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HO-1 inducers and CO-releasing molecule inhibit LPS-induced HMGB 1 release in vitro and improves survival of mice in LPS- and CLP-induced sepsis model in vivo

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Running title: CO inhibits HMGB1 release in LPS- and CLP-induced sepsis model

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Abbreviations: BSA, bovine serum albumin; 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, desferoxamine mesylate; PVDF, polyvinylidene difluoride; TLR, toll-like receptor; L-NAME, N^{ω}-nitro-L-arginine methyl ester hydrochloride; iNOS, inducible nitric oxide synthase; PCNA, proliferating cell nuclear Ag protein;

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Abstract

We examined our hypothesis that HO-1 derived carbon monoxide (CO) inhibits release of HMGB1 in RAW264.7 cells activated with 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, CoPPIX), or transfection of HO-1 significantly inhibited HMGB1 release, which was blocked by HO-1 siRNA, in cells activated by LPS. 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 TNF-α, IL-1β, INF-β, and L-NAME but not NS-398 significantly inhibited HMGB1 release in LPS-activated cells. Production of TNF-α, IL-1β and IFN-β 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 administration of HO-1 inducers or CORM-2 which was accompanied by either reduction (pretreatment) or no change (delayed administration) of serum TNF-α and IL-1β 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.

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1. Introduction

Sepsis is defined as a systemic inflammatory response syndrome from a microbial infection which results from excessive stimulation of the host immune system by pathogen components to produce various pro-inflammatory cytokines, and their overproduction causes systemic inflammation that can lead to the lethal multiple organ damage (Oberholzer et al., 2001). High mobility group box1 (HMGB1) is a chromatin-binding protein, which participates in maintaining nucleosome structure and regulation of gene transcription (Landsman and Bustin, 1993). Various evidences 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, Toll-like receptor (TLR) 2 and TLR 4, and mediate various cellular responses, chemotactic cell movement and release of proinflammatory cytokines (Andersson et al., 2000; Park et al., 2004; Rouhiainen et al., 2007). The administration of anti-HMGB1 Abs or inhibitors (e.g., ethyl pyruvate, nicotine) significantly protected mice from lethal sepsis (Wang et al., 1999; Yang et al., 2004; Wang et al., 2004a; Ulloa et al., 2002). Thus, inhibition of HMGB1 release is 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: Fe^{++}, biliverdin, and carbon monoxide (CO). Biliverdin is subsequently converted into bilirubin by biliverdin reductase. The increased level of Fe^{2+} stimulates synthesis of ferritin (iron-bound compound) which is known as cytoprotective, antioxidant protein. HO-1 system exerts anti-apoptotic, anti-oxidant 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

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induction improves animal survival in lethal sepsis. Thus, the aim of present study was to investigate 1) HO-1 inducers or HO-1 gene transfection inhibit the expression and release of HMGB1 in murine RAW264.7 macrophages when activated with LPS, and 2) is CO responsible for the inhibition of expression and release of HMBG1 by HO-1 induction? Finally, 3) dose CO improve survival in LPS- or CLP-treated mice? In order to do this, we used pharmacological agents such as hemin, cobalt protoporphyrin (CoPP)IX as HO-1 inducers and tricarbonyldichlororuthenium (II) dimmer ($[Ru(CO)_3Cl_2]_2$, CORM-2) as CO-releasing molecule. While we are submitting this manuscript, 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.

2. Materials and methods

Materials. Anti-HMGB1 was purchased from Abcam (Cambridge, MA), anti-iNOS from Transduction Laboratories (Lexington, KY), anti-β-actin and anti-heme oxygenase-1 from Santa Cruz Biotechnology (Santa Cruz, CA). Neutralizing antibodies to TNF-α, IL-1β and INF-β were provided from R&D systems (Minneapolis, MN), Enhanced chemiluminescence (ECL) Western blotting detection reagent was from Amersham (Buckinghamshire, UK). All other chemicals including lipopolysaccharide (LPS, Escherechia coli 0111:B4), cobalt protoporphyrin IX, hemin, tricarbonyldichlororuthenium (II) dimmer ($[Ru(CO)_3Cl_2]_2$) (CORM-2), NS-398 and N^{ω}-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 (ATCC, Rockville, MD). The cells were grown in RPMI-1640 medium supplemented with 25

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mM N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid (HEPES), 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum.

Cell stimulation. RAW 264.7 cells were plated at density of 1×10^7 cells per 100mm dish. The cells were rinsed with fresh medium and stimulated with LPS (1 μg/ml) in the presence of different concentration of pharmacological agents. At 16 h after stimulation levels of HMGB1 or iNOS were determined. Oxyhemoglobin (HbO_2) was prepared by reduction of bovine hemoglobin with sodium hydrosulfite followed by gel filtration with a prepacked disposable column (PD-10, Pharmacia, Uppsala, Sweden), previously equilibrated with 50 mM Tris/HCl at pH 7.4 (Salyemini et al., 1989). The concentration of HbO_2 was determined using a Perkin-Elmer Lambda 5 spectrophotometer at 576 nm wavelength, according to Kondo et al (1989).

NO assay. Nitric oxide was measured as its stable oxidative metabolite, nitrite (NOx), as described by Kang et. al. (1999). At the end of incubation, 100 μl 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-α, INF-β and IL-β in the culture medium were determined using commercial available ELISA kits from R&D systems (Minneapolis, MN) according to the provided manuals.

HMGB1 analysis. Culture medium samples were briefly centrifuged. Same volumes of samples were then concentrated 40-fold with Amicon Ultra-4-10000 NMWL (Millipore). Centrifugation conditions were fixed angle (35 degree) and 7500g for 20 min at 4 °C. Blood was collected by cardiac puncture into sodium heparin containing tube. After centrifugation, plasma samples were

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filtered and concentrated through Centricon YM-100 and YM-10 (Millipore, Billerica, MA) respectively. The concentrated samples were subjected to SDS-PAGE electrophoresis. Ponceus staining was used as loading control.

Western blot. The cytoplasmic/nuclear fractionation was performed using nuclear/cytosol fractionation kit (Cat # K266-25, BioVision, Mountain view, CA) according to manufacture 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, β-actin, 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 (PVDF) membranes by semidry electrophoretic transfer at 15 V for 60 to 75 min. The membranes was stained with Ponceau S solution (2μg/ml) for 5 min to determine efficiency of transfer or/and protein loading levels per track. Then the PVDF membranes were blocked overnight at 4 °C in 5% bovine serum albumin (BSA). The cells were incubated with primary antibodies diluted 1:500 in Tris/buffered saline/Tween 20 (TBS-T) containing 5% BSA for overnight in 4 °C and then incubated with secondary antibody at room temperature for 1 h. The signals were detected by ECL.

Transfection of HO-1 gene into RAW267.4 cells. For HO-1 transfecion, cells were washed twice with serum free media and 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 was removed and the cells were incubated in appropriate media for 16 h.

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siRNA technique. HO-1 small interference RNA (siRNA) and scramble siRNA were purchased from Invitrogen (Carlsbad, CA). The sequence of mouse HO-1 siRNA (5 end prime to 3 end prime) is as follows: UUACAUGGCAUAAAUUCCCACUGCC. 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 FBS, and cells were washed and pretreated with our without Hemin or CoPPIX, following LPS stimulation.

Animal model of endotoxemia and sepsis. Endotoxemia was induced in BALB/c mice (male 7-8 wk, 20-25g) by i.p. 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 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 (NIH publication 85-23, revised 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 ± SD 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.

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3. Results

Upregulation 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, confirming that HMGB1 is the late proinflammatory mediator (Wang et al., 1999). To determine whether HO-1 protein overexpression can downregulate HMGB1 release, we pretreated macrophages with CoPPIX or hemin (potent HO-1 inducers, Figure 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 (Figure 1C). Next, we asked whether inhibitory effect of hemin and CoPPIX is mediated by HO-1 induction or not. As shown in Figure 1D, HO-1 inducers failed to inhibit HMGB1 levels in the presence of siHO-1 RNA. Finally, in mHO-1 transfected cells HMGB1 release was inhibited in RAW264.7 cells activated with LPS (Figure 1E). These results suggest that upregulation 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 Fe^{2+}, bilirubin and CO (Ryter et al., 2006). Thus to clarify the role of each product of HO-1, we used bililubin, CORM-2, CO releasing molecule, and desferoxamine mesylate (iron chelator, DFO). Our results showed that only CO can modulate LPS-induced HMGB1 release (Figure 2A) suggesting that inhibitory effect HO-1 on HMGB1 release may be due to CO release. Whereas, bilirubin (Figure 2 B) or Fe^{2+} (Figure 2 C) did not show their involvement in HO-1-mediated inhibitory effect. To confirm CO derived from

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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 (HbO_2, CO chelator). As shown in Figure 3, inhibitory effect of hemin and CoPPIX was reversed by 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 nucleus into cytosol (Gardella et al., 2002). Keeping in mind that hemin, CoPPIX and CORM-2 downregulated HMGB-1 release into extracellular milieu, we were interested in whether these agents can affect relocalization 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 (Figure 4), thereby inhibited HMGB1 release.

Effect of early phase cytokines, iNOS and COX-2 on the endotoxin-induced HMGB1 release. LPS is specific ligand for TLR4, which can activate induction of proinflammatory cytokines (TNF-α, IL-1β) and INF-β 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, COX-2 are linked with HMGB1 release in LPS-stimulated macrophages. As shown in Figure 5, neutralizing antibodies to TNF-α, IL-1β and INF-β significantly prevented HMGB1 release by LPS. In addition, treatment with L-NAME (iNOS inhibitor) but not NS-398 (COX-2 inhibitor) showed reduction in HMGB1 level in cell culture medium after LPS stimulation in RAW264.7 cells. This data

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proposes that TNF-α, IL-1β, INF-β and iNOS are involved in HMGB1 release but not COX-2 (Figure 5).

