured in Dulbecco's modified Eagle medium, supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin at 37°C in 5% CO_2 and 95% air. Cells were cultured to 80 to 90% confluence and then split at a starting density of 2×10^5 cells per milliliter in 96-well plates. The cells were rinsed with a fresh medium and stimulated with lipopolysaccharide (1 μg/ml) in the presence or absence of CORM2 (100 μM), ZnPP (10 μM), or hemin (20 μM).[21,22] After incubation for 24 h, the cells were harvested for Western blotting and real-time polymerase chain reaction (PCR).

Cell Viability

The effects of various experimental manipulations on cell viability were determined with the MTT assay, as previously reported.[23] In brief, RAW 264.7 cells (2×10^4 cells per milliliter) were seeded in 96-well plates and incubated overnight. Approximately, 10 μl of 5 mg/ml MTT was added to each well during the last 2 h of culture, and the medium was aspirated. Then, 150 μl dimethyl sulfoxide was added to solubilize blue formazan crystals for 30 min at 37°C. The absorbance of each well was measured at 570 nm by using a microplate reader. The viability of treated cells was calculated as follows: ($absorbance_{570\ nm}$ of the therapeutic group/$absorbance_{570\ nm}$ of the control group) ×100%.

ROS Production

ROS generation by lipopolysaccharide-activated RAW 264.7 cells was determined spectrofluorometrically using DCFH-DA as a fluorescent dye. DCFH-DA is cleaved by

intracellular esterase to yield nonfluorescent DCFH, which is oxidized by peroxides to highly fluorescent dichlorofluorescein (DCF).[24] Briefly, cells were plated in 96-well plates and cultured to confluence. The cell suspension was loaded with 10 μM DCFH-DA for 30 min at 37°C. DCF fluorescence was monitored at an excitation wavelength of 480 nm and emission wavelength of 530 nm, respectively, using a Chameleon microplate reader (Hidex, Finland). The results were reported as the differences relative to the initial fluorescence.

Mitochondrial Respiratory Function

Mitochondria were isolated according to the methods described by Takamura *et al.*[25] and Carlson *et al.*[26] with slight modifications. The final mitochondrial pellet was stored at 0°C in a buffer comprising 250 mM sucrose, 10 mM Tris–hydrochloric acid (HCl), 0.5 mM EDTA, and 0.5 g/L fatty acid–free bovine serum albumin (pH 7.2). In brief, mitochondrial respiratory function was assessed by monitoring the oxygen consumption of isolated mitochondria using a Clark-type oxygen electrode (Hansatech, United Kingdom). A 60-μl mitochondrial suspension (0.5 mg/ml) in 105 mM KCl, 5 mM KH_2PO_4, 20 mM Tris–HCl, 1 mM diethylenetriamine pentaacetic acid, and 1 mg/ml fatty acid–free bovine serum albumin (pH 7.4) was incubated in the chamber for 10 min at 25°C. The reaction was initiated with 10 mM succinate (state 4 [ST_4]) and the addition of 0.2 mM adenosine diphosphate (state 3 [ST_3]). Both the ST_3 and ST_4 respiration rates were expressed as nanomoles of monomeric oxygen per minute per milligram mitochondrial protein. Based on the definition of Jacoby *et al.*,[27] the ratio of ST_3/ST_4 was deemed the RCR.

Western Blot Analysis

At the indicated times after being pretreated with various chemicals, cells were washed with phosphate-buffered saline and scraped in a lysis buffer (50 mM Tris base [pH 8.0], 0.1% sodium dodecyl sulfate, and 150 mM NaCl). Mitochondrial cytoplasmic fractionation was prepared by centrifugation as delineated previously.[25] Cell proteins were quantified by the bicinchoninic acid assay (Sigma, USA). Equal amounts of soluble protein were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Bio-Rad, USA). Membranes were blocked with 5% nonfat powdered milk in Tris-buffered saline and Tween 20 (TBST) and incubated further at 4°C overnight with primary antibodies for HO-1 (1:500), Mfn1 (1:400), Mfn2 (1:300), OPA1 (1:1,000), Drp1 (1:500), and β-actin (1:500). After being washed three times with TBST, the blots were incubated with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (1:3,000) at room temperature for 1 h. Protein bands were visualized by the enhanced chemiluminescence reagent (Bio-Rad), and the image densities of specific bands were normalized to β-actin.

Real-time Reverse Transcription PCR

Total RNA was isolated from cultured cells or mouse lung tissues using a high-purity RNA kit (Roche, Germany) and was quantified by absorbance at 260 nm using a spectrophotometer. Then, 5 μl total RNA was reverse-transcribed into complementary DNA by using a reverse transcriptase (Promega, USA). The resulting complementary DNA was subjected to reverse transcription PCR with SYBR Green Master Mix on an ABI Prism 7000 sequence detector system (Applied Biosystems, USA). The predegeneration of the PCR mix was performed at 95°C for 10 min, followed by 40 thermal cycles consisting of denaturing for 30 s at 95°C, annealing for 5 s at 95°C, and extension for 34 s at 60°C. The primers used for the study were as follows: for HO-1, forward: 5′-GAGCGAAACAAGCAGAACCC-3′ and reverse: 5′-ACCTCGTGGAGACGCTTTAC-3′; for Mfn1, forward: 5′-CTTTCGGGAGGGAGAGAACAC-3′ and reverse: 5′-GCGAGGGTACTTGTGCTTGT-3′; for Mfn2, forward: 5′-TTGAAGCCCCATGCTCCTAC-3′ and reverse: 5′-TTGAAGCCCCATGCTCCTAC-3′; for OPA1, forward: 5′-GGTTGCTTGGGAGACCCTAC-3′ and reverse: 5′-GCCGCTTGATACTCTCCTCC-3′; for Drp1, forward: 5′-AGGCAACTGGAGAGGAATGC-3′ and reverse: 5′-ACAATCTCGCTGTTCTCGGG-3′; and for β-actin, forward: 5′-CAGGGCTGCCTTCTCTTGTG-3′ and reverse: 5′-TCTCGCTCCTGGAAGATGGT-3′. β-actin was used as an internal loading control to normalize all PCR products. The quantification of target gene expression was determined by the comparative cycle threshold (C_T) methods.[2,28]

