

# Heme-Oxygenase-1 Induction and Carbon Monoxide-Releasing Molecule Inhibit Lipopolysaccharide (LPS)-Induced High-Mobility Group Box 1 Release in Vitro and Improve Survival of Mice in LPS- and Cecal Ligation and Puncture-Induced Sepsis Model in Vivo

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## ABSTRACT

We examined our hypothesis that heme-oxygenase-1 (HO-1)-derived carbon monoxide (CO) inhibits the release of high-mobility group box 1 (HMGB1) in RAW264.7 cells activated with lipopolysaccharide (LPS) in vitro and in LPS- or cecal ligation and puncture (CLP)-induced septic mice in vivo, so that HO-1 induction or CO improves survival of sepsis in rodents. We found that pretreatment with HO-1 inducers (hemin, cobalt protoporphyrin IX) or transfection of HO-1 significantly inhibited HMGB1 release, which was blocked by HO-1 small interfering RNA, in cells activated by LPS. Carbon monoxide-releasing molecule 2 (CORM-2) but not bilirubin or deferoxamine inhibited HMGB1 release in LPS-activated macrophages. Oxyhemoglobin reversed the effect of HO-1 inducers on HMGB1 release. Translocation of HMGB1 from nucleus to cytosol was significantly inhibited by HO-1 inducers, CORM-2, or HO-1 transfection. Neutralizing antibodies to tumor necrosis factor (TNF)- $\alpha$ ,

interleukin (IL)-1 $\beta$ , interferon- $\beta$ , and *N*<sup>ω</sup>-nitro-L-arginine methyl ester hydrochloride but not *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methane sulfonamide (NS-398) significantly inhibited HMGB1 release in LPS-activated cells. Production of TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\beta$  was significantly reduced by pretreatment of HO-1 inducers, CORM-2, or HO-1 transfection in LPS-activated cells. Plasma levels of HMGB1 in mice challenged with LPS or CLP were significantly reduced by the administration of HO-1 inducers or CORM-2, which was accompanied by either reduction (pretreatment) or no change (delayed administration) of serum TNF- $\alpha$  and IL-1 $\beta$  levels. Regardless of pretreatment or delayed administration, CORM-2 and hemin rescued mice from lethal endotoxemia and sepsis induced by LPS or CLP. Taken together, we concluded that HO-1-derived CO reduces HMGB1 release in LPS-activated cells and LPS- or CLP-induced animal model of sepsis.

Sepsis is defined as a systemic inflammatory response syndrome from a microbial infection that results from excessive stimulation of the host immune system by pathogen components to produce various proinflammatory cytokines, and their overproduction causes systemic inflammation that

can lead to the lethal multiple organ damage (Oberholzer et al., 2001). High-mobility group box 1 (HMGB1) is a chromatin-binding protein that participates in maintaining nucleosome structure and regulation of gene transcription (Landsman and Bustin, 1993). Various evidence indicated that HMGB1 is a necessary and sufficient late mediator of severe sepsis (Wang et al., 1999; Yang et al., 2004). Once released, HMGB1 can bind to cell-surface receptors, such as the receptor for advanced glycation end products and Toll-like receptors 2 and 4, and mediate various cellular responses, chemotactic cell movement, and release of proinflammatory

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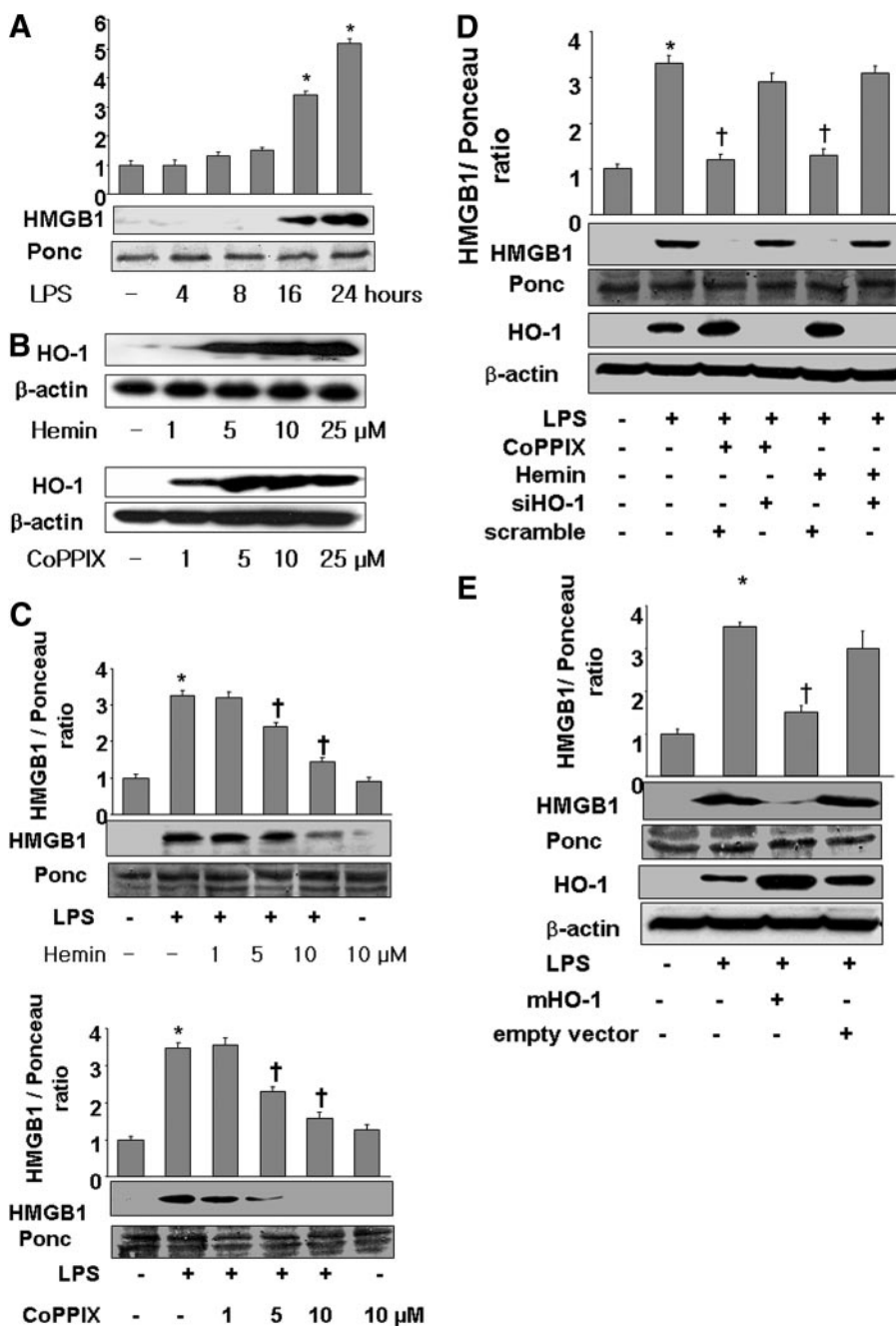
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**ABBREVIATIONS:** HMGB1, high-mobility group box 1; HO-1, heme-oxygenase-1; CORM-2, carbon monoxide-releasing molecule II; LPS, lipopolysaccharide; CLP, cecal ligation and puncture; CoPPIX, cobalt protoporphyrin IX; COX, cyclooxygenase; DFO, deferoxamine mesylate; L-NAME, *N*<sup>ω</sup>-nitro-L-arginine methyl ester hydrochloride; iNOS, inducible nitric-oxide synthase; NS-398, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methane sulfonamide; siRNA, small interfering RNA; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; INF- $\beta$ , interferon- $\beta$ ; ELISA, enzyme-linked immunosorbent assay; HbO<sub>2</sub>, oxyhemoglobin; DMSO, dimethyl sulfoxide.

cytokines (Andersson et al., 2000; Park et al., 2004; Rouhiainen et al., 2007). The administration of anti-HMGB1 antibodies or inhibitors (e.g., ethyl pyruvate, nicotine) significantly protected mice from lethal sepsis (Wang et al., 1999, 2004a; Ulloa et al., 2002; Yang et al., 2004). Thus, inhibition of HMGB1 release is a prospective target for therapeutic intervention against sepsis.