Effect of HO-1/CO upregulation on the expression of TNF-α, IL-1β, INF-β and iNOS (NO) in LPS stimulated macrophages. In previous experiment, we determined that HMGB-1 is dependent on TNF-α, IL-1β, INF-β, and NO (iNOS) production. It was interesting for us to know whether HO-1 upregulation or CO can affect the production of these molecules. LPS greatly induced production of cytokines and NO by induction of iNOS in murine macrophages. However, these effects were abrogated in the presence of CoPPIX, hemin, mHO-1 and CORM-2 (Figures 6A, B, C, D) suggesting that inhibitory effect of HO-1 upregulation and CO releasing molecule on HMGB1 release can be due to inhibition of production of early phase cytokines and iNOS (NO) induction in macrophages.

Hemin, CORM-2 reduces 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 (Figure 7A). Although endotoxemia is useful to investigate the complex cytokine cascades of sepsis, more clinically relevant animal model is CLP. We asked whether HO-1 inducer or CORM-2 can attenuate animal death stimulated with CLP. As it is shown in Figure 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 form death. These experiments suggest that HO-1 upregulation and CO protect mice from lethal endotoxemia and sepsis. Furthermore, no protection was observed against lethal endotoxemia or CLP-induced sepsis when administered $RuCl_3$, a negative control for CORM-2 (Figures 7A and B). Several studies persuade that HMGB1 is necessary and sufficient late mediator of the lethal

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multiple organ failure associated with severe sepsis (Wang et al., 1999; Yang et al., 2004). Therefore, we next investigated whether 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 initiation of sepsis (Degryse et al., 2001). To confirm 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 (eg., intestine and thoracic aorta) of CLP-sepsis mice. As shown in figures 8A 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 (Figure 8A). Likewise, administration of CORM-2 but not $RuCl_3$ significantly reduced serum levels of HMGB1 in CLP-induced sepsis animals (Figure 8B)

Hemin, CORM-2 downregulate TNF-α, IL-1β levels in septic mice. We also observed significant reduction of serum levels of TNF-α and IL-1β in hemin- or CORM-2-treated mice 24 h after CLP stimulation (Figure 8C and D), 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 downregulation of HMGB1 release.

Delayed hemin and CORM-2 administrations down-regulate HMGB1 levels without affecting TNF-α and IL-1β and protect animal from death by lethal LPS- and CLP-induced sepsis. In previous figure, 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 inhibition of HMGB1 or TNF-α and IL-1β together. Keeping in mind that TNF-α and IL-1β are induced within hours, whereas HMGB1 starts to be detected at 16-24 h after induction of sepsis (data not shown), we treated mice with hemin or CORM-2 about 12 h after initiation of endotoxemia by LPS or CLP-induced sepsis. HMGB1

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levels were significantly decreased in hemin- and CORM-2-treated groups without significant inhibitory effect on TNF-α and IL-1β and showed significantly improved survival (Figures 9 and 10). The improved survival was related with HO-1/CO, we measured the levels of HO-1 protein expression in the organs (intestine and thoracic aorta) of CLP-sepsis mice, as expected, and increased expression of HO-1 was shown although it was treated 12 h after initiation of CLP. In contrast, hemin in combination with ZnPPIX failed to upregulate HO-1 as well as to downregulate HMGB1 levels. Thus, we can conclude that inhibitory effect of HO-1/CO on HMGB1 levels is necessary action to rescue animals from sepsis.

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4. Discussion

We demonstrated that induction of HO-1 is able to downregulate HMGB1 release via CO in activated macrophages. Both upregulation of HO-1 and CORM-2 also can protect mice from lethal effect of LPS- and CLP-induced sepsis model and this protection is paralleled by decrease in the systemic levels of HMGB1. CORMs are now being used as very useful pharmacological tools for investigation of HO-1 derived CO effect since Motterlini et al (2002) firstly reported. We confirmed the beneficial effect of HO-1 or CORM-2 against sepsis in mice by showing of improved survival by treatment 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 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; Andersson et al., 2000; Ulloa et al., 2002; Park et al., 2004; Wang et al., 2004a; Yang et al., 2004; Sunden-Cullberg et al., 2005). Although HMGB1 was firstly implicated as an important endogenous signaling molecule (Wang et al., 1999) as a late mediator of endotoxin related lethality in mice, the relationship between inhibition of HMGB1 release and HO-1 upregulation has recently been reported in LPS-induced acute lung injury (Gong et al., 2008). While we are preparing this manuscript, the relationship between HO-1 and HMGB1 in experimental model of sepsis and septic shock also has been reported (Takamiya et al., 2008). The authors reported that circulating levels of HMGB1 were higher in HO-$1^{-/-}$ mice when treated with low dose of LPS (5 mg/kg), a dose in which wild-type mice do not exhibit increased circulating levels of HMGB1, indicating that a release of HMGB1 from inflammatory cells is very vulnerable in HO-1 null mice. But, how HO-1 is responsible for the inhibition of HMGB1 release? To resolve this question, we tested each component of HO-1 metabolites. Firstly, we

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used DFO, iron chelator to see whether iron ion is responsible for the reduction of HMGB1 release. If 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 iron ion in the inhibition of HMGB1 release by HO-1. Secondly we examined whether bilirubin reduces HMGB1 release in cells activated with LPS, since bilirubin, another product of HO-1, documented as a potent antioxidant in many inflammatory disorders (Ryter et al., 2006). In particular, bilirubin reduced iNOS as well as TNF-α 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. Lastly, we examined CO. To do so, we used CORM-2. CORMs already have shown their anti-inflammatory properties in many pathological, experimental models including septic ones (Bani-Hani et al., 2006; Masini et al., 2008; Cepinkas et al., 2008). It has been demonstrated that CO mediates improved survival in septic animal models by its inhibitory effect on proinflammatory cytokines production (Bani-Hani et al., 2006; Masini et al., 2008; Cepinkas 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 (Riedmann et al., 2003). Therefore, it seems not convincing that CO improves survival during sepsis by the inhibition of only inflammatory cytokines. Recently, it has been reported that systemic administration of CORM-2 attenuated inflammation or adhesion molecules expression by inhibition of activation of NF-kB in CLP-induced liver of sepsis mice and LPS-treated HUVECs, which underscores again the importance of anti-inflammatory action of CO (Cepinkas et al., 2008). Whereas, 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 CORMs significantly suppressed the inflammatory

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response elicited by LPS in cultured macrophages (Sawle et al., 2005), there were no report about 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-α or IL-1β, suggesting that inhibition of HMGB1 is a clue for HO-1 and/or CO-mediated improved survival. Consistent with 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) found that biliverdin, another by product 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 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 survival of mice with sepsis, also reduced HMGB1 in LPS-treated RAW 264.7 cells due to CO through induction of HO-1 (Ha et al., submitted). 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 (Li et al., 2007a; 2007b; Wu et al., 2006; Chen et al., 2007). Although molecular mechanism by which CO prevents release of HMGB1 in activated

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macrophages and septic animal model has to be elucidated, it can be speculated that early proinflammatory cytokine production such as TNF-α can be inhibited by CO which can, in turn, affect later the translocation of HMGB1. In fact, proinflammatory cytokines producing 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-α, IL-1β, INF-β, NO and HMGB1 release. Moreover, HO-1 inducers and CORM-2 blocked the expression of early phase cytokines and NO (iNOS) production suggesting that inhibition of HMGB1 release can go through inhibition of TNF-α, IL-1β, INF-β and NO in LPS stimulated macrophages. Most importantly, however, it was observed that delayed administration of CORM-2 still inhibited CLP- or LPS-induced HMGB1 release and improved survival without attenuation of early phase cytokine (TNF-α or IL-1β) levels. This may clearly reflect that plasma level of TNF-α and IL-1β is already declined 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 plasma level of HMGB1 is critical for survival of endotoxemia or sepsis. Although it is difficult to explain how CO inhibits release HMGB1 at the present time, CO can inhibit NF-kB activity or p38 MAPK (Ulloa et al., 2002; Bonaldi et al. 2003; Aneja et al., 2008) or activate PPAR-γ (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. Probably it is explained why specific COX-2 inhibition did not improve 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 necessary step of CO in protection against sepsis. In this way, CO

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releasing molecules can be target for the development of therapeutic agents against systemic inflammatory disorders, such as sepsis.

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Footnotes

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Legends for figures

Fig. 1. Hemin, CoPPIX and mHO-1 attenuated endotoxin-induced HMGB1 release in RAW 264.7 cells. (A) Cells were stimulated with LPS (1μg/ml) for 4, 8, 16, 24 hours and culture medium samples were concentrated for HMGB1 detection as described in the Materials and Methods. (B) Cells were treated with hemin or CoPPIX at 1, 5, 10 25 μM for 16 hours, then HO-1 protein levels were determined by Western blot. (C) LPS (1μg/ml) stimulated macrophages in the presence or absence of hemin or CoPPIX in various doses for 16 hours, and HMGB1 release was identified, main ponceau band was used as a loading control. (D) Cells were transfected with siHO-1 or scramble as described in Materials and Methods, after transfection, cells were pretreated with Hemin (10μM) or CoPPIX (10μM) for 1 h, and stimulated with LPS (1μg/ml), HMGB1 and HO-1 levels were determined. (E) Cells were transfected with mHO-1 or empty vector as described in Materials and Methods and then stimulated with LPS, after 16 hours culture medium were subjected to HMGB1 levels determination, cells were subjected to whole lysis for HO-1 detection by Western blot. Data are presented as ± SD 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, $^{\dagger}P < 0.05$).

Fig. 2. Effect of CORM-2, bilirubin, 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 (1μg/ml) for 16 h, $RuCl_3$ was used as negative control for CORM-2. (C) DFO (500 μg/ml) were administrated for 1 h and later hemin (10 μg/ml) or CoPPIX (10 μg/ml) was administrated.

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After 1 h cells were stimulated with LPS (1μg/ml) for 16 h. HMGB1 levels in culture medium were determined by Western blot. Data are presented as ± SD of three independent experiments. One-way analysis of variance was used to compare multiple group means followed by Newman-Keuls test (significance campared with control, $*P < 0.05$; significance compared with LPS, $^{\dagger}P < 0.05$).