Animal Model of Endotoxin-induced ALI

Studies were performed in accordance with National Institutes of Health guidelines and were preapproved by the Institutional Animal Use and Care Committee of Tianjin Nankai Hospital, Tianjin, China. Two-month-old male Sprague-Dawley rats (160 to 185 g) were provided by the Laboratory Animal Center of the Nankai Clinical Institution of Tianjin Medical University, Tianjin, China. Rats were caged individually at 30 to 70% humidity (23 to 25°C) and acclimatized to a 12-h light–dark cycle with access to food and water *ad libitum*. Animals were anesthetized and operated on as previously described.[17] Lipopolysaccharide (5 mg/kg, caudal vein injection) reconstituted with 1 ml sterile sodium chloride, 0.9%, was applied to replicate the experimental model of endotoxin-induced ALI. The rats were pretreated intraperitoneally with 50 mg/kg hemin (dissolved in 0.1 M sodium hydroxide) and 10 μmol/kg ZnPP (in 50 mM sodium bicarbonate) 1 h before lipopolysaccharide injection.[29,30] The blood samples were collected at 6 h after lipopolysaccharide administration, and then the rats were euthanized. The lung tissue was harvested, snap-frozen in liquid nitrogen, and stored at −70°C for analytical examinations.

Lung Histology

The middle lobe of the right lung was fixed in 10% formaldehyde and routinely processed in paraffin sections (4 μm) for hematoxylin and eosin staining. Ten different fields from each

slice were visualized by light microscopy (×400). As described in detail previously,[31,32] scoring criteria to grade the degree of lung injury were used based on the following pathologic features: alveolar edema, airway congestion, interstitium widening or hyaline membrane formation, and neutrophil margination or infiltration. Grading was assessed by a blinded pathologist.

Lung Mitochondrial Malondialdehyde Content and Manganese Superoxide Dismutase Activity

Mitochondria were extracted by using a mitochondrial fraction kit (Beyotime, China), and protein concentration was determined by using a Bio-Rad protein assay kit according to the manufacturer's protocols. The enzymatic activity of manganese superoxide dismutase (MnSOD) and the content of malondialdehyde in lung mitochondria were assessed separately by means of xanthine oxidase and thiobarbituric acid, whose related commercial assay kits were supplied by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). MnSOD activities were expressed as units per milligram protein, while malondialdehyde values were represented as nanomoles per milligram protein.[33] All the procedures complied with the manufacturer's instructions.

Statistical Analysis

All values are expressed as mean ± SD, except for lung injury scores, for which the Mann–Whitney U test was used as appropriate. For comparisons among multiple groups, one-way ANOVA was used, followed by the Bonferroni correction *post hoc* test. Graph Prism 5.0 software (GraphPad Software, USA) was used for statistical analysis. Of note, the sample sizes were estimated based on our previous experiences rather than a formal statistical power calculation.[24,34] Randomization methods were used to assign all animals to treatment groups and blinded assessments. The null hypothesis was rejected for *P* values less than 0.05 with the two-tailed test. No experimental data were missing or lost to statistical analysis.

Results

Time-dependent Viability Losses in RAW 264.7 Cells Induced by Lipopolysaccharide

The results of the time–response study in which RAW 264.7 cells were subjected to 1 μg/ml lipopolysaccharide up to 48 h are shown in figure 1. Gradual losses of cell viability, as assessed by MTT assay, were observed from 12 to 48 h after lipopolysaccharide exposure. The magnitude of cell injury peaked at 24 h after incubation with lipopolysaccharide (1 μg/ml), and cell viability was close to 54 ± 11% ($P = 0.001$). Therefore, cells were treated with 1 μg/ml of lipopolysaccharide for 24 h or vehicle as a control.

Carbon Monoxide and HO-1 Induction Protected Alveolar Macrophages from Lipopolysaccharide-driven ROS Production and RCR Reduction

To further understand the role of the HO-1 system in the production of ROS stimulated by lipopolysaccharide, RAW 264.7

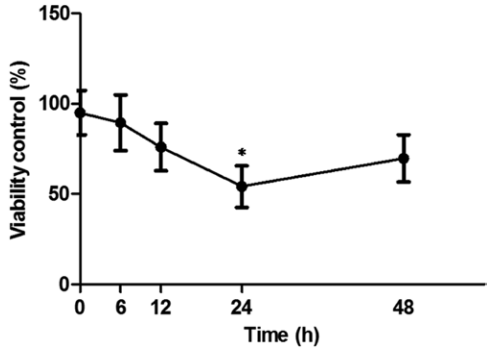

Fig. 1. Viability losses in RAW 264.7 cells induced by 1 μg/ml lipopolysaccharide for different time periods. Survival was monitored daily for 2 days. The magnitude of cell injury peaked at 24 h after lipopolysaccharide exposure. Values were analyzed using the Mann–Whitney U test with Bonferroni correction for five individual experiments. Of note, the predefined significance level ($P < 0.05$) was reset as *P* value less than 0.005 after correction. Significant differences were indicated with an *asterisk* (*$P < 0.005$).