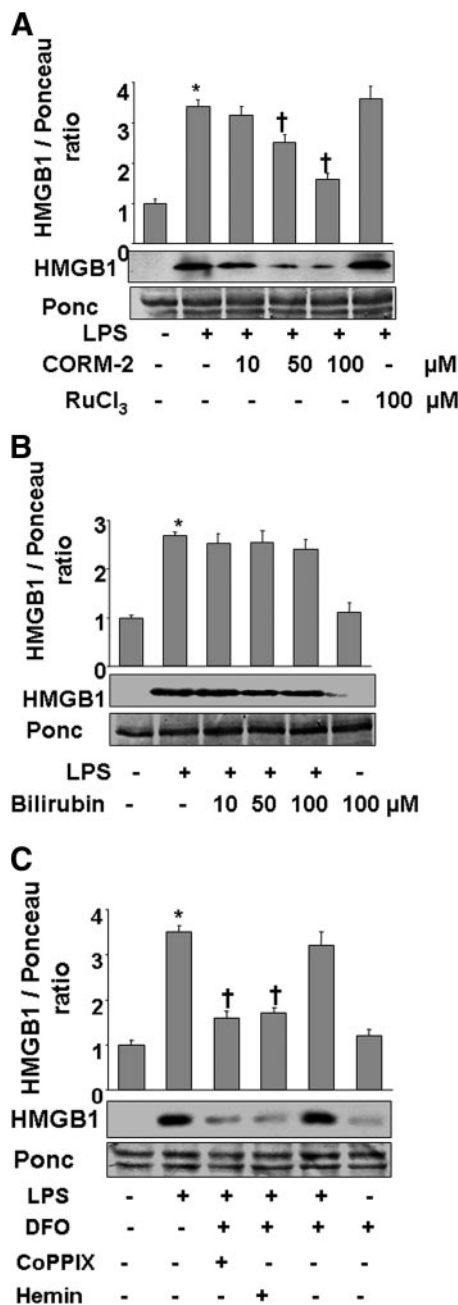
Heme oxygenase-1 (HO-1), a stress-responsive protein that can be induced by stimulants such as inflammatory cytokines, heat shock, heavy metals, and oxidants, degrades heme into three products:  $\text{Fe}^{2+}$ , biliverdin, and carbon monoxide (CO). Biliverdin is subsequently converted into bilirubin by biliverdin reductase. The increased level of  $\text{Fe}^{2+}$  stimulates the synthesis of ferritin (iron-bound compound), which is known as a cytoprotective, antioxidant protein. HO-1 sys-

tem exerts antiapoptotic, antioxidant, and immunomodulatory functions in various situations (Ryter et al., 2006). It has already been reported that HO-1 induction improves animal survival in lethal endotoxemia (Otterbein et al., 1995; Yu and Yao, 2008). We hypothesized that HO-1-derived CO may inhibit HMGB1, a necessary and sufficient late mediator of severe sepsis, so that HO-1 induction improves animal survival in lethal sepsis. Thus, the aim of the present study was to investigate the following questions: 1) how HO-1 inducers or HO-1 gene transfection inhibit the expression and release of HMGB1 in murine RAW264.7 macrophages when activated with LPS; 2) whether CO is responsible for the inhibition of expression and release of HMGB1 by HO-1 induction; and 3) whether CO improves survival in LPS- or CLP-treated mice. To do this, we used pharmacological agents such as



**Fig. 1.** Hemin, CoPPiX, and mHO-1 attenuated the endotoxin-induced HMGB1 release in RAW 264.7 cells. **A**, cells were stimulated with LPS (1  $\mu\text{g/ml}$ ) for 4, 8, 16, and 24 h, and culture medium samples were concentrated for HMGB1 detection as described under *Materials and Methods*. **B**, cells were treated with hemin or CoPPiX at 1, 5, 10, and 25  $\mu\text{M}$  for 16 h, and then HO-1 protein levels were determined by Western blot. **C**, LPS (1  $\mu\text{g/ml}$ ) stimulated macrophages in the presence or absence of hemin or CoPPiX in various doses for 16 h, HMGB1 release was identified, and the main Ponceau band was used as a loading control. **D**, cells were transfected with siHO-1 or scramble as described under *Materials and Methods*. After transfection, cells were pretreated with hemin (10  $\mu\text{M}$ ) or CoPPiX (10  $\mu\text{M}$ ) for 1 h and stimulated with LPS (1  $\mu\text{g/ml}$ ); HMGB1 and HO-1 levels were determined. **E**, cells were transfected with mHO-1 or empty vector as described under *Materials and Methods* and then stimulated with LPS. After 16 h, culture medium was subjected to HMGB1 levels determination, and cells were subjected to whole lysis for HO-1 detection by Western blot. Data are presented as mean  $\pm$  S.D. of three independent experiments. One-way analysis of variance was used to compare multiple group means followed by Newman-Keuls test (significance compared with control, \*,  $P < 0.05$ ; significance compared with LPS, †,  $P < 0.05$ ).

hemin and cobalt protoporphyrin IX (CoPPIX) as HO-1 inducers and tricarbonyldichlororuthenium (II) dimer ( $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ , CORM-2) as a CO-releasing molecule. Very recently, Takamiya et al. (2008) reported that elevated circulating levels of HMGB1 contributed to LPS-induced mortality in the absence of HO-1 rodents, which further reinforces our hypothesis.



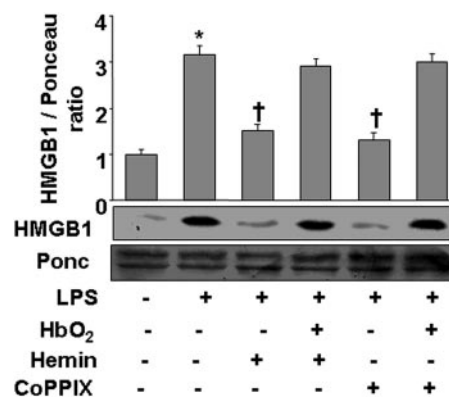
**Fig. 2.** Effect of CORM-2, bilirubin, and DFO on endotoxin-induced HMGB1 release in RAW264.7 cells. Cells were pretreated with CORM-2 (A) or bilirubin (B) for 1 h and then stimulated with LPS (1  $\mu\text{g}/\text{ml}$ ) for 16 h.  $\text{RuCl}_3$  was used as a negative control for CORM-2. C, DFO (500  $\mu\text{g}/\text{ml}$ ) was administered for 1 h, and later hemin (10  $\mu\text{g}/\text{ml}$ ) or CoPPIX (10  $\mu\text{g}/\text{ml}$ ) was administered. After 1 h, cells were stimulated with LPS (1  $\mu\text{g}/\text{ml}$ ) for 16 h. HMGB1 levels in culture medium were determined by Western blot. Data are presented as mean  $\pm$  S.D. of three independent experiments. One-way analysis of variance was used to compare multiple group means followed by Newman-Keuls test (significance compared with control, \*,  $P < 0.05$ ; significance compared with LPS, †,  $P < 0.05$ ).