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_2 (20 μg/ml) and then hemin (10 μg/ml) or CoPPIX (10 μg/ml) were administrated. After that, cells were stimulated with LPS (1 μg/ml). Culture medium was collected and subjected to HMGB1 analysis by Western blot. Data are presented as ± SD 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, $^{\dagger}P < 0.05$).

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

Fig. 5. Effect of TNF-α, IL-1β, INF-β neutralization and NO (iNOS), COX-2 inhibition on the endotoxin-induced HMGB1 release in macrophages. Cells were pretreated with Nab TNF-α (100 μg/ml), Nab IL-1β (100 μg/ml), Nab INF-β (500 μg/ml) or L-name (300 μM), NS-398 (50 μM) for 1h and treated with LPS (1 μg/ml) for 16 h. After incubation culture medium samples were subjected to HMGB1 levels determination as described in Materials and Methods. Data are presented as ± SD of three independent experiments. One-way analysis of variance was used to

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compare multiple group means followed by Newman-Keuls test (significance compared with control, $*P < 0.05$; significance compared with LPS, $^{\dagger}P < 0.05$).

Fig. 6. Effect of HO-1 upregulation and CORM-2 on the release of various cytokines and NO (iNOS) in LPS-activated macrophages. (A-D) Cells were incubated with hemin (10 μM), CoPPIX (10 μM), CORM-2 (100 μM) for 1 h or transfected with mHO-1, and later activated with LPS for 16 h. After incubation time culture medium samples were subjected TNF-α (A), INF-β (B) and IL-1β (C) determination by appropriate ELISA kit, NO production (D) was measured by NO assay as described in Materials and Methods. Cells were harvested by total lysis and iNOS protein (D) levels were determined by Western blot. Data are presented as ± SD 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, $^{\dagger}P < 0.05$ or $^{\dagger\dagger}P<0.01$).

Fig. 7. Hemin and CORM-2 administration protect mice from lethal endotoxemia and sepsis. (A, B) BALB/c mice were pretreated for 2h with hemin (10 mg/kg, i.p., n=20), hemin+ZnPPIX (10 and 5 mg/kg respectively, i. p., 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 i.p. administered with DMSO, hemin (10mg/kg), or CORM-2 (30 mg/kg). Survival was monitored daily, up to two weeks, The results are the mean ± SD of three independent experiment. $**P < 0.01$compared to DMSO treated animals.

Fig. 8. Hemin and CORM-2 pretreatments inhibit HMGB1, TNF-α, IL-1β release during sepsis. (A, B) DMSO, hemin (10 mg/kg), hemin+ZnPPIX (10 and 5 mg/kg respectively, i. p., n=20),

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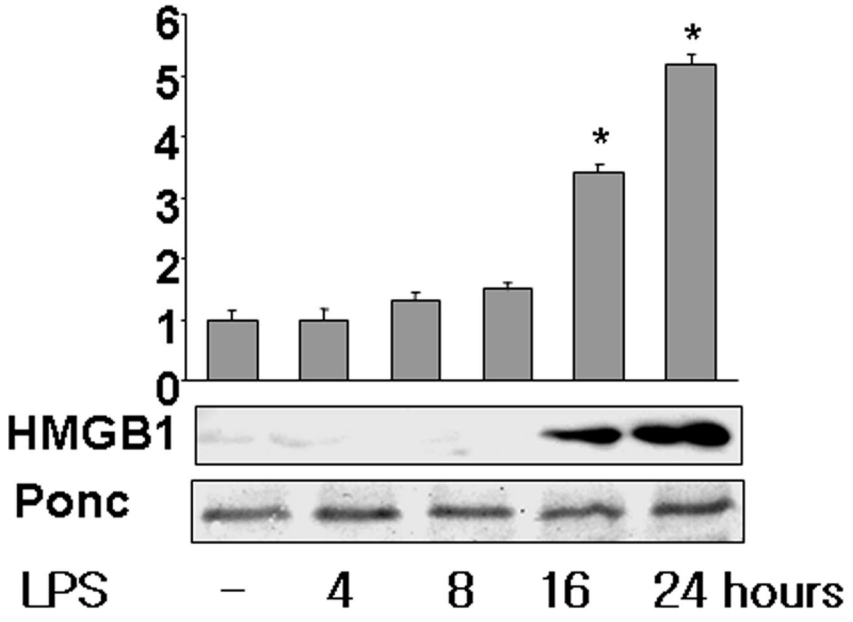
CORM-2 (30 mg/kg), Ruthenium chloride (30 mg/kg, i.p., n=20) were administrated 2 h before and 12 h after LPS stimulation (15 mg/kg) (A) or CLP (B). Plasma was collected by cardiac puncture 24 h after LPS or CLP induction, the HMGB1 levels were determined by Western blot, cytokines levels were determined by ELISA. Thoracic aorta and intestine were homogenized, subjected to Western blot for HO-1 detection. Data are presented as ± SD 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, $^{\dagger}P < 0.05$).

Fig. 9. Delayed Hemin, 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 treated with hemin (10 mg/kg, i.p), hemin+ZnPPIX (10 and 5 mg/kg respectively, i. p., 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 h, 48 h, 72 h, and 96h after induction of endotoxemia or sepsis. Survival was monitored daily, up to two weeks. The results are the mean ± SD of three independent experiment $**P < 0.01$ compared to DMSO treated animals.

Fig. 10. Delayed Hemin and CORM-2 administration reduce HMGB1 but not TNF-α or IL-1β levels in septic mice. Hemin (10 mg/kg, i.p.) or CORM-2 (30 mg/kg, i.p.), 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, subjected to Western blot for HO-1 detection. Data are presented as ± SD of three independent experiments. Significance compared with control or sham, $*P < 0.05$; significance compared with LPS or CLP, $^{\dagger}P < 0.05$.

A)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HMGB1
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B)

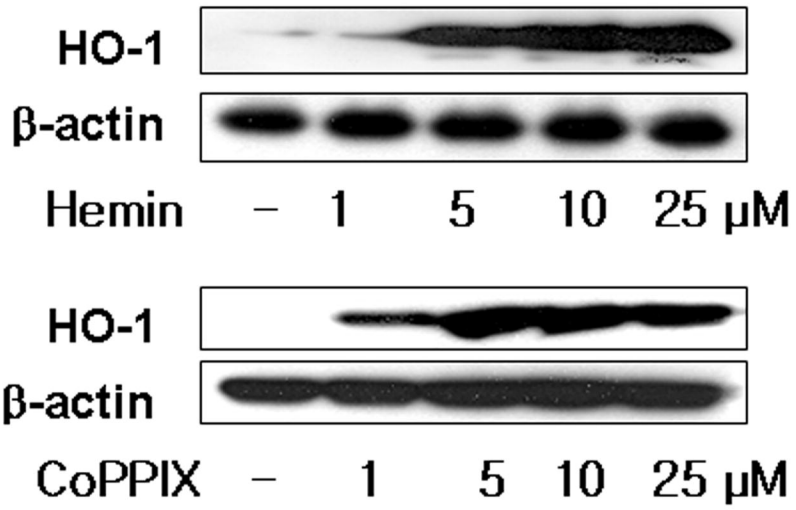
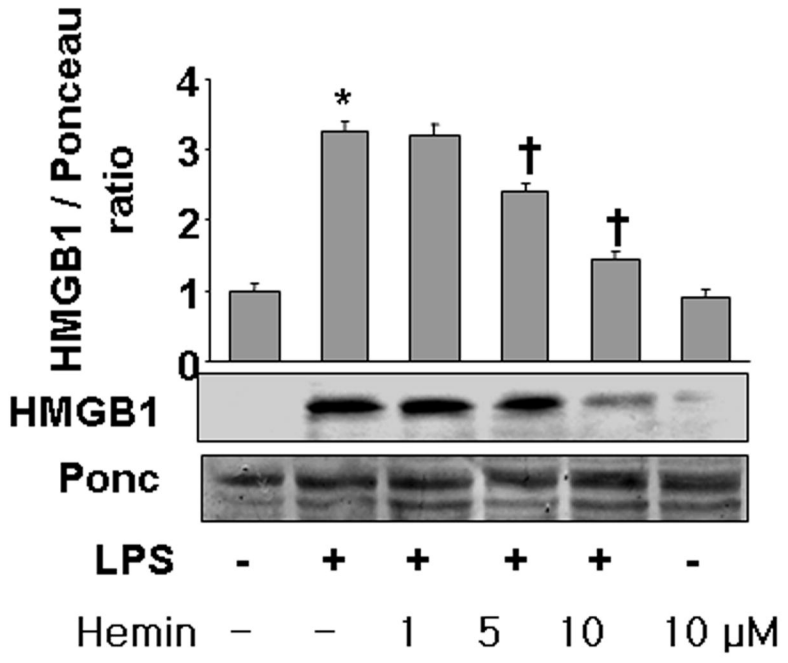

HO-1
β-actin
Hemin – 1 5 10 25 μM
HO-1
β-actin
CoPPIX – 1 5 10 25 μM

Figure 1

C)