cells were pretreated with 100 μM CORM2 or 20 μM hemin for 1 h with 10 μM ZnPP for 0.5 h before lipopolysaccharide treatment and further incubated for 24 h. CORM2 was reported to be a useful pharmacologic tool to mimic partial protective actions attributed to HO-1 and carbon monoxide.[21] The results shown in figure 2A indicate that ROS levels were markedly increased (approximately 31%) by treatment of cells with lipopolysaccharide compared to the control ($P < 0.001$), and the above inductions (lipopolysaccharide-driven ROS production) were dramatically diminished by pretreatment with CORM2 (CORM2 *vs.* lipopolysaccharide, 116.62 ± 3.54 *vs.* 132.21 ± 8.07; **$P = 0.004$; n = 5), while CORM2 alone had no effect (fig. 1A, Supplemental Digital Content, http://links.lww.com/ALN/B313), which confirmed the involvement of carbon monoxide. Furthermore, to evaluate the role of carbon monoxide–induced HO-1 in ROS generation, the HO-1 inhibitor ZnPP was used for 0.5 h ahead of lipopolysaccharide. As expected, the inclusion of ZnPP reversed the protection conferred by CORM2 and resulted in 12% enhanced ROS levels in lipopolysaccharide-stimulated macrophages (*$P = 0.034$). In addition, to address the direct effects of HO-1 on ROS generation, macrophages were pretreated with hemin for 1 h and then incubated with lipopolysaccharide for 24 h (fig. 2B). Interestingly, hemin, known as a substrate and potent inducer of HO-1, significantly inhibited lipopolysaccharide-mediated excessive ROS (hemin *vs.* lipopolysaccharide, 118.08 ± 3.48 *vs.* 133.83 ± 7.56; **$P = 0.003$), while the addition of ZnPP inversely promoted ROS production in the presence of hemin (*$P = 0.025$). Of note, the vehicle, dimethyl sulfoxide, or sodium bicarbonate alone was proven to have no effect (fig. 1, A and B, Supplemental Digital Content, http://links.lww.com/ALN/B313, which presents the effects of CORM2 or ZnPP-alone pretreatment on ROS production).

To gain insight into the integrity and intactness of isolated mitochondria, the RCR was evaluated (fig. 2, C and D). A

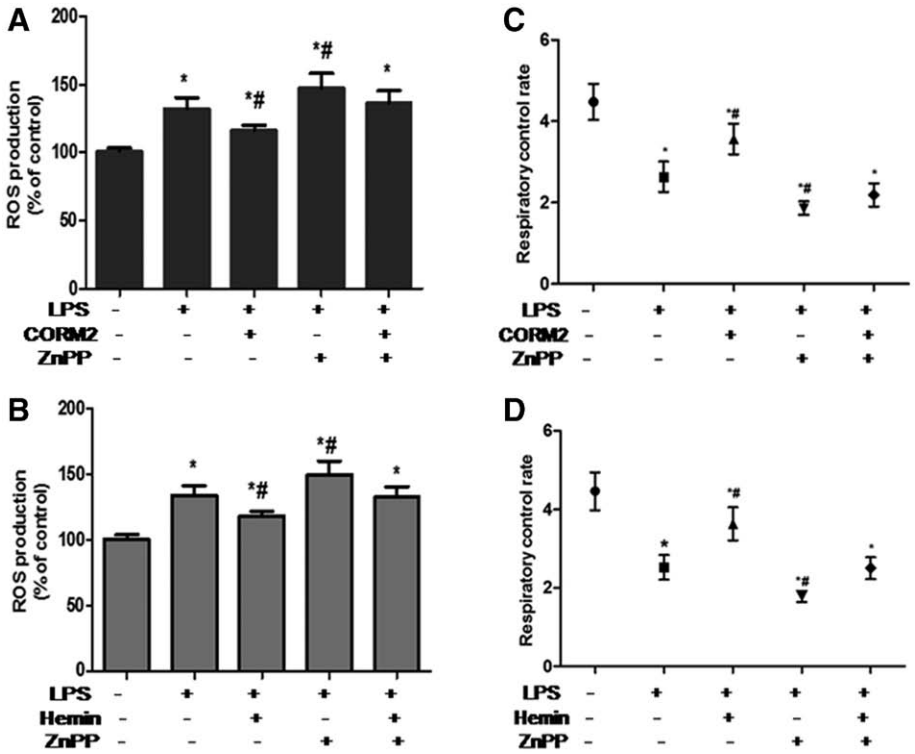

Fig. 2. Effect of carbon monoxide and heme oxygenase (HO)-1 induction on reactive oxygen species (ROS) production (*A*, *B*) and respiratory control ratio (RCR) levels (*C*, *D*). RAW 264.7 cells were pretreated with 100 μM carbon monoxide–releasing molecule-2 (CORM2) or 20 μM hemin for 1 h with 10 μM zinc protoporphyrin IX (ZnPP) for 0.5 h and then stimulated with 1 μg/ml lipopolysaccharide (LPS) for 24 h. Note that CORM2 or hemin pretreatment protected macrophages against LPS-derived ROS overproduction and RCR depression. In contrast, pretreatment with ZnPP, the HO-1 inhibitor, reversed the above favorable effects in the presence of CORM2 or hemin and further increased ROS and decreased RCR levels in LPS-stimulated cells. Values are represented as mean ± SD from five individual samples using one-way ANOVA and the Bonferroni test for multiple comparisons. *Significance compared with control cells ($P < 0.05$). #Significance compared with LPS-exposed cells ($P < 0.05$).

significant reduction in RCR levels (approximately 41%) coupled with excessive ROS production was observed in RAW 264.7 cells subjected to challenge with lipopolysaccharide (**$P < 0.001$), which were further depressed by pretreatment with HO-1 inhibitor, ZnPP (lipopolysaccharide *vs.* ZnPP, 2.63 ± 0.37 *vs.* 1.86 ± 0.17; **$P = 0.003$). In contrast, lipopolysaccharide-exposed cells with CORM2 pretreatment dramatically restored the levels of RCR, which suggested that carbon monoxide exerted a protective effect against the lipopolysaccharide-induced loss of RCR (**$P = 0.004$). In support, hemin treatment obviously increased lipopolysaccharide-induced RCR levels (hemin *vs.* lipopolysaccharide, 3.63 ± 0.42 *vs.* 1.78 ± 0.14), whereas the effect was abrogated by ZnPP administration (**$P = 0.001$). Together, the results suggested that the HO-1/carbon monoxide improved mitochondrial function in lipopolysaccharide-exposed macrophages as reflected by reduced mitochondrial ROS generation and elevated RCR.