## Materials and Methods

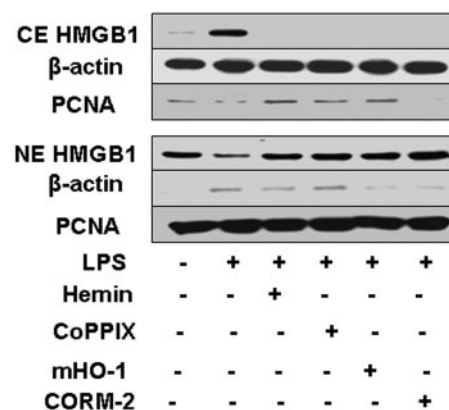
**Materials.** Anti-HMGB1 was purchased from Abcam (Cambridge, MA), anti-iNOS from Transduction Laboratories (Lexington, KY), anti- $\beta$ -actin and anti-heme oxygenase-1 from Santa Cruz Biotechnology (Santa Cruz, CA). Neutralizing antibodies to TNF- $\alpha$ , IL-1 $\beta$ , and INF- $\beta$  were provided by R&D Systems (Minneapolis, MN). Enhanced chemiluminescence Western blotting detection reagent was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). All other chemicals, including lipopolysaccharide (LPS, *Escherichia coli* 0111:B4), cobalt protoporphyrin IX, hemin, CORM-2, NS-398, and *N*<sup>ω</sup>-nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell Culture.** RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in RPMI 1640 medium supplemented with 25 mM HEPES, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 10% heat-inactivated fetal calf serum.

**Cell Stimulation.** RAW 264.7 cells were plated at a density of  $1 \times 10^7$  cells per 100-mm dish. The cells were rinsed with fresh medium and stimulated with LPS (1  $\mu\text{g}/\text{ml}$ ) in the presence of different



**Fig. 3.** Inhibitory effect of HO-1 on LPS-induced HMGB1 release in RAW264.7 cells is dependent on CO. Cells were treated with HbO<sub>2</sub> (20  $\mu\text{g}/\text{ml}$ ), and then hemin (10  $\mu\text{g}/\text{ml}$ ) or CoPPIX (10  $\mu\text{g}/\text{ml}$ ) was administered. After that, cells were stimulated with LPS (1  $\mu\text{g}/\text{ml}$ ). Culture medium was collected and subjected to HMGB1 analysis by Western blot. Data are presented as the mean  $\pm$  S.D. of three independent experiments. One-way analysis of variance was used to compare multiple group means followed by Newman-Keuls test (significance compared with control, \*,  $P < 0.05$ ; significance compared with LPS, †,  $P < 0.05$ ).



**Fig. 4.** Hemin, CoPPIX, and CORM-2 impair cytoplasmic translocation of HMGB1 in activated macrophages. Cells were pretreated with hemin (10  $\mu\text{M}$ ), CoPPIX (10  $\mu\text{M}$ ), or CORM-2 (100  $\mu\text{M}$ ) for 1 h and then stimulated with LPS (1  $\mu\text{g}/\text{ml}$ ) for 16 h. After treatment, cells were subjected to nuclear/cytosol fractionation and immunoblotted against HMGB1 antibody as described under *Materials and Methods*. The data are from two independent experiments.



concentration of pharmacological agents. At 16 h after stimulation, levels of HMGB1 or iNOS were determined. Oxyhemoglobin (HbO<sub>2</sub>) was prepared by the reduction of bovine hemoglobin with sodium hydrosulfite followed by gel filtration with a prepacked disposable column (PD-10; Pfizer, New York, NY), previously equilibrated with 50 mM Tris/HCl at pH 7.4 (Salvemini et al., 1989). The concentration of HbO<sub>2</sub> was determined using a spectrophotometer (Lambda 5; PerkinElmer Life and Analytical Sciences, Waltham, MA) at 576 nm wavelength, according to Kondo et al. (1989).

**NO Assay.** Nitric oxide was measured as its stable oxidative metabolite, nitrite, as described by Kang et al. (1999). At the end of

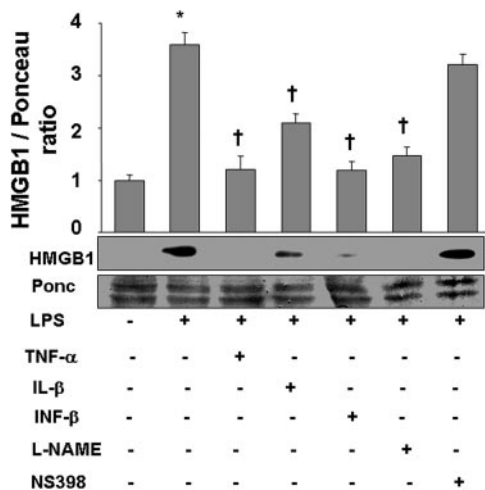
incubation, 100  $\mu$ l of the culture medium was mixed with the same volume of Griess solution (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% phosphoric acid). Light absorbance was measured at 550 nm, and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

**ELISA.** The levels of TNF- $\alpha$ , INF- $\beta$ , and IL- $\beta$  in the culture medium were determined using commercially available ELISA kits from R&D Systems according to the provided manuals.

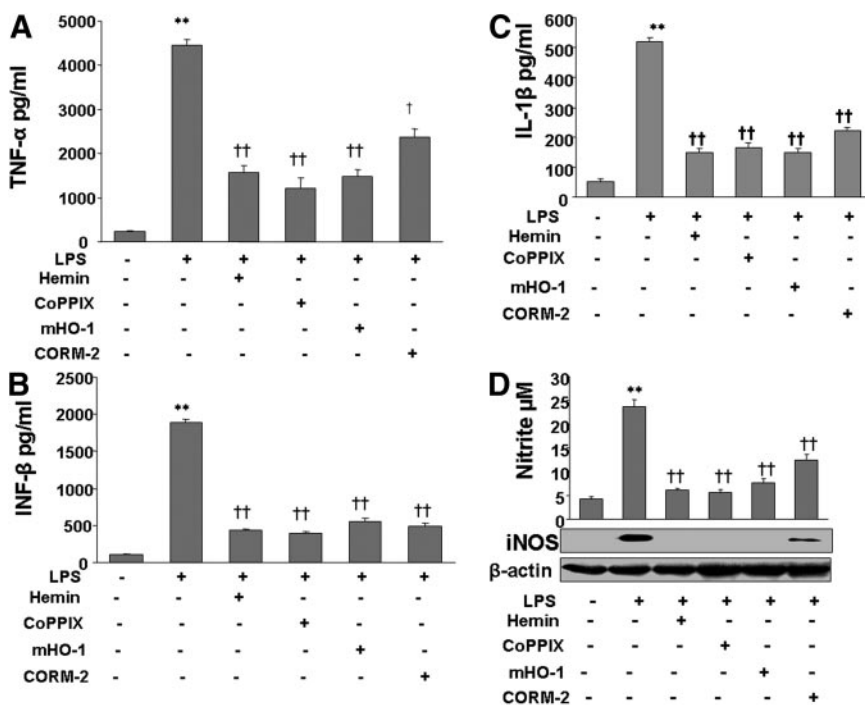
**HMGB1 Analysis.** Culture medium samples were briefly centrifuged. The same volumes of samples were then concentrated 40-fold with Amicon Ultra-4-10000 NMWL (Millipore, Billerica, MA). Centrifugation conditions were fixed-angle (35°) and 7500g for 20 min at 4°C. Blood was collected by cardiac puncture into a sodium heparin-containing tube. After centrifugation, plasma samples were filtered and concentrated through Centricon YM-100 and YM-10 (Millipore), respectively. The concentrated samples were subjected to SDS-polyacrylamide gel electrophoresis. Ponceau staining was used as loading control.