HMGB1 / Ponceau ratio
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HMGB1
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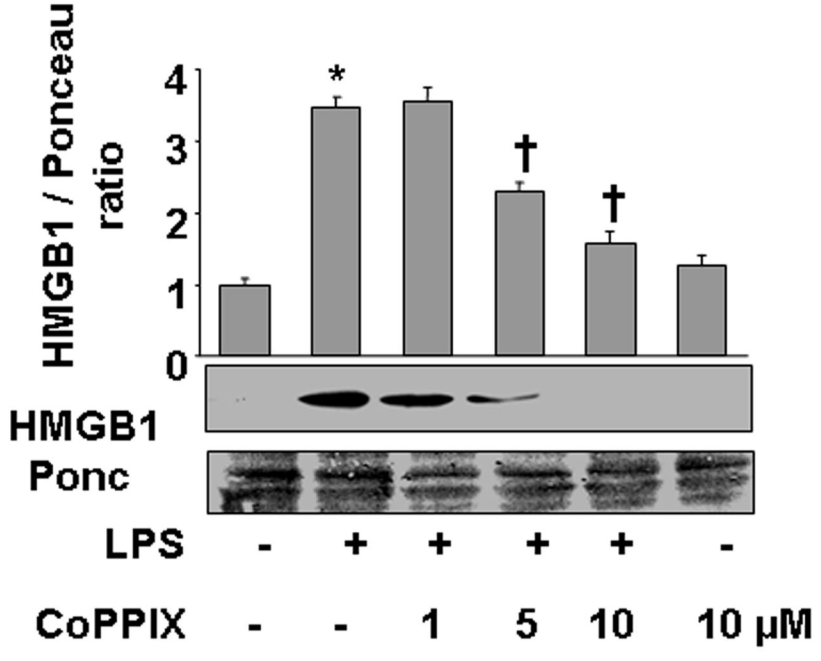
LPS - + + + + -
Hemin - - 1 5 10 10 μM


HMGB1 / Ponceau ratio
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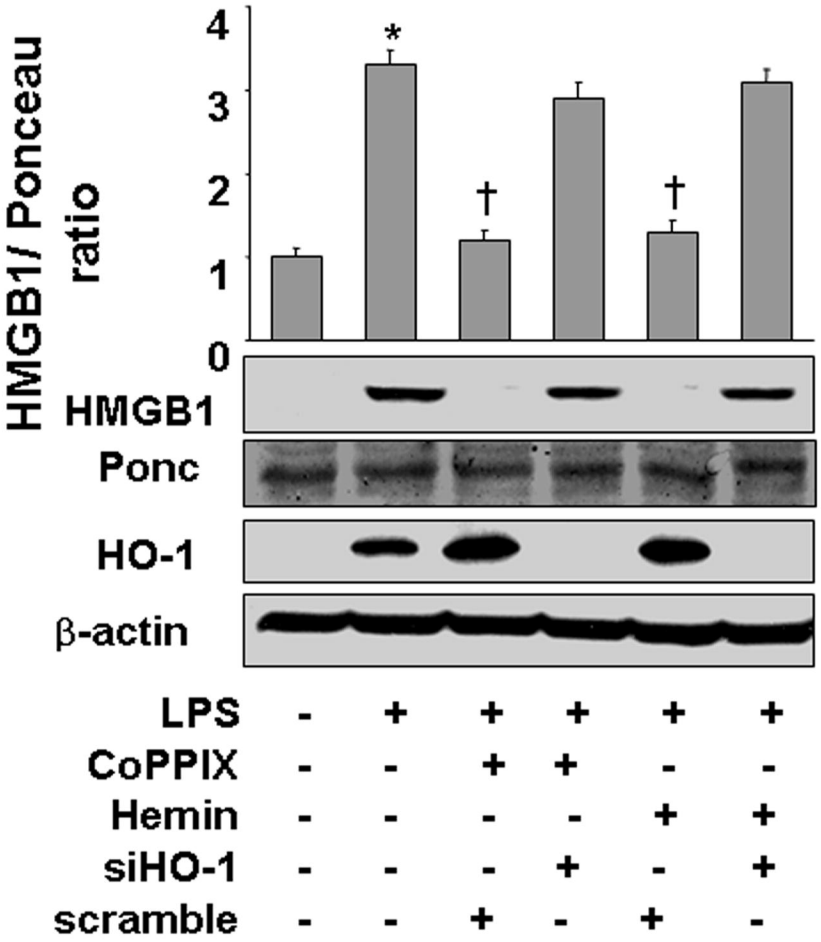
HMGB1
Ponc
LPS - + + + + -
CoPPIX - - 1 5 10 10 μM

Figure 1

D)


HMGB1/ Ponceau ratio
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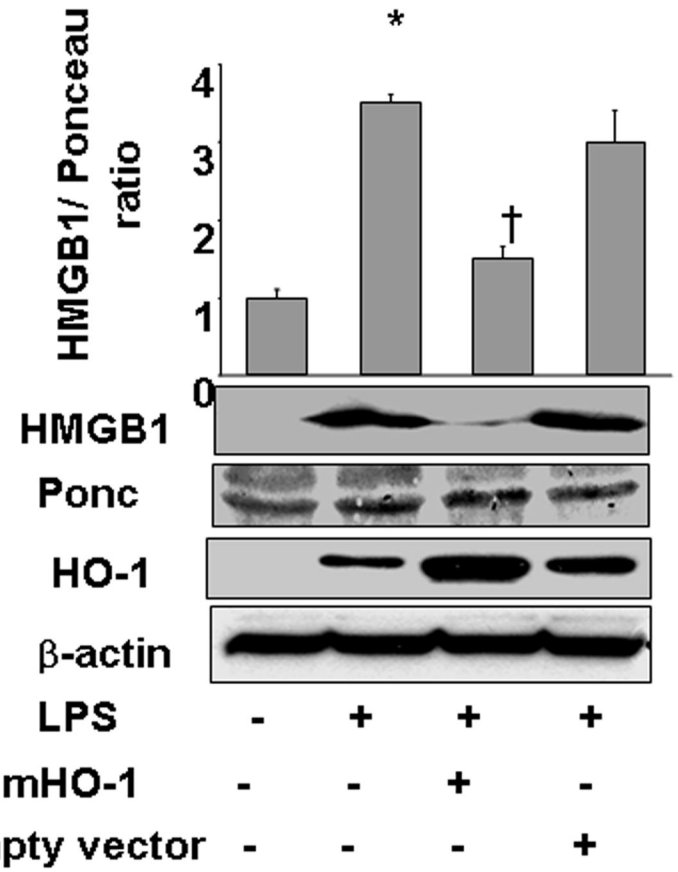
HMGB1
Ponc
HO-1
β-actin
LPS - + + + + +
CoPPIX - - + + - -
Hemin - - - - + +
siHO-1 - - - + - +
scramble - - + - + -

E)


HMGB1/ Ponceau ratio
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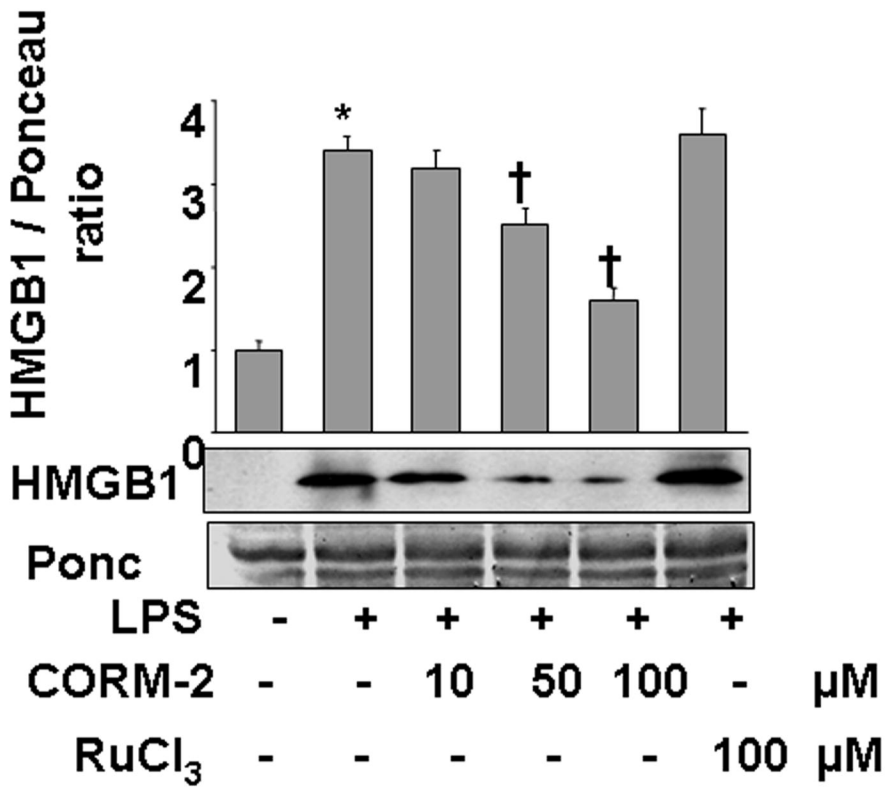
Ponc
HO-1
β-actin
LPS - + + +
mHO-1 - - + -
empty vector - - - +

Figure 1

A)


HMGB1 / Ponceau ratio
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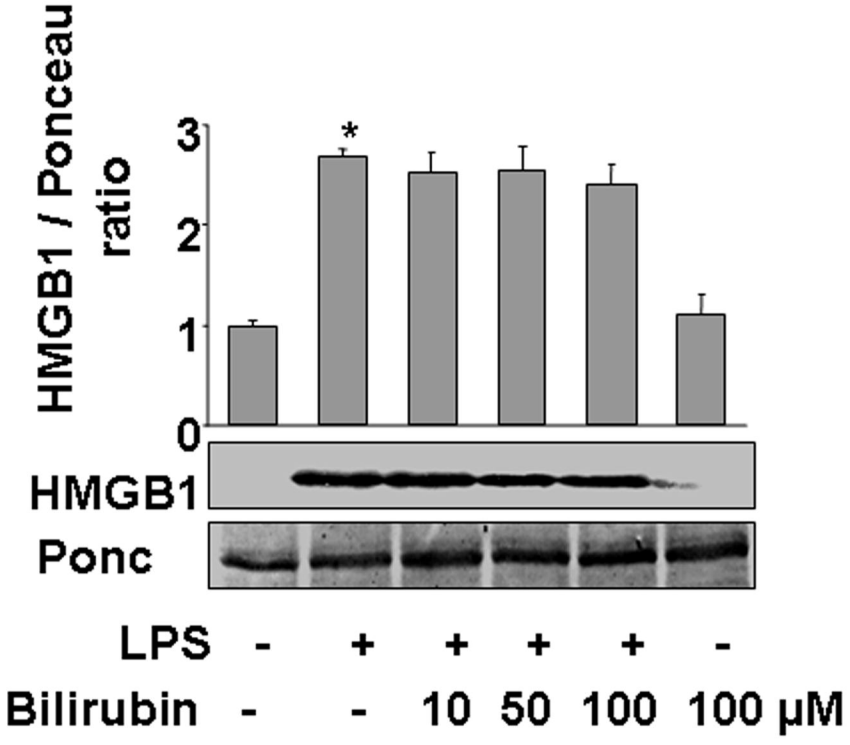
HMGB1
Ponc
LPS - + + + + +
CORM-2 - - 10 50 100 - μM
RuCl3 - - - - - 100 μM