Carbon Monoxide and HO-1 Induction Upregulated Mitochondrial Mfn1/2 and OPA1 and Downregulated Drp1 Expression in RAW 264.7 Cells

As shown in figure 3, exposure to lipopolysaccharide after 24 h greatly diminished the expressions of mitochondrial fusion genes and proteins Mfn1, Mfn2, and OPA1 but enhanced the levels of mitochondrial fission gene and protein Drp1 (Mfn1: control *vs.* lipopolysaccharide, 1.065 ± 0.078 *vs.* 0.493 ± 0.043; Mfn2: control *vs.* lipopolysaccharide, 1.096 ± 0.081 *vs.* 0.531 ± 0.044; OPA1: control *vs.* lipopolysaccharide, 1.194 ± 0.079 *vs.* 0.512 ± 0.040; and Drp1: control *vs.* lipopolysaccharide, 0.550 ± 0.049 *vs.* 1.032 ± 0.069; n = 5; mean ± SD). In parallel with the previous indexes, higher levels of the messenger RNA (mRNA) and proteins of HO-1, Mfn1, Mfn2, and OPA1 and lower levels of mRNA and protein Drp1 were confirmed by real-time PCR and Western blot in CORM2 or hemin-pretreated cells elicited by lipopolysaccharide ($P < 0.05$). Conversely, HO-1, Mfn1, Mfn2, and OPA1 mRNA and proteins expression exhibited a striking reduction, while Drp1 mRNA and protein levels were increased in response to preincubation with ZnPP of RAW 264.7 cells exposed to lipopolysaccharide ($P < 0.05$). Of note, CORM2 alone had no effect on the expression of HO-1 and above-mentioned mitochondrial dynamic markers (Mfn1/2, OPA1, and Drp1; fig. 2, Supplemental Digital Content, http://links.lww.com/ALN/B314, which presents the effects of CORM2 or ZnPP alone on mRNA levels of HO-1 and mitochondrial fusion/fission markers in RAW

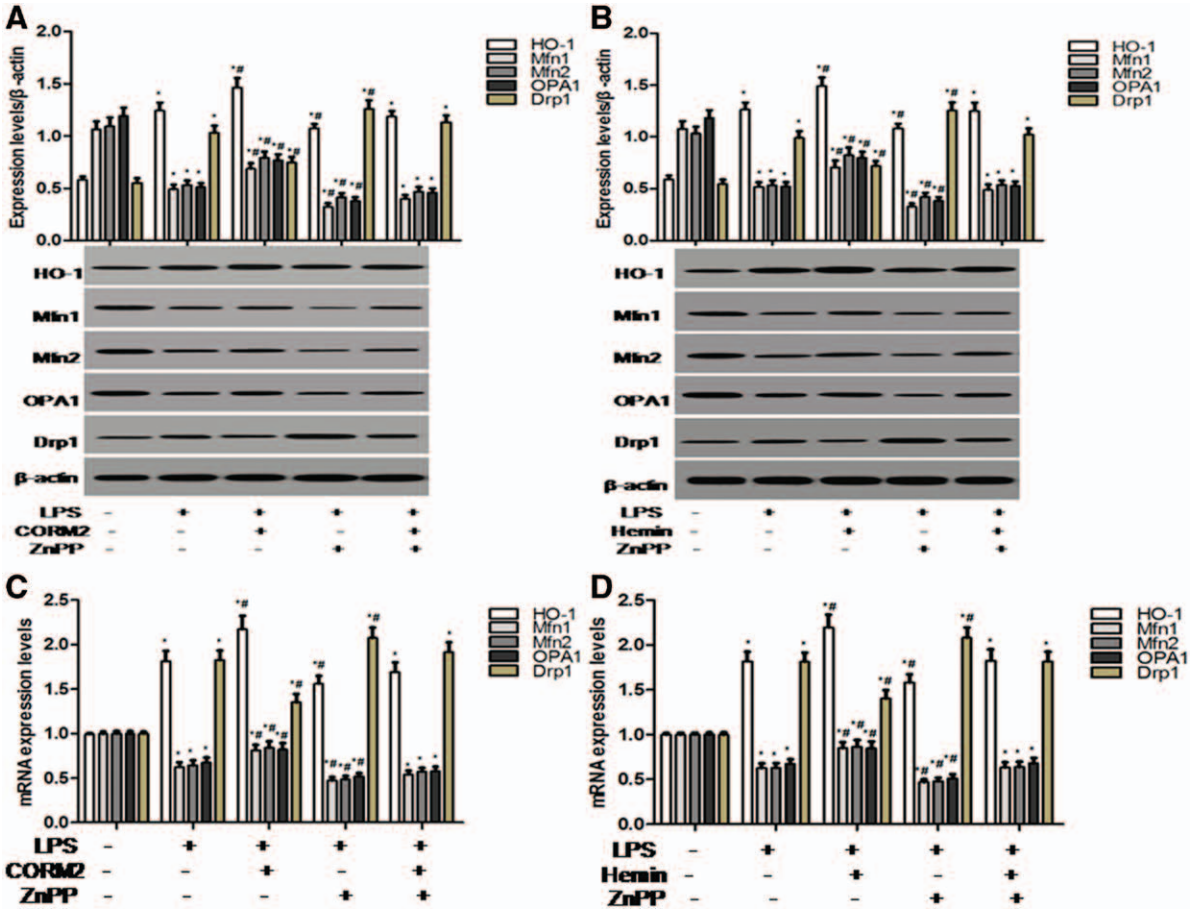

Fig. 3. Effect of carbon monoxide and heme oxygenase (HO)-1 induction on the protein (*A*, *B*) and messenger RNA (mRNA; *C*, *D*) expression of mitochondrial fusion/fission markers in RAW 264.7 cells subjected to lipopolysaccharide (LPS). Pretreatment with carbon monoxide–releasing molecule-2 (CORM2) or hemin markedly attenuated LPS-induced increases in the mRNA and protein levels of dynamin-related protein 1 (Drp1) and decreases in the mRNA and protein levels of mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy 1 (OPA1) in macrophages, along with up-regulated HO-1. Conversely, the levels of the above fusion proteins and mRNA (Mfn1, Mfn2, and OPA1) were further restricted, while the fission protein and mRNA (Drp1) were increased in cells preincubated with zinc protoporphyrin IX (ZnPP) *via* HO-1 down-regulation. β-actin served as an internal standard to ensure similar gel loading of the staring material in each sample. Values are expressed as mean ± SD of five independent experiments using one-way ANOVA and the Bonferroni test for multiple comparisons. *Significant difference from control cells ($P < 0.05$). #Significant difference from LPS-exposed cells ($P < 0.05$).