**Western Blot.** The cytoplasmic/nuclear fractionation was performed using nuclear/cytosol fractionation kit (BioVision, Mountain View, CA) according to the manufacturer's manual. Whole-cell lysates were performed using buffer containing 0.5% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5, and protease inhibitors. Concentrated supernatants, to detect HMGB1, and whole-cell lysates, to detect iNOS,  $\beta$ -actin, and HO-1 as well as nuclear, cytosol lysates were subjected to electrophoresis in different-percentage polyacrylamide gels depending on the size of interested protein. The gels were transferred to polyvinylidene difluoride membranes by semidry electrophoretic transfer at 15 V for 60 to 75 min. The membranes were stained with Ponceau S solution (2  $\mu$ g/ml) for 5 min to determine the efficiency of transfer or/and protein loading levels per track. Then the polyvinylidene difluoride membranes were blocked overnight at 4°C in 5% bovine serum albumin. The cells were incubated with primary antibodies diluted 1:500 in Tris-buffered saline/Tween 20 containing 5% bovine serum albumin for overnight in 4°C and then incubated with secondary antibody at room temperature for 1 h. The signals were detected by enhanced chemiluminescence.

**Transfection of HO-1 Gene into RAW264.7 Cells.** For HO-1 transfection, cells were washed twice with serum-free media and



**Fig. 5.** Effect of TNF- $\alpha$ , IL-1 $\beta$ , and INF- $\beta$  neutralization and NO (iNOS) and COX-2 inhibition on the endotoxin-induced HMGB1 release in macrophages. Cells were pretreated with neutralizing antibody (Nab) TNF- $\alpha$  (100  $\mu$ g/ml), Nab IL-1 $\beta$  (100  $\mu$ g/ml), Nab INF- $\beta$  (500  $\mu$ g/ml), L-NAME (300  $\mu$ M), or NS-398 (50  $\mu$ M) for 1 h and treated with LPS (1  $\mu$ g/ml) for 16 h. After incubation, culture medium samples were subjected to HMGB1 levels determination as described under *Materials and Methods*. Data are presented as the mean  $\pm$  S.D. of three independent experiments. One-way analysis of variance was used to compare multiple group means followed by Newman-Keuls test (significance compared with control, \*,  $P < 0.05$ ; significance compared with LPS, †,  $P < 0.05$ ).



**Fig. 6.** Effect of HO-1 up-regulation and CORM-2 on the release of various cytokines and NO (iNOS) in LPS-activated macrophages. A to D, cells were incubated with hemin (10  $\mu$ M), CoPPiX (10  $\mu$ M), and CORM-2 (100  $\mu$ M) for 1 h or were transfected with mHO-1 and later activated with LPS for 16 h. After incubation time, culture medium samples were subjected to TNF- $\alpha$  (A), INF- $\beta$  (B), and IL-1 $\beta$  (C) determination by the appropriate ELISA kit, and NO production (D) was measured by NO assay as described under *Materials and Methods*. Cells were harvested by total lysis, and iNOS protein (D) levels were determined by Western blot. Data are presented as the mean  $\pm$  S.D. of three independent experiments. One-way analysis of variance was used to compare multiple group means followed by Newman-Keuls test (significance compared with control, \*\*,  $P < 0.01$ ; significance compared with LPS, †,  $P < 0.05$  or ††,  $P < 0.01$ ).

incubated with HO-1 cDNA (from Dr. Augustine M. K. Choi; University of Pittsburgh, Pittsburgh, PA) that had been subcloned into the mammalian pcDNA 3 expression vector and Superfect transfection reagent (QIAGEN, Valencia, CA). After 4 h of incubation, the media were removed, and the cells were incubated in appropriate media for 16 h.

**siRNA Technique.** HO-1 small interfering RNA (siRNA) and scramble siRNA were purchased from Invitrogen (Carlsbad, CA). The sequence of mouse HO-1 siRNA (5'-end to 3'-end) is UUACAUG-GCAUAAAUUCCACUGCC. The siRNA was transfected into RAW264.7 cells according to the manufacturer's protocol using transfection reagent SuperFect from QIAGEN. The cells were incubated with 100 nM HO-1 or scramble siRNA for 48 h in serum- and antibiotic-free media. Then the cells were incubated for 12 h in media containing antibiotics and fetal bovine serum, and cells were washed and pretreated with or without Hemin or CoPPiX after LPS stimulation.

**Animal Model of Endotoxemia and Sepsis.** Endotoxemia was induced in BALB/c mice (male aged 7–8 weeks, 20–25 g) by intraperitoneal injection of bacterial endotoxin (LPS 15 mg/kg). To induce sepsis, BALB/c mice were anesthetized with ketamine (30 mg/kg) and xylazine (6 mg/kg). Next, a 2-cm midline incision was performed to allow exposure of the cecum with adjoining intestine. The cecum was tightly ligated with a 3.0-silk suture at 5.0 mm from the cecal tip and punctured once with a 22-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation sites and returned to the peritoneal cavity. The laparotomy site was then stitched with 4.0 silk. In sham control animals, the cecum was exposed but not ligated or punctured and then returned to the abdominal cavity. Mice were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and were treated ethically. The protocol was approved in advance by the Animal Research Committee of the Gyeongsang National University.

**Statistical Evaluation.** Data are expressed as the mean  $\pm$  S.D. of results obtained from the number of replicate treatments. Differences between data sets were assessed by one-way analysis of variance followed by Newman-Keuls tests.  $P < 0.05$  or  $P < 0.01$  was accepted as statistically significant.