B)


HMGB1 / Ponceau ratio
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*
HMGB1
Ponc
LPS - + + + + -
Bilirubin - - 10 50 100 100 μM

Figure 2

C)

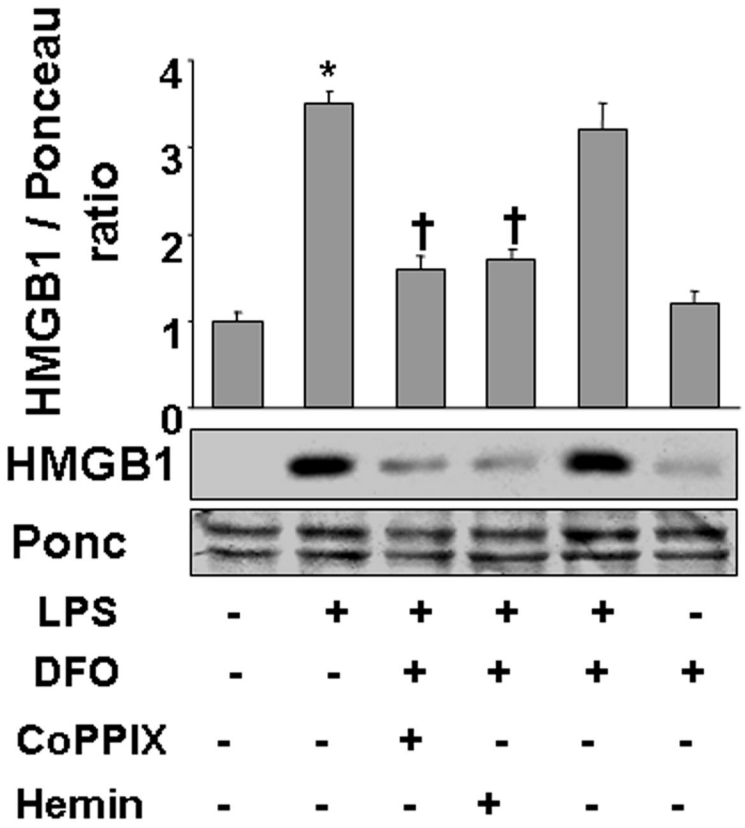


Figure 2

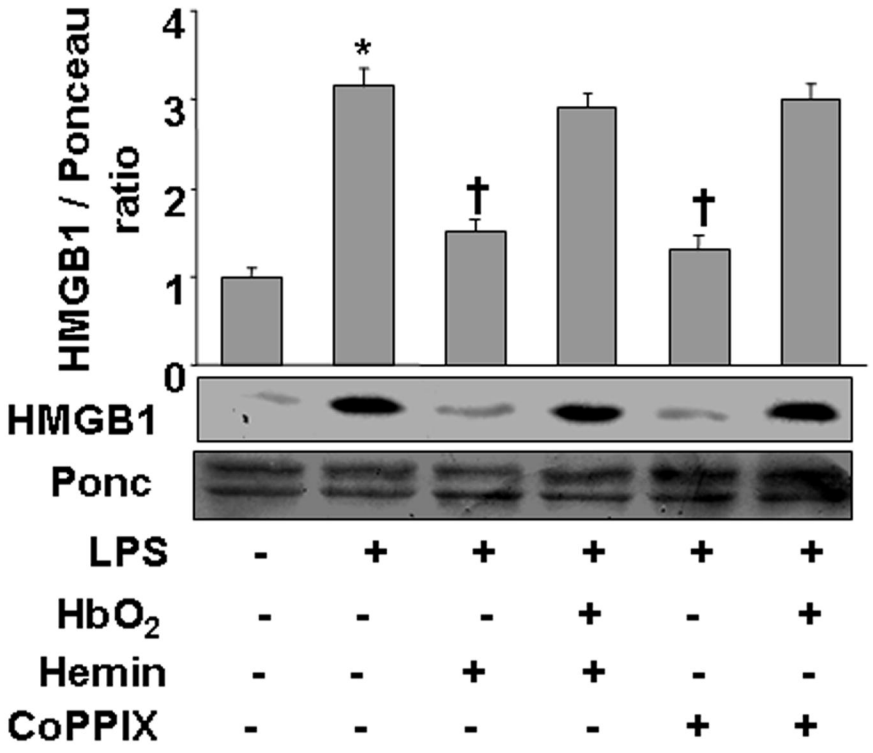


Figure 3

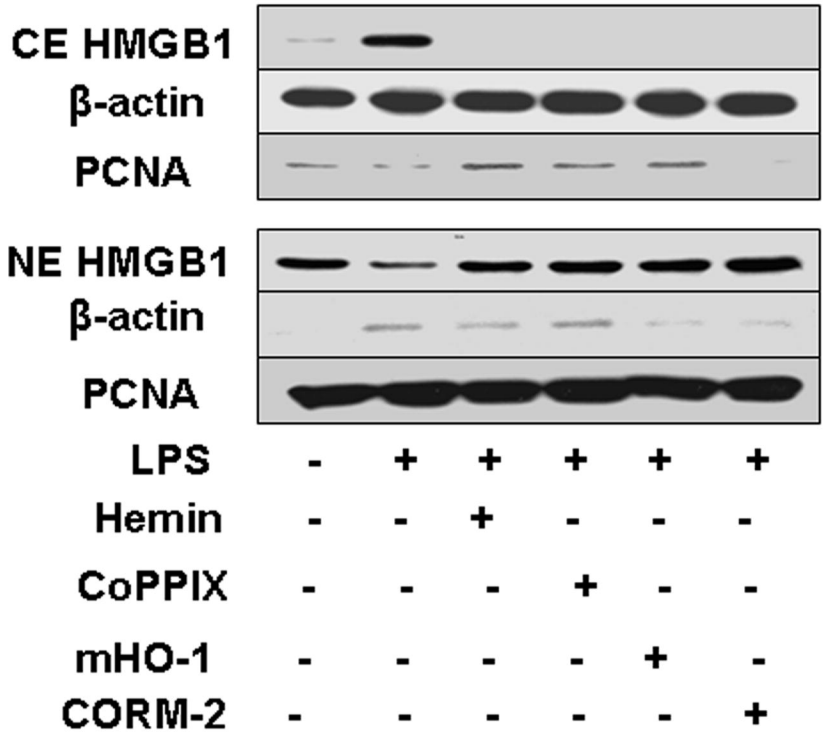

CE HMGB1
β-actin
PCNA
NE HMGB1
β-actin
PCNA
LPS - + + + + +
Hemin - - + - - -
CoPPIX - - - + - -
mHO-1 - - - - + -
CORM-2 - - - - - +