264.7 cells elicited by lipopolysaccharide). Collectively, the data support a key role for carbon monoxide and HO-1 induction in regulating mitochondrial dynamics.

Induction of HO-1 Attenuated the Septic Lung Injury of Rats

In light of the impact of HO-1 in ameliorating lipopolysaccharide-induced lung injury of rats, hemin (50 mg/kg, intraperitoneally) or ZnPP (10 μmol/kg, intraperitoneally) was administered 1 h before lipopolysaccharide injection. Photomicrographs of rat lung slices revealed that intravenous lipopolysaccharide elicited thickening of the alveolar wall, infiltration of inflammatory cells into alveolar spaces, hemorrhage, and formation of hyaline membrane, as shown in figure 4A. In comparison, the pathologic changes were less pronounced with hemin pretreatment but were reinforced with ZnPP pretreatment. Semiquantitative assessment using the lung injury scores is summarized in figure 4B. The degree of lung injury increased at 6 h after lipopolysaccharide induction (10.2 [8 to 11]); however, hemin pretreatment improved the levels of lung injury scores (5.4 [4 to 6]), while ZnPP preconditioning aggravated the lung injury scores (13.8 [12 to 15]). Respectively, the vehicle, sodium hydroxide, or sodium bicarbonate alone did not affect the above variables (the lung injury scores) in sham-operated rats.

Activation of HO-1 Increased MnSOD Activity and Decreased Malondialdehyde Content while Restoring RCR Levels in the Lung Mitochondria of Rats

As the primary antioxidant enzyme of mitochondria, MnSOD scavenged the superoxide anion to protect against ROS-mediated oxidative damage.[35] However, as a reliable marker of the

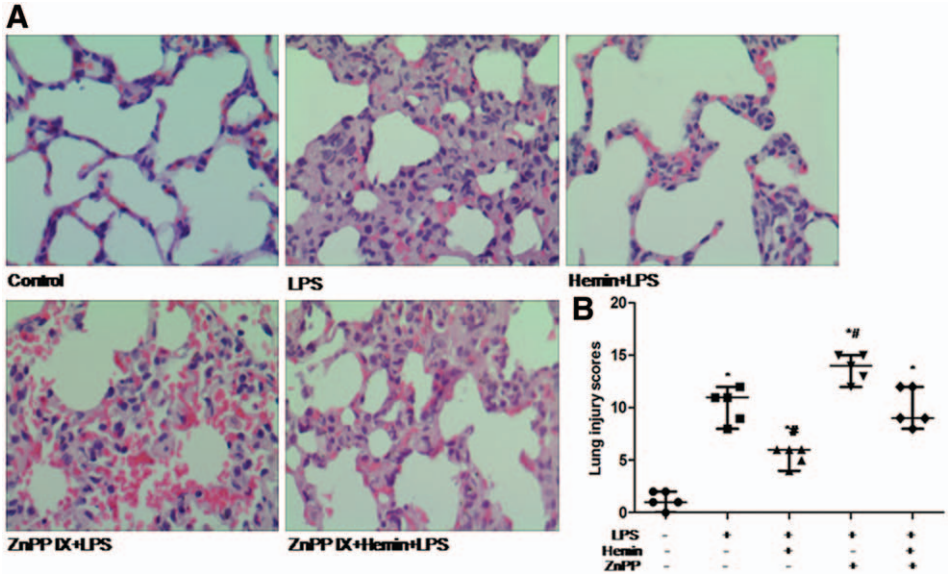

Fig. 4. Effect of heme oxygenase (HO)-1 induction on lung histology during lipopolysaccharide (LPS)–induced lung injury. (*A*) Representative photomicrographs of lung sections stained with hematoxylin and eosin at 6 h after LPS injection (original magnification, ×400). Pretreatment with hemin, a HO-1 inducer, suppressed the histopathologic damage caused by LPS, shown as alveolar edema, hemorrhage, the infiltration of inflammatory cells, and thickened alveolar septum, which were reversed by zinc protoporphyrin IX (ZnPP). (*B*) Semiquantitative analysis of lung tissues by lung injury scores. A five-point scale was used to grade the degrees of lung injury: scores of 0 = minimal damage, 1+ = mild damage, 2+ = moderate damage, 3+ = severe damage, and 4+ = maximal damage. Values are presented as medians (range) using the Mann–Whitney U test (n = 5 per group). *Significance compared to control ($P < 0.05$). #Significance compared to LPS-treated rats ($P < 0.05$).

extent of lipid peroxidation, malondialdehyde was applied to reflect the cumulative oxidative damage to membrane lipids.[36] As shown in figure 5, A and B, lung mitochondrial MnSOD activity was significantly decreased, while lung mitochondrial malondialdehyde content was increased in Sprague-Dawley rats subjected to lipopolysaccharide (MnSOD [U/mg protein] and malondialdehyde [nmol/mg protein]: control, 36.48 ± 4.98 and 2.50 ± 0.21; lipopolysaccharide, 20.82 ± 2.31 and 5.27 ± 0.46; $^{**}P < 0.001$; n = 5). HO-1 induction by hemin increased the enzymatic activities of MnSOD by 34% and depleted the contents of malondialdehyde by 26% in the lung mitochondria. Specifically, higher malondialdehyde contents and lower MnSOD activities were observed after pretreatment with ZnPP at 6 h during lipopolysaccharide exposure ($P < 0.05$), which implied that the HO-1 system provided protection against endotoxin-mediated mitochondrial oxidative damage.