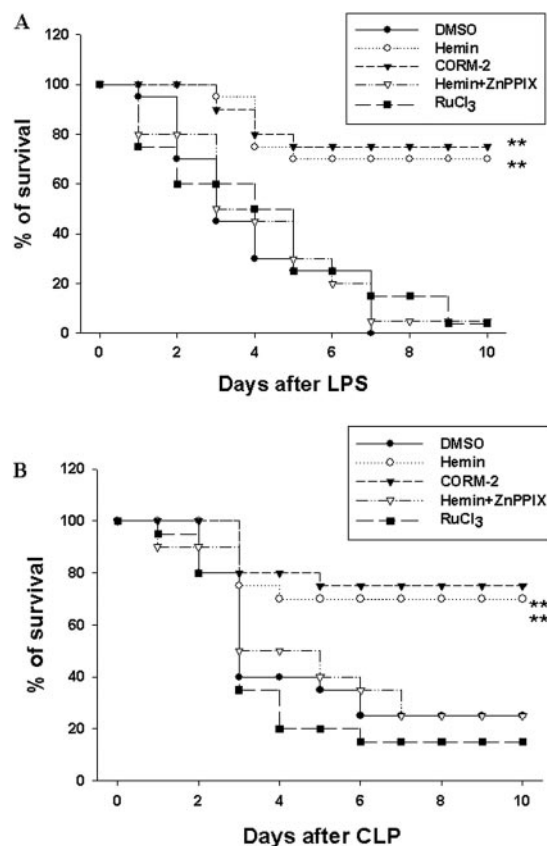
## Results

**Up-Regulation of HO-1 Attenuates Endotoxin-Induced Release of HMGB1.** HO-1 is known as a potent regulator of inflammatory cytokines, but its effect on HMGB1 in macrophages was not yet determined. Figure 1A shows that HMGB1 release becomes detectable at 16 h after LPS administration in RAW264.7 cells, confirming that HMGB1 is the late proinflammatory mediator (Wang et al., 1999). To determine whether HO-1 protein overexpression can down-regulate HMGB1 release, we pretreated macrophages with CoPPiX or hemin (potent HO-1 inducers, Fig. 1B) for 1 h and then stimulated with LPS for 16 h. HMGB1 extracellular expression was significantly induced by LPS, but HO-1 inducers could overwhelm this induction in a dose-dependent manner (Fig. 1C). Next, we asked whether the inhibitory effect of hemin and CoPPiX is mediated by HO-1 induction or not. As shown in Fig. 1D, HO-1 inducers failed to inhibit HMGB1 levels in the presence of si-HO-1 RNA. Finally, in mHO-1-transfected cells, HMGB1 release was inhibited in RAW264.7 cells activated with LPS (Fig. 1E). These results suggest that up-regulation of HO-1 can decrease HMGB1 extracellular level in macrophages stimulated with LPS.

**Effect of HO-1 Metabolites on HMGB1 Release by LPS.** Protective effect of HO-1 has been associated with its

main metabolites such as  $\text{Fe}^{2+}$ , bilirubin, and CO (Ryter et al., 2006). Thus, to clarify the role of each product of HO-1, we used bilirubin, CORM-2, CO-releasing molecule, and deferoxamine mesylate (iron chelator, DFO). Our results showed that only CO can modulate LPS-induced HMGB1 release (Fig. 2A), suggesting that the inhibitory effect HO-1 on HMGB1 release may be due to CO release. Bilirubin (Fig. 2B) or  $\text{Fe}^{2+}$  (Fig. 2C) did not show its involvement in the HO-1-mediated inhibitory effect. To confirm that CO derived from HO-1 induction decreases HMGB1 release in cells activated with endotoxin, we analyzed HMGB1 release by Western blot from the media in the presence of oxyhemoglobin ( $\text{HbO}_2$ , CO chelator). As shown in Fig. 3, inhibitory effect of hemin and CoPPiX was reversed by  $\text{HbO}_2$ .

**CoPPiX, Hemin, and CORM-2 Inhibit the Translocation of HMGB1 from the Nucleus to the Cytoplasm.** Consistent with previous reports, quiescent macrophages constitutively express HMGB1 and maintain it in the nucleus. However, activation of monocytes and macrophages by LPS results in the translocation of HMGB1 from the nucleus into the cytosol (Gardella et al., 2002). Keeping in mind that hemin, CoPPiX, and CORM-2 down-regulated HMGB1 re-



**Fig. 7.** Hemin and CORM-2 administration protect mice from lethal endotoxemia and sepsis. A and B, BALB/c mice were pretreated for 2 h with hemin (10 mg/kg i.p.,  $n = 20$ ), hemin + ZnPPiX (10 and 5 mg/kg i.p., respectively,  $n = 20$ ) CORM-2 (30 mg/kg i.p.,  $n = 20$ ), ruthenium chloride (30 mg/kg i.p.,  $n = 20$ ), or DMSO (i.p.,  $n = 20$ ) for 2 h. Then mice were subjected to lethal endotoxemia (LPS, 15 mg/kg i.p.) (A) or sepsis (induced by CLP) (B). At +12, +24, +48, +72, and +96 h after the onset of endotoxemia or sepsis, animals were intraperitoneally administered with DMSO, hemin (10 mg/kg), or CORM-2 (30 mg/kg). Survival was monitored daily for up to 2 weeks. The results are the mean  $\pm$  S.D. of three independent experiments. \*\*,  $P < 0.01$  compared with DMSO-treated animals.

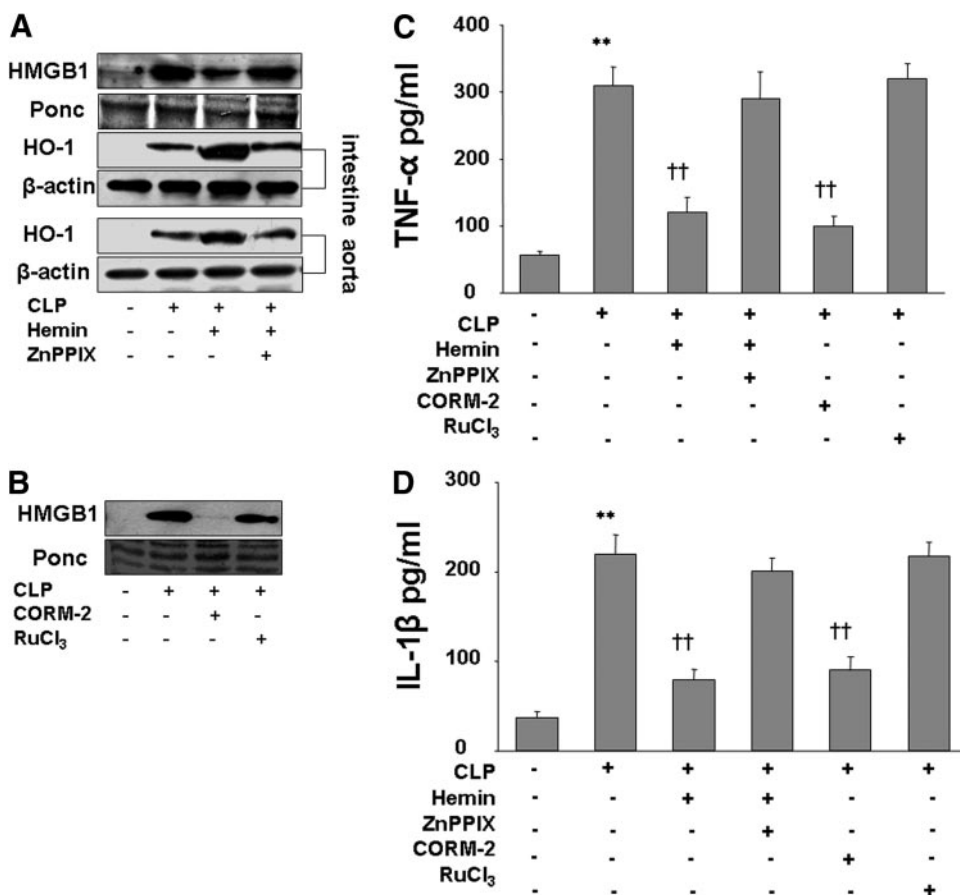
lease into extracellular milieu, we were interested in whether these agents can affect relocation of HMGB1 in LPS-stimulated RAW 264.7 cells. Cells were incubated for 16 h with LPS in the absence or presence of hemin, CoPPiX, or CORM-2. After incubation, cells were subjected to nuclear/cytosol fractionation. All three pharmacological agents significantly prevented HMGB1 translocation from nucleus into cytoplasm (Fig. 4), thereby inhibiting HMGB1 release.