Figure 4

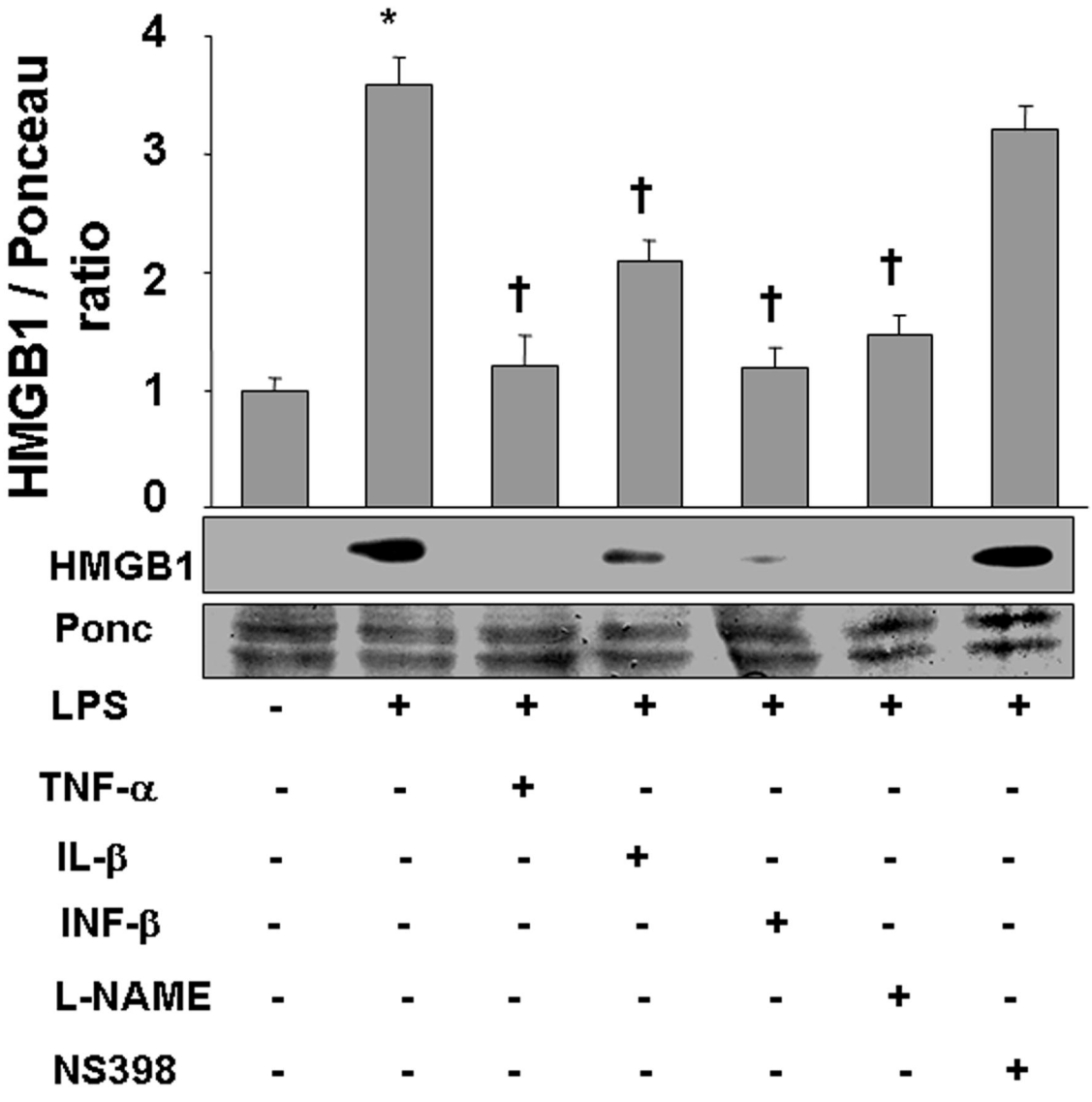
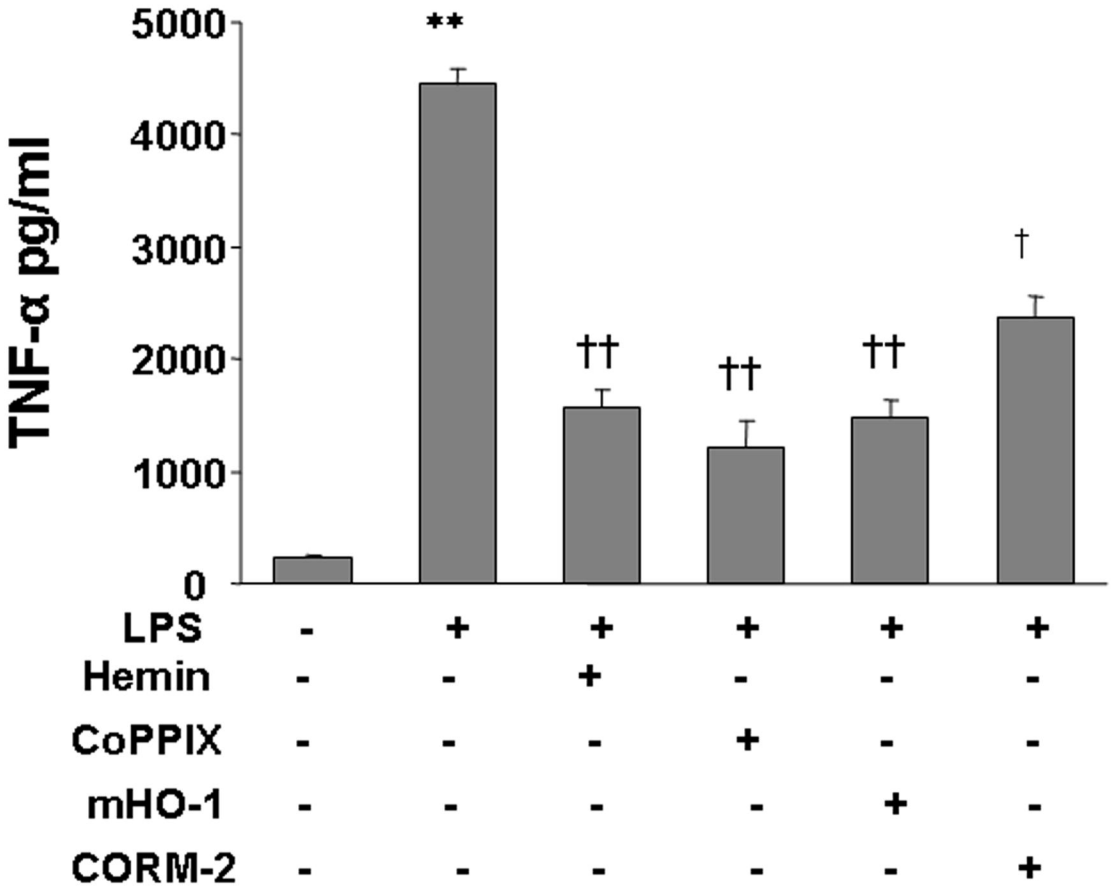

HMGB1 / Ponceau ratio
4
3
2
1
0
*
†
†
†
†
HMGB1
Ponc
LPS - + + + + + +
TNF-α - - + - - - -
IL-β - - - + - - -
INF-β - - - - + - -
L-NAME - - - - - + -
NS398 - - - - - - +

Figure 5

A)


TNF-α pg/ml
5000
4000
3000
2000
1000
0
**
††
††
††
†
LPS - + + + + +
Hemin - - + - - -
CoPPIX - - - + - -
mHO-1 - - - - + -
CORM-2 - - - - - +

B)

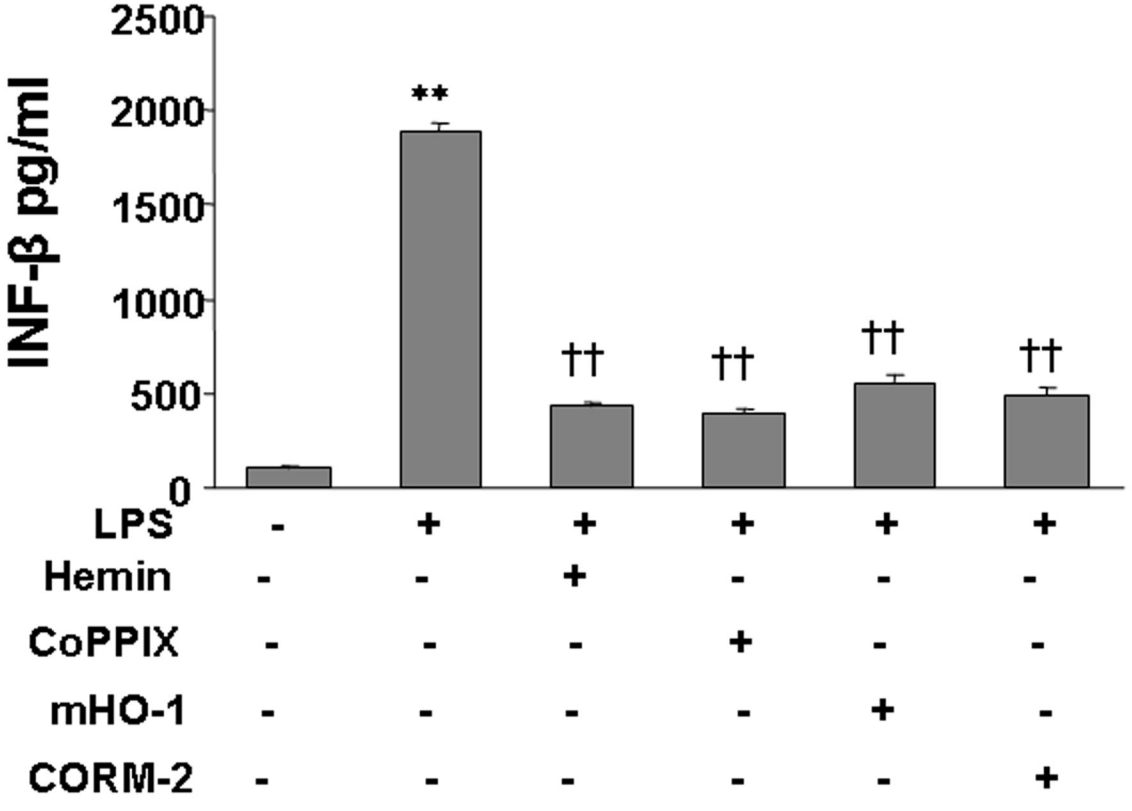
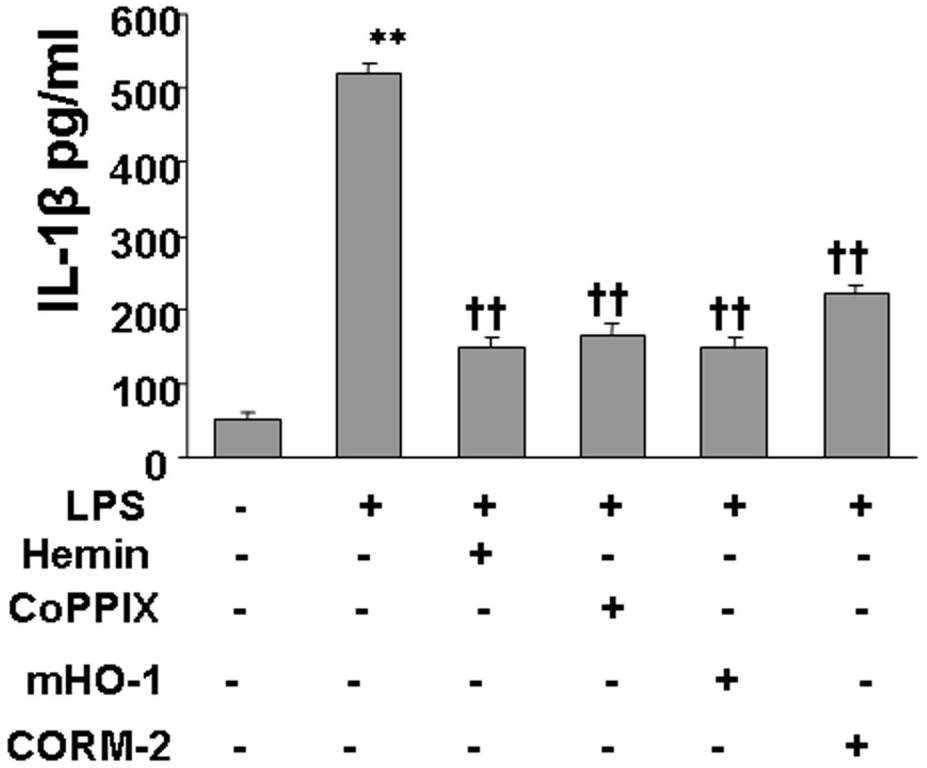