Oxygen consumption was measured to determine alterations in the mitochondrial respiratory function. Therein,

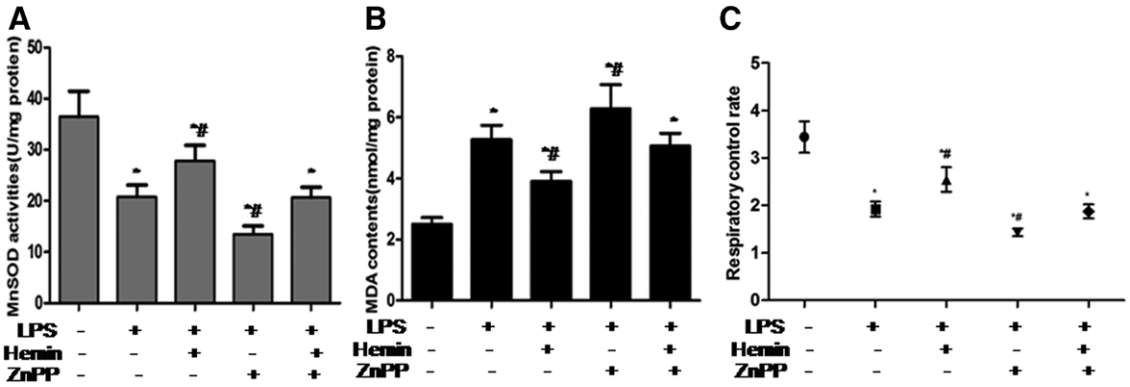

Fig. 5. Effect of heme oxygenase (HO)-1 induction on the activities of manganese superoxide dismutase (MnSOD; *A*), content of malondialdehyde (MDA; *B*), and level of respiratory control ratio (RCR; *C*) in the lung mitochondria of rats. Pretreatment with hemin *via* HO-1 induction significantly increased RCR levels and MnSOD activity and decreased MDA content in the rat lung mitochondria subjected to lipopolysaccharide (LPS). However, zinc protoporphyrin IX (ZnPP)–pretreated rats counteracted the above protective efficacy manifested as reduced RCR levels, lower MnSOD activity, and higher MDA content. Values are representative of mean ± SD from five individual samples using one-way ANOVA and the Bonferroni test for multiple comparisons. $^{*}P < 0.05$ *versus* control and #$P < 0.05$ *versus* LPS-treated rats.

RCR was considered to reflect the functional ability of mitochondrial oxidative phosphorylation.[37] Its function was markedly compromised in the lung mitochondria of Sprague-Dawley rats elicited by lipopolysaccharide, as indicated by the lower RCR levels compared to controls (1.92 ± 0.16 *vs.* 3.44 ± 0.33) (fig. 5C). Additionally, along with ZnPP pretreatment, RCR levels were further decreased at 6 h in lipopolysaccharide-stimulated rats (ZnPP *vs.* lipopolysaccharide, 1.43 ± 0.08 *vs.* 1.92 ± 0.16; $^{**}P < 0.001$). However, the induction of HO-1 by hemin was effective in attenuating the depression in mitochondrial function by restoring RCR levels ($^{**}P < 0.001$).

HO-1 Protected Against Septic ALI by Regulating the Expression of Mitochondrial Fusion/Fission Genes

To ascertain whether HO-1 conferred defense against septic lung injury through the preservation of mitochondrial dynamics, specific fusion/fission markers, including Mfn1, Mfn2, OPA1, and Drp1, in the lungs of rats were detected by real-time PCR and Western blot at 6 h after lipopolysaccharide stimulation, as shown in figure 6, A and B. The results showed that lipopolysaccharide shifted the balance toward a fission phenotype, characterized by elevated levels of Drp1 mRNA and protein, and decreased the levels of Mfn1, Mfn2, and OPA1 mRNA and proteins (Drp1: control *vs.* lipopolysaccharide, 0.618 ± 0.050 *vs.* 1.079 ± 0.071; Mfn1: control *vs.* lipopolysaccharide, 1.387 ± 0.096 *vs.* 0.891 ± 0.069; Mfn2: control *vs.* lipopolysaccharide, 1.381 ± 0.094 *vs.* 0.951 ± 0.080; OPA1: control *vs.* lipopolysaccharide, 1.454 ± 0.103 *vs.* 1.089 ± 0.067; n = 5; mean ± SD). Notably, hemin pretreatment 1 h ahead of lipopolysaccharide induced a dramatic decrease of Drp1 mRNA and protein and a distinct increase in HO-1, Mfn1, Mfn2, and OPA1 mRNA and proteins ($P < 0.05$). However, Drp1 mRNA and protein were apparently up-regulated, while the levels of HO-1, Mfn1, Mfn2, and OPA1 mRNA and proteins were down-regulated in the presence of the HO-1 inhibitor ZnPP, which deteriorated the lipopolysaccharide-mediated mitochondrial dynamic imbalance ($P < 0.05$).

Discussion

In the current study, we illustrated the paramount role of HO-1/carbon monoxide in the modulation of mitochondrial dynamics–related proteins, including Mfn1/2, OPA1, and Drp1, to preserve the balance of fusion and fission to mitigate lung injury in rats or RAW 264.7 cells exposed to endotoxin. We reported that (1) lipopolysaccharide injured alveolar macrophages by initiating intrinsic mitochondria-dependent dysfunction, which involved elevated ROS production, depleted RCR levels, and altered mitochondrial dynamic equilibrium; (2) as a vital endogenous antioxidant, the HO-1 system, located partly in the mitochondria, conferred protection against oxidative cellular injury through reduced ROS generation, increased RCR levels, and sustained the balance of mitochondrial dynamics; (3) lipopolysaccharide exposure reduced RCR levels, inhibited the