**Effect of Early-Phase Cytokines, iNOS, and COX-2 on the Endotoxin-Induced HMGB1 Release.** LPS is a specific ligand for Toll-like receptor 4, which can activate the induction of proinflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ ) and INF- $\beta$  through MyD88-dependent and -independent pathways, respectively (Sato et al., 2002). iNOS and COX-2 are inducible isoforms of nitric-oxide synthase and cyclooxygenase, respectively. They were both documented as potent proinflammatory genes. In this experiment, we tested whether early-phase cytokines and/or iNOS and COX-2 are linked with HMGB1 release in LPS-stimulated macrophages. As shown in Fig. 5, neutralizing antibodies to TNF- $\alpha$ , IL-1 $\beta$ , and INF- $\beta$  significantly prevented HMGB1 release by LPS. In addition, treatment with L-NAME (iNOS inhibitor) but not NS-398 (COX-2 inhibitor) showed a reduction in HMGB1 level in cell culture medium after LPS stimulation in RAW264.7 cells. These data propose that TNF- $\alpha$ , IL-1 $\beta$ , INF- $\beta$ , and iNOS are involved in HMGB1 release but not COX-2 (Fig. 5).

**Effect of HO-1/CO Up-Regulation on the Expression of TNF- $\alpha$ , IL-1 $\beta$ , INF- $\beta$ , and iNOS (NO) in LPS-Stimulated Macrophages.** In a previous experiment, we deter-

mined that HMGB1 is dependent on TNF- $\alpha$ , IL-1 $\beta$ , INF- $\beta$ , and NO (iNOS) production. It was interesting for us to know whether HO-1 up-regulation or CO can affect the production of these molecules. LPS greatly induced the production of cytokines and NO by the induction of iNOS in murine macrophages. However, these effects were abrogated in the presence of CoPPiX, hemin, mHO-1, and CORM-2 (Fig. 6, A–D), suggesting that the inhibitory effect of HO-1 up-regulation and CO-releasing molecule on HMGB1 release can be due to the inhibition of production of early-phase cytokines and iNOS (NO) induction in macrophages.

**Hemin and CORM-2 Reduced HMGB1 Release and Protected Against Lethal Endotoxemia and Sepsis.** In light of the capacity of CoPPiX, hemin, and CORM-2 in attenuating LPS-induced HMGB1 release, we explored their efficacy in the animal model of lethal endotoxemia and sepsis. Administration of hemin and CORM-2 significantly attenuated animal death during lethal endotoxemia (Fig. 7A). Although endotoxemia is useful to investigate the complex cytokine cascades of sepsis, a more clinically relevant animal model is CLP. We asked whether HO-1 inducer or CORM-2 can attenuate animal death stimulated with CLP. As shown in Fig. 7B, hemin and CORM-2 also provided protection against CLP-induced mice mortality, whereas coadministration of hemin with ZnPPiX (HO-1 inhibitor) failed to protect animals from death. These experiments suggest that HO-1 up-regulation and CO protect mice from lethal endotoxemia and sepsis. Furthermore, no protection was observed against lethal endotoxemia or CLP-induced sepsis when administered RuCl<sub>3</sub>, a negative control for CORM-2 (Fig. 7, A and B).



**Fig. 8.** Hemin and CORM-2 pretreatments inhibit HMGB1, TNF- $\alpha$ , and IL-1 $\beta$  release during sepsis. A and B, DMSO, hemin (10 mg/kg), hemin + ZnPPiX (10 and 5 mg/kg i.p., respectively,  $n = 20$ ), CORM-2 (30 mg/kg), and ruthenium chloride (30 mg/kg i.p.,  $n = 20$ ) were administered 2 h before and 12 h after LPS stimulation (15 mg/kg) (A) or CLP (B). Plasma was collected by cardiac puncture 24 h after LPS or CLP induction, the HMGB1 levels were determined by Western blot, and cytokine levels were determined by ELISA. Thoracic aorta and intestine were homogenized and subjected to Western blot for HO-1 detection. Data are presented as the mean  $\pm$  S.D. of three independent experiments. One-way analysis of variance was used to compare multiple group means followed by Newman-Keuls test (significance compared with control or sham, \*,  $P < 0.05$ ; significance compared with LPS or CLP, †,  $P < 0.05$ ).



Several studies persuade that HMGB1 is a necessary and sufficient late mediator of the lethal multiple organ failure associated with severe sepsis (Wang et al., 1999; Yang et al., 2004). Therefore, we next investigated whether the administration of CORM-2 and hemin can attenuate circulating HMGB1 levels during sepsis. HMGB1 serum level starts to increase from 24 to 72 h after the initiation of sepsis (Degryse et al., 2001). To confirm that the reduced serum levels of HMGB1 by hemin in CLP-sepsis mice are related with HO-1 protein induction (HO-1 activity), we measured the levels of HO-1 protein expression in the organs (e.g., intestine and thoracic aorta) of CLP-sepsis mice. As shown in Fig. 8, A and B, hemin significantly increased HO-1 protein expression in those organs, which was reduced by ZnPPiX. To match with this, the increased serum levels of HMGB1 by CLP were significantly reduced by hemin, which was reversed by ZnPPiX (Fig. 8A). Likewise, the administration of CORM-2 but not RuCl<sub>3</sub> significantly reduced the serum levels of HMGB1 in CLP-induced sepsis animals (Fig. 8B).

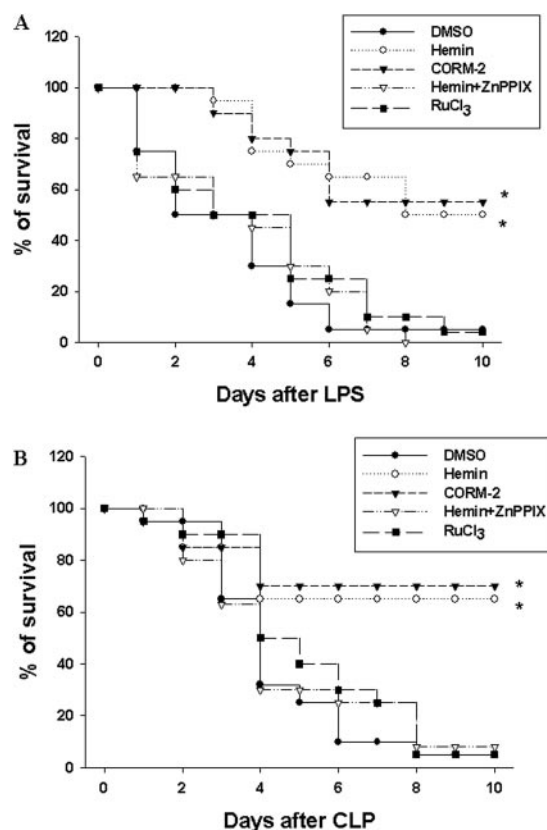
**Hemin and CORM-2 Down-Regulate TNF- $\alpha$  and IL-1 $\beta$  Levels in Septic Mice.** We also observed a significant reduction of serum levels of TNF- $\alpha$  and IL-1 $\beta$  in hemin- or CORM-2-treated mice 24 h after CLP stimulation (Fig. 8, C and D), and similar results were obtained in LPS-treated animals (data not shown). Taken together, these results indicate that induction of HO-1 and CO rescue mice from septic death probably through down-regulation of HMGB1 release.