INF-β pg/ml
2500
2000
1500
1000
500
0
**
††
††
††
††
LPS - + + + + +
Hemin - - + - - -
CoPPIX - - - + - -
mHO-1 - - - - + -
CORM-2 - - - - - +

Figure 6

C)


IL-1β pg/ml
600
500
400
300
200
100
0
**
††
††
††
††
LPS - + + + + +
Hemin - - + - - -
CoPPIX - - - + - -
mHO-1 - - - - + -
CORM-2 - - - - - +

D)

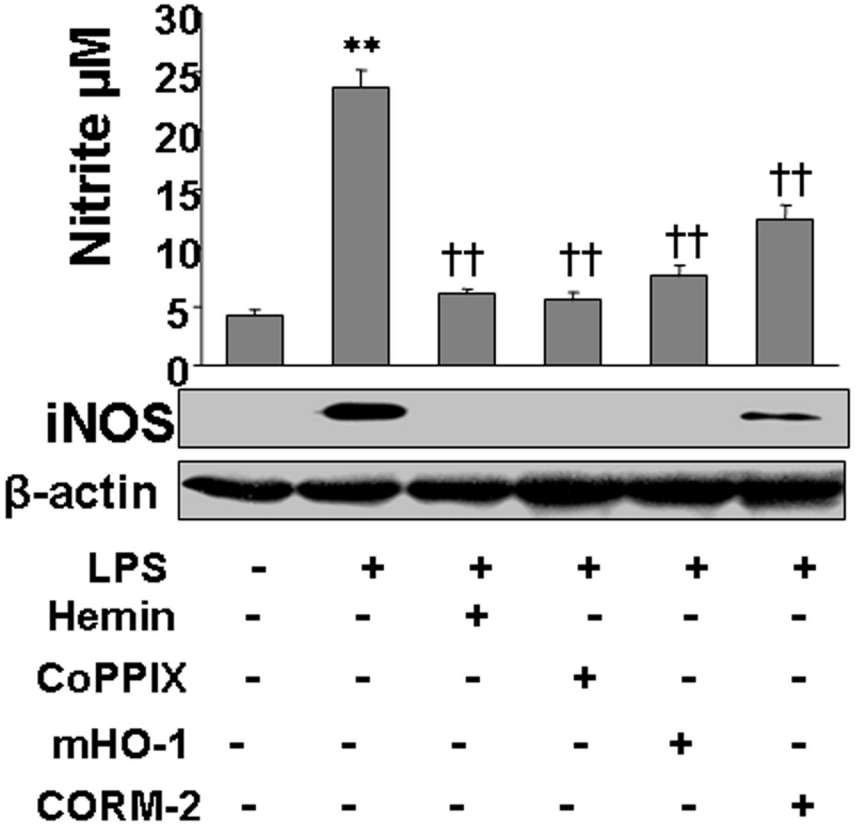
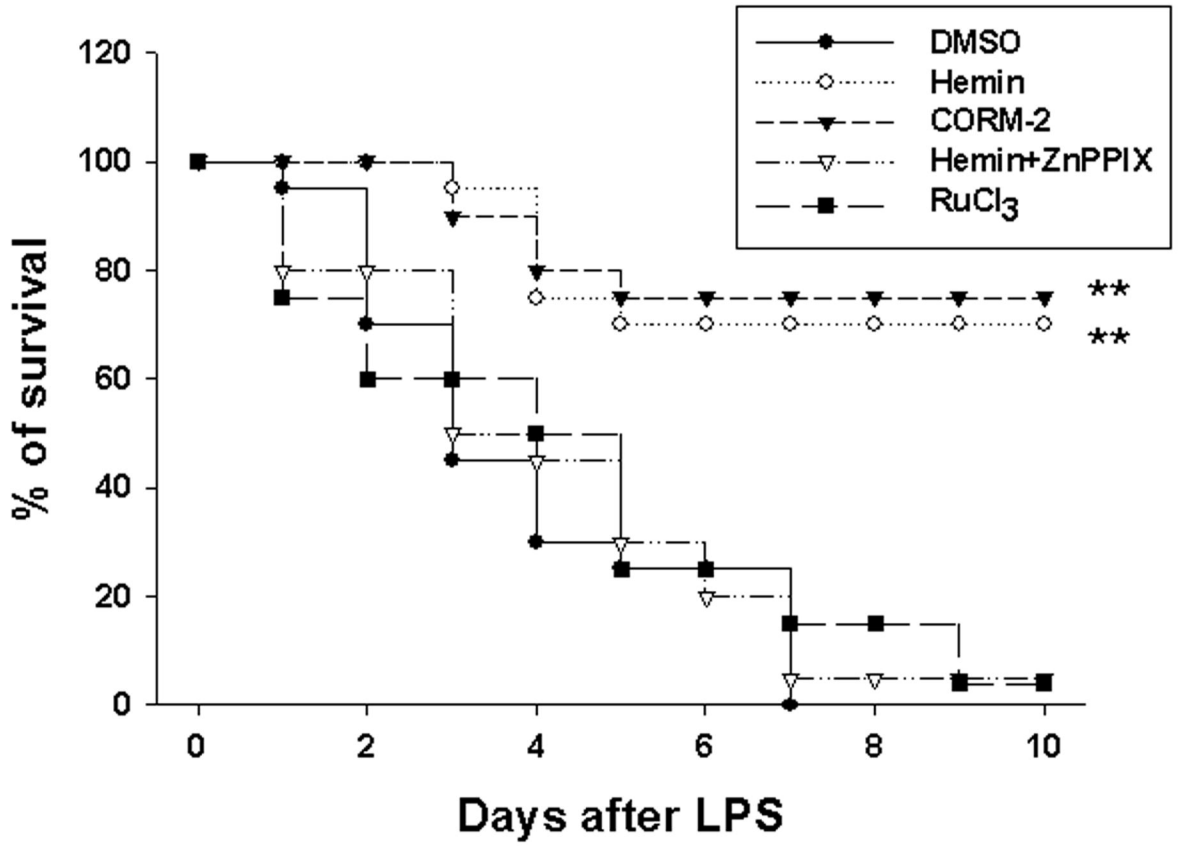

Nitrite μM
30
25
20
15
10
5
0
**
††
††
††
††
iNOS
β-actin
LPS - + + + + +
Hemin - - + - - -
CoPPIX - - - + - -
mHO-1 - - - - + -
CORM-2 - - - - - +

Figure 6

A)


DMSO
Hemin
CORM-2
Hemin+ZnPPIX
RuCl3
% of survival
Days after LPS
**
**

B)

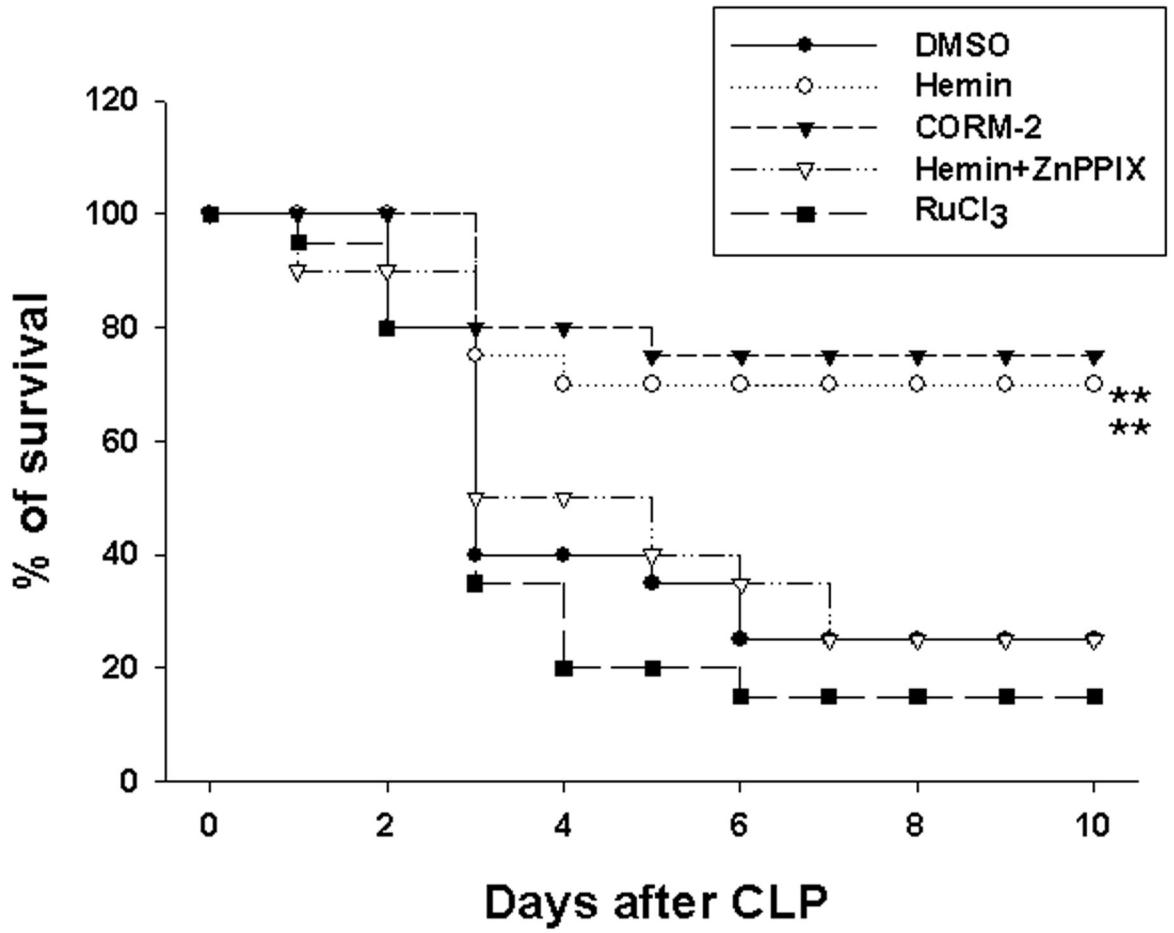
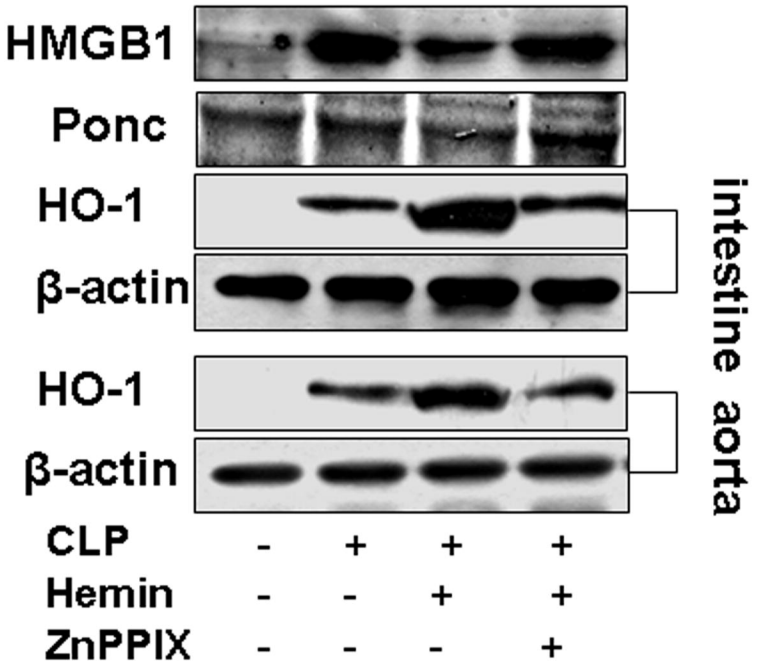