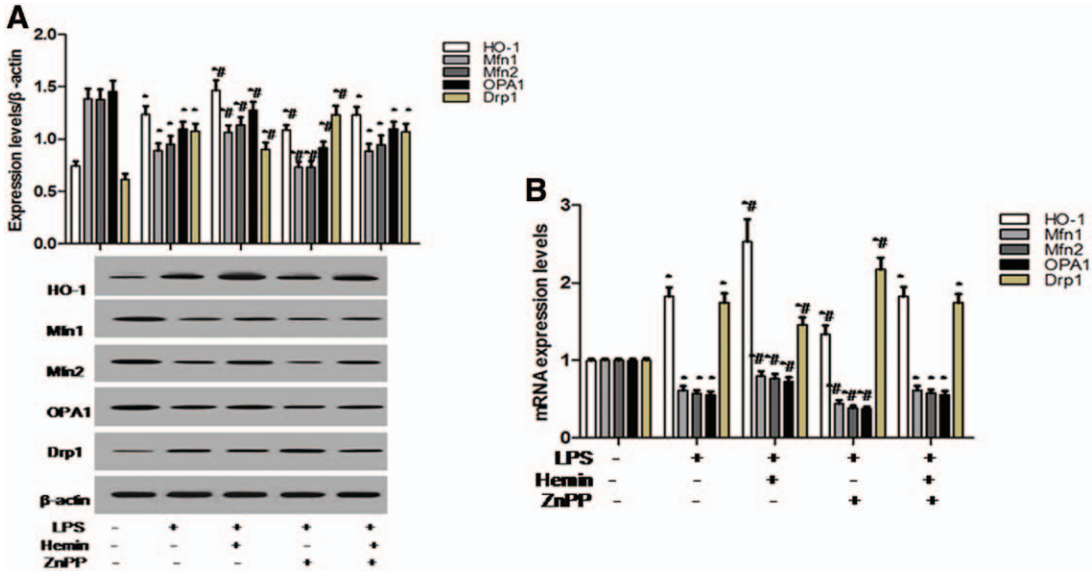

Fig. 6. Effect of heme oxygenase (HO)-1 induction on the proteins (*A*) and messenger RNA (mRNA; *B*) levels of mitochondrial fusion/fission markers in the lungs of rats. Rats pretreated with hemin showed notably increased expression of mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy 1 (OPA1) mRNA and protein and decreased dynamin-related protein 1 (Drp1) mRNA and protein levels *via* the up-regulation of HO-1 expression. However, the levels of HO-1, Mfn1, Mfn2, and OPA1 mRNA and proteins were apparently reduced, but Drp1 mRNA and proteins were enhanced in response to zinc protoporphyrin IX (ZnPP) pretreatment before lipopolysaccharide (LPS) exposure. β-actin was monitored as a standard for protein loading. The blots are representative of five separate experiments using one-way ANOVA and the Bonferroni test for multiple comparisons. Data are expressed as mean ± SD. *Significant difference from controls ($P < 0.05$). #Significant difference from LPS-treated rats ($P < 0.05$).

activities of MnSOD, and increased the content of malondialdehyde in the rat lung mitochondria, which contributed to lung injury and was counteracted by HO-1 induction; (4) the up-regulation of HO-1 defended against lipopolysaccharide-induced ALI in rats, which was associated with the restored expression of mitochondrial fusion/fission markers.

Sepsis-related ALI/acute respiratory distress syndromes are devastating clinical syndromes with a mortality rate as high as 35.1 to 46.1%.[38] In succession, the complexity of the ultimate mechanisms of endotoxin-induced ALI determines the limitations of therapeutic regimens, such as antiinfection and mechanical ventilation.[39] To date, mitochondrial functional impairment has been experimentally and clinically recognized in systemic organs during sepsis.[40] More specifically, ROS were generated excessively in exacerbated oxidative stress, defined as the imbalance between the production of cellular oxidant species and antioxidants, which involves the direct or indirect impairment of mitochondrial functions correlated with the depletion of RCR levels.[6,41] RCR, as the single most useful general measure of function in isolated mitochondria, indicated the functional ability of mitochondrial oxidative phosphorylation.[13] In this regard, the malondialdehyde content and the MnSOD activity of lung mitochondria were both applied in our study to reflect the metabolism of oxygen free radicals. In the current study, we show that 1 μg/ml lipopolysaccharide and 24-h incubation of macrophages, namely, RAW 264.7 cells, were sufficient to detect oxidant-induced cell injury based on the cell viability in the MTT assay. Notably, the augmentation of intracellular ROS, along with decreased levels of RCR, was found in lipopolysaccharide-exposed RAW 264.7 cells. In accordance with the findings *in vivo*, the scores of lung injury were decreased, accompanied by lower RCR levels, reduced activities of MnSOD, and an increased malondialdehyde content in lung mitochondria in a lipopolysaccharide-treated sepsis rat model. Taken together, lipopolysaccharide, an outer membrane component of Gram-negative bacteria, specifically triggered the cell inviability and ALI of rats primarily through oxidative stress–mediated mitochondrial dysfunction, which was in agreement with studies by Chuang *et al.*[42]

As double-membrane organelles, mitochondria participate in energy production, intermediary metabolism, calcium signaling, and apoptosis.[43] Predictably, the dysfunction of mitochondria gives rise to a plethora of defects in all tissues. In turn, the coordination of mitochondrial function is dependent on the dynamic nature of mitochondria, which is controlled by the delicate balance between two opposing processes, mitochondrial fission and fusion.[44] Recently, a published report by Gonzalez *et al.*[45] showed that the abnormal balance of mitochondrial fusion/fission, probably evoked by a massive increase of ROS, contributed to the progression of sepsis-associated multiple organ failure. In accordance with that study, we demonstrated herein that lipopolysaccharide exposure shifted the balance toward a fission phenotype, characterized by elevated Drp1 mRNA and protein, accompanied by decreased mRNA and protein levels of Mfn1, Mfn2, and OPA1 *in vitro* and *in vivo*. Therefore, in conjunction with excessive levels of ROS and malondialdehyde content, as well as depressed levels of RCR and activities of MnSOD, it was plausible that the balance of mitochondrial fusion and fission was upset, leading to abnormal alterations in mitochondrial function in the impaired lung tissue of rats or RAW 264.7 cells exposed to endotoxin. Interestingly, the resolution of disturbed mitochondrial dynamics by an intrinsic antioxidant may be fundamental to the recovery from sepsis-related ALI.