**Delayed Hemin and CORM-2 Administrations Down-Regulate HMGB1 Levels without Affecting TNF- $\alpha$  and IL-1 $\beta$  and Protect Animal from Death by Lethal LPS- and CLP-Induced Sepsis.** In Fig. 8, we showed that HO-1 inducers and CORM-2 negatively regulate inflammatory cytokines and subsequently improved survival during sepsis. But it remains unclear whether improved survival is due to the inhibition of HMGB1 or TNF- $\alpha$  and IL-1 $\beta$  together. Keeping in mind that TNF- $\alpha$  and IL-1 $\beta$  are induced within hours, whereas HMGB1 starts to be detected at 16 to 24 h after the induction of sepsis (data not shown), we treated mice with hemin or CORM-2 approximately 12 h after the initiation of endotoxemia by LPS- or CLP-induced sepsis. HMGB1 levels were significantly decreased in hemin- and CORM-2-treated groups without significant inhibitory effect on TNF- $\alpha$  and IL-1 $\beta$  and showed significantly improved survival (Figs. 9 and 10). The improved survival was related to HO-1/CO, and we measured the levels of HO-1 protein expression in the organs (intestine and thoracic aorta) of CLP-sepsis mice; as expected, increased expression of HO-1 was shown, although it was treated 12 h after the initiation of CLP. In contrast, hemin in combination with ZnPPiX failed to up-regulate HO-1 and to down-regulate HMGB1 levels. Thus, we can conclude that inhibitory effect of HO-1/CO on HMGB1 levels is a necessary action to rescue animals from sepsis.

## Discussion

We demonstrated that induction of HO-1 is able to down-regulate HMGB1 release via CO in activated macrophages. Up-regulation of HO-1 and CORM-2 also can protect mice from the lethal effect of LPS- and CLP-induced sepsis model, and this protection is paralleled by a decrease in the systemic levels of HMGB1. CORMs are now very useful pharmacological tools for the investigation of HO-1-derived CO effect

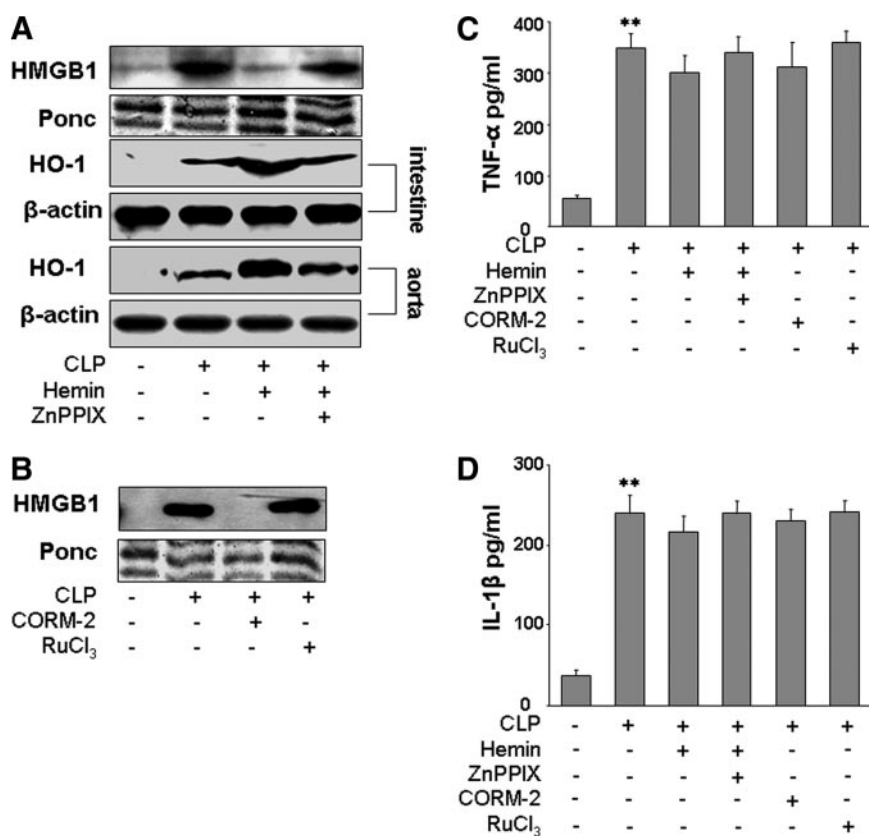
since Motterlini et al. (2002) first reported. We confirmed the beneficial effect of HO-1 or CORM-2 against sepsis in mice by showing the improved survival by treatment with CoPPiX and hemin during LPS- and CLP-induced sepsis (Foresti et al., 2005). These facts emphasize the importance of HO-1 against sepsis; however, it needs to be clarified further how HO-1 induction is beneficial for sepsis. There is growing evidence that the release of HMGB1 contributes to the pathology and mortality of sepsis, and subsequent inhibition of HMGB1 protects from sepsis-induced mortality in animals and humans (Wang et al., 1999, 2004a; Andersson et al., 2000; Ulloa et al., 2002; Park et al., 2004; Yang et al., 2004; Sundén-Cullberg et al., 2005). Although HMGB1 was first implicated as an important endogenous signaling molecule (Wang et al., 1999) and as a late mediator of endotoxin related lethality in mice, the relationship between inhibition of HMGB1 release and HO-1 up-regulation has recently been reported in LPS-induced acute lung injury (Gong et al., 2008). The relationship between HO-1 and HMGB1 in an experimental model of sepsis and septic shock has also been reported (Takamiya et al., 2008). These authors reported that circulating levels of HMGB1 were higher in HO-1(-/-) mice when treated with a low dose of LPS (5 mg/kg), a dose in which wild-type mice do not exhibit increased circulating



**Fig. 9.** Delayed hemin and CORM-2 administration protect against established endotoxemia and sepsis. BALB/c mice were subjected to lethal endotoxemia (LPS, 15 mg/kg i.p.) or sepsis (CLP) and 12 h later were treated with hemin (10 mg/kg i.p.), hemin + ZnPPiX (10 and 5 mg/kg i.p., respectively,  $n = 20$ ), CORM-2 (30 mg/kg i.p.), ruthenium chloride (30 mg/kg i.p.,  $n = 20$ ), or DMSO. Treatment was repeated at 24, 48, 72, and 96 h after induction of endotoxemia or sepsis. Survival was monitored daily for up to 2 weeks. The results are the mean  $\pm$  S.D. of three independent experiments. \*\*,  $P < 0.01$  compared with DMSO-treated animals.