DMSO
Hemin
CORM-2
Hemin+ZnPPIX
RuCl3
% of survival
Days after CLP
**
**

Fig 7

A)


HMGB1
Ponc
HO-1
β-actin
HO-1
β-actin
intestine
aorta
CLP - + + +
Hemin - - + +
ZnPPIX - - - +

B)

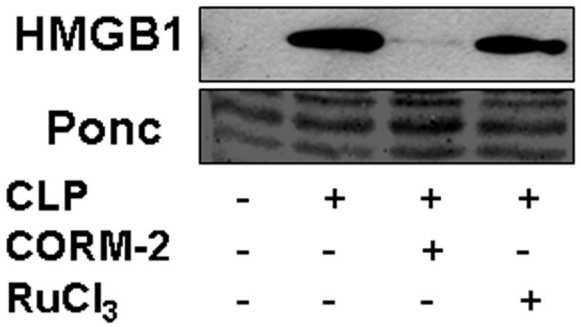
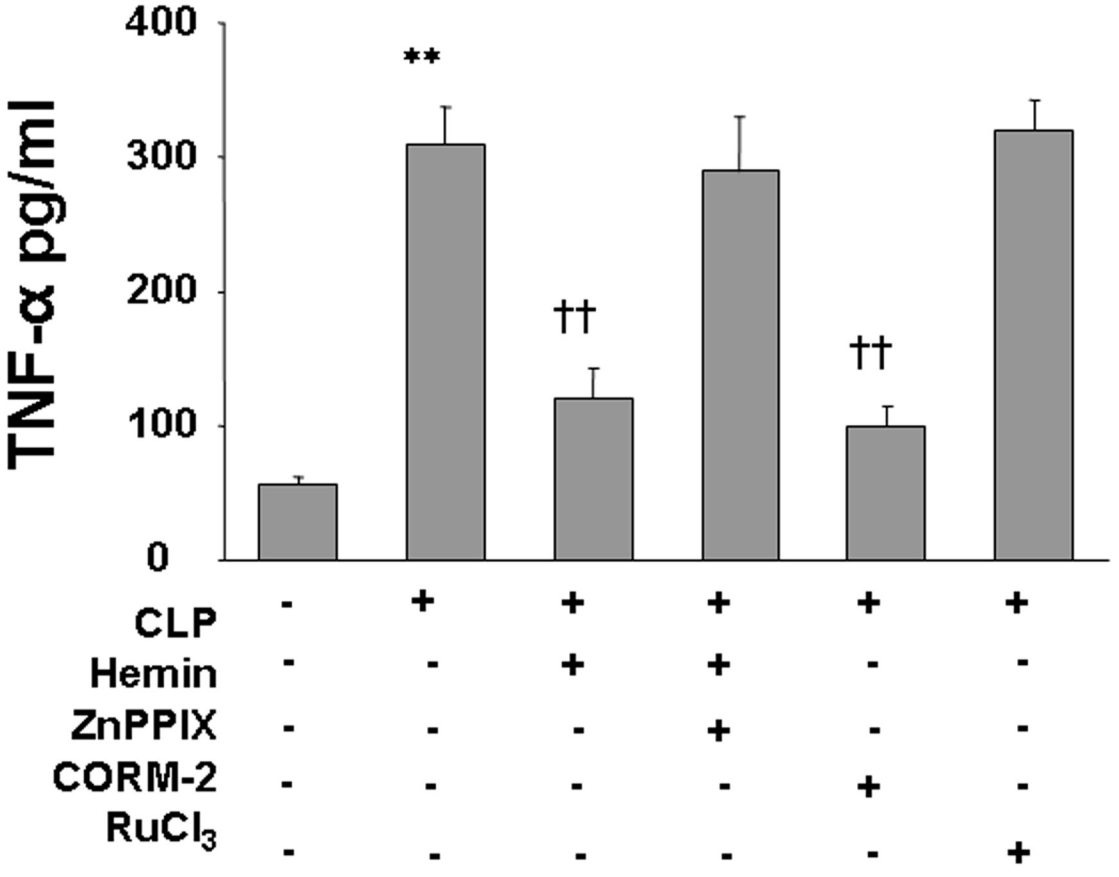

HMGB1
Ponc
CLP - + + +
CORM-2 - - + -
RuCl3 - - - +

Figure 8

C)


TNF-α pg/ml
400
300
200
100
0
**
††
††
CLP - + + + + +
Hemin - - + + - -
ZnPPIX - - - + - -
CORM-2 - - - - + -
RuCl3 - - - - - +

D)

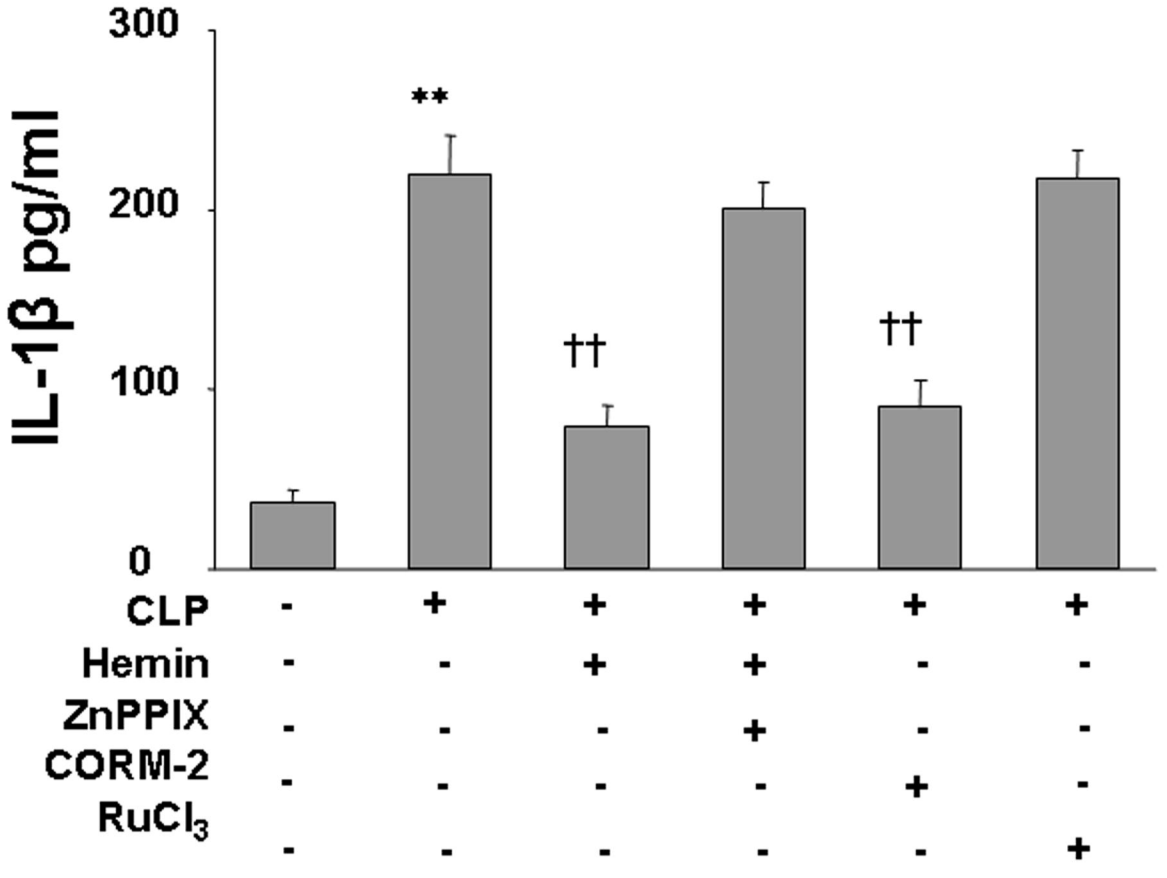