The stress-inducible protein HO-1 and the byproducts of heme catabolism serve as a basis for cytoprotection against oxidative stress.[46] Importantly, the localization of HO-1 in mitochondria was indicative of the protective effects of HO-1 against conditions such as sepsis, ischemia–reperfusion, or hypoxia.[19] As one of the products of heme degradation, carbon monoxide, whether it came from either carbon monoxide–releasing molecules or HO-1 induction, appeared to have broad physiologic functions that mimicked the beneficial effects of HO-1.[22] Therefore, to ascertain the intimate links between the HO-1/carbon monoxide system and mitochondrial dynamics, we pretreated lipopolysaccharide-exposed septic rats with hemin, a potent HO-1 inducer, and ZnPP, a selective HO-1 inhibitor, while CORM2, a potent carbon monoxide–releasing reagent, or hemin and ZnPP were preincubated in lipopolysaccharide-stimulated macrophages. CORM2 or hemin pretreatment before lipopolysaccharide challenge in cultured RAW 264.7 cells up-regulated the expressions of HO-1 mRNA and protein, increased the levels of Mfn1, Mfn2, OPA1 mRNA, and protein, and decreased the levels of Drp1 mRNA and protein. Additionally, excessive ROS was inhibited, but regressive RCR in lipopolysaccharide-exposed RAW 264.7 cells was partially promoted by CORM2 or hemin pretreatment. Based on these observations, exogenously delivered carbon monoxide from CORM2 and HO-1 induction by hemin could affect mitochondrial fusion or fission, resulting in the prevention of mitochondrial dysfunction. Interestingly, the administration of ZnPP markedly reversed carbon monoxide/HO-1–mediated incremental levels of HO-1 mRNA and protein, along with increased ROS and decreased RCR levels, which further deteriorated the mitochondrial fusion/fission afforded by lipopolysaccharide. As such, CORM2 in combination with ZnPP failed to up-regulate HO-1 expression and restored the balance of mitochondrial dynamics, which further implicate that HO-1 induction may be a prerequisite for cytoprotection from the endogenous carbon monoxide by CORM2. These findings were consistent with previous studies by Jamal Uddin *et al.*[16] Altogether, the current study was in agreement with those that showed that the induction of HO-1, a paramount antioxidant, abrogated the lipopolysaccharide-mediated oxidative damage of mitochondrial function by modulating the imbalances in mitochondrial fusion and fission.[25,47] Similarly, the blockade of

the HO-1 system by ZnPP markedly down-regulated HO-1 mRNA and protein expression in parallel to reduced levels of Mfn1, Mfn2, and OPA1 mRNA and proteins. It enhanced Drp1 mRNA and protein accompanied by reduced RCR levels, decreased MnSOD activity, and increased malondialdehyde content in the lung mitochondria of rats exposed to lipopolysaccharide for 6 h. In the reverse, pretreatment with hemin ameliorated this dampening of mitochondrial dynamics in a rat model of septic lung injury. In particular, the importance of the endogenous protective effects of HO-1 has been underscored *in vivo*, which was in line with previous studies that showed that the up-regulation of HO-1 was involved in sepsis-induced organ injury by electroacupuncture.[34,48] Collectively, our results identified the pivotal role of HO-1/carbon monoxide in preserving the mitochondrial dynamic equilibrium due to the attenuation of lipopolysaccharide-induced lung injury in rats and in lipopolysaccharide-activated RAW 264.7 cells.

The current study has certain limitations. First, recently published reports have established that the activation of P38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase signal transduction pathways is facilitated by the cytoprotective efficacy of HO-1.[49,50] Thus, the underlying mechanism by which the HO-1 system modulates the balance of mitochondrial fusion and fission is not fully expounded here and requires further exploration. Second, in the context of septic ALI, previous studies noted that electroacupuncture-induced protection was coincident with HO-1 up-regulation.[34] Therefore, elucidating the relationship among electroacupuncture, the HO-1 system, and mitochondrial dynamics may have potential value for clinical applications. Third, an in-depth study has revealed that HO-1 expression was increased in bronchial epithelial cells in diseases due to aggravated oxidative stress, which is a clue for comprehensive exploration of the endogenous cytoprotection of HO-1 in the future.[51] Fourth, it has been shown that mitochondrial morphology is a vital determinant of its function and is governed by the balance of dynamic behavior.[52] Therefore, it is of great value to discuss the structural implications of mitochondrial dynamics in the protective effects of HO-1 against sepsis-related mitochondrial dysfunction in the future.

In summary, the current study demonstrated that HO-1-derived carbon monoxide was endowed with cytoprotective effects against oxidative stress–mediated lung injury during sepsis, primarily by improving mitochondrial dysfunction *via* preserving the balance of mitochondrial fusion and fission *in vivo* and *in vitro*. In this way, pharmacologic agents or acupuncture adjuvants that up-regulate HO-1 expression may be a promising strategy for preventing sepsis-related lung injury.

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Competing Interests

The authors declare no competing interests.

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"Dr. Dewees's Anaesthetic Inhaler" for Ether or Chloroform

After earning his A.B. and A.M. degrees at Pennsylvania's Ursinus College, William Bushy Dewees (1854 to 1911) completed his M.D. thesis on obstetrical anesthesia in 1877 at the University of Pennsylvania. Following his move to Salina, Kansas, Dr. Dewees applied for a patent on a double-valved inhaler (*right*) for ether or chloroform. The "patent applied for" designation (*left*) dates the box to sometime between the September 13, 1900, filing and the November 12, 1901, "granting" dates on US Patent No. 686270. The ease with which the all-metal "DR. DEWEES'S Anaesthetic Inhaler" was sanitized was a selling point exploited by its Conshohocken, Pennsylvania, manufacturer, the J. Elwood Lee Company. That firm was better known to veteran anesthesiologists by its acronym "JELCO," which was used on many products, including intravenous catheters. (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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