levels of HMGB1, indicating that a release of HMGB1 from inflammatory cells is very vulnerable in HO-1-null mice. But how is HO-1 responsible for the inhibition of HMGB1 release? To resolve this question, we tested each component of HO-1 metabolites. First, we used DFO, an iron chelator, to determine whether the iron ion is responsible for the reduction of HMGB1 release. If the iron ion is responsible for the inhibition of HMGB1 release, DFO treatment should block or at least reverse the action of HO-1 inducers on HMGB1 release. However, DFO does not affect HMGB1 release by HO-1 inducers in the present study. Hence, we can eliminate the possibility of involvement of the iron ion in the inhibition of HMGB1 release by HO-1. Second, we examined whether bilirubin reduces HMGB1 release in cells activated with LPS, because bilirubin, another product of HO-1, is documented as a potent antioxidant in many inflammatory disorders (Ryter et al., 2006). In particular, bilirubin reduced iNOS and TNF- $\alpha$  and protected animals from hepatotoxicity during endotoxemia (Wang et al., 2004b). Despite its valuable properties, bilirubin failed to inhibit LPS-induced HMGB1 release in our study. Finally, we examined CO. To do so, we used CORM-2. CORMs already have shown their anti-inflammatory properties in many pathological and experimental models, including septic ones (Bani-Hani et al., 2006; Cepinskas et al., 2008; Masini et al., 2008). It has been demonstrated that CO mediates improved survival in septic animal models by its inhibitory effect on proinflammatory cytokine production (Bani-Hani et al., 2006; Cepinskas et al., 2008; Masini et al., 2008). However, inhibition of inflammatory cytokines by neutralizing antibodies or receptor antagonists did not show improved survival in animal septic models and clinical trials (Riedemann et al., 2003). Therefore, it

does not seem convincing that CO improves survival during sepsis by the inhibition of only inflammatory cytokines. Recently, it was reported that systemic administration of CORM-2 attenuated inflammation or adhesion molecules expression by inhibition of activation of nuclear factor- $\kappa$ B in CLP-induced liver of sepsis mice and LPS-treated human umbilical vein endothelial cells, which underscores again the importance of anti-inflammatory action of CO (Cepinskas et al., 2008). However, several reports suggested that HMGB1 inhibition is enough and sufficient to save animals from death initiated by sepsis or endotoxemia (Wang et al., 1999; Yang et al., 2004). We found, for the first time, that CO liberated by CORM-2 can attenuate HMGB1 release in LPS-treated RAW 264.7 cells. Although it has been reported that CORMs significantly suppressed the inflammatory response elicited by LPS in cultured macrophages (Sawle et al., 2005), there was no report about the effect of CORMs on HMGB1 release. We also demonstrated that administration of HO-1 inducers (hemin, CoPPiX) or CORM-2 significantly reduced circulating HMGB1 and protected animals from not only LPS-induced endotoxemia but also CLP-induced sepsis. Moreover, delayed administration of CORM-2 (12 h after LPS or CLP) significantly inhibited HMGB1 levels without alterations in serum levels of TNF- $\alpha$  or IL-1 $\beta$ , suggesting that inhibition of HMGB1 is a clue for HO-1 and/or CO-mediated improved survival. Consistent with a recent report that CORM-2 was able to suppress the increased circulating HMGB1 in HO-1(-/-) mice and rescued HO-1(-/-) mice from the lethality of endotoxemia (Takamiya et al., 2008), the finding of reduced serum levels of HMGB1 by CO and/or HO-1 inducers implies that CO is important regulator of HMGB1 release. However, they (Takamiya et al., 2008)



**Fig. 10.** Delayed hemin and CORM-2 administration reduce HMGB1 but not TNF- $\alpha$  or IL-1 $\beta$  levels in septic mice. Hemin (10 mg/kg i.p.), CORM-2 (30 mg/kg i.p.), or ruthenium chloride (30 mg/kg i.p.,  $n = 20$ ) were administrated 12 h after CLP or LPS induction. Plasma was collected 24 h after initiation of sepsis by heart puncture and subjected to HMGB1 analysis or ELISA. Thoracic aorta and intestine were homogenized and subjected to Western blot for HO-1 detection. Data are presented as mean  $\pm$  S.D. of three independent experiments. Significance compared with control or sham, \*,  $P < 0.05$ ; significance compared with LPS or CLP, †,  $P < 0.05$ .



found that biliverdin, another byproduct of HO-1, also significantly reduced the circulating HMGB1 and rescued HO-1(−/−) mice from the mortality of endotoxemia. In the present study, bilirubin did not reduce HMGB1 release in LPS-treated RAW 264.7 cells. Based on this result of in vitro study, we did not further investigate the effect of bilirubin in in vivo study. As to whether bilirubin reduces circulating HMGB1 in LPS- or CLP-induced sepsis animals remains to be elucidated. Although anti-inflammatory effect of CO is important for attenuation of sepsis-related events (Bani-Hani et al., 2006), reduction of HMGB1 release is also critical for the alleviation of septic symptoms and increase of survival as shown in the present study. Thus, CO, whether it comes from either HO-1 induction or CO-releasing molecules, may have a great potential for treatment of sepsis. We found that ascorbic acid, known to improve the survival of mice with sepsis, also reduced HMGB1 in LPS-treated RAW 264.7 cells due to CO through the induction of HO-1 (Y. M. Ha, unpublished observations). It is interesting to note that potent HO-1-inducing agents, such as (−)-epigallocatechin-3-gallate and tanshinone IIA, were identified as potent inhibitors of endotoxin-induced HMGB1 release and protected mice from death by lethal sepsis (Wu et al., 2006; Chen et al., 2007; Li et al., 2007a,b). Although the molecular mechanism by which CO prevents release of HMGB1 in activated macrophages and septic animal model has to be elucidated, it can be speculated that early proinflammatory cytokine production such as TNF- $\alpha$  can be inhibited by CO, which can, in turn, affect later the translocation of HMGB1. In fact, proinflammatory cytokines produced by endotoxin-stimulated macrophages/monocytes can be roughly subdivided into two parts, early- (e.g., TNF and IL-1) and late-phase cytokines (e.g., HMGB1). However, reciprocal regulation of early- and late-phase cytokines has been documented (Jiang and Pisetsky, 2006). In agreement with previous studies, we demonstrated relationships between TNF- $\alpha$ , IL-1 $\beta$ , INF- $\beta$ , NO, and HMGB1 release. Moreover, HO-1 inducers and CORM-2 blocked the expression of early-phase cytokines and NO (iNOS) production, suggesting that the inhibition of HMGB1 release can go through the inhibition of TNF- $\alpha$ , IL-1 $\beta$ , INF- $\beta$ , and NO in LPS-stimulated macrophages. Most important, however, was the observation that delayed administration of CORM-2 still inhibited CLP- or LPS-induced HMGB1 release and improved survival without attenuation of early-phase cytokine (TNF- $\alpha$  or IL-1 $\beta$ ) levels. This may clearly reflect that the plasma level of TNF- $\alpha$  and IL-1 $\beta$  is already decreased to the minimal level at the time of CORM-2 administration (12 h after LPS or CLP challenge), so no changes have been observed. Alternately, it may underscore that the plasma level of HMGB1 is critical for the survival of endotoxemia or sepsis. Although it is difficult to explain how CO inhibits the release HMGB1 at the present time, CO can inhibit nuclear factor- $\kappa$ B activity or p38 mitogen-activated protein kinase (Ulloa et al., 2002; Bonaldi et al., 2003; Aneja et al., 2008) or activate peroxisome proliferator-activated receptor- $\gamma$  (Hoetzel et al., 2008). Therefore, further study is needed to determine whether possible mechanism(s) of CO exerts on the HMGB1 release by LPS- or CLP-induced sepsis. Finally, we found that inhibition of COX-2 by NS-398, a selective COX-2 inhibitor, did not affect HMGB1 release. It probably explains why specific COX-2 inhibition did not im-

prove survival during lethal sepsis or endotoxemia in mice (Reddy et al., 2001).

In conclusion, here we report that CO can significantly attenuate HMGB1 release during sepsis, and this inhibition is a necessary step of CO in protection against sepsis. In this way, CO-releasing molecules can be targeted for the development of therapeutic agents against systemic inflammatory disorders, such as sepsis.

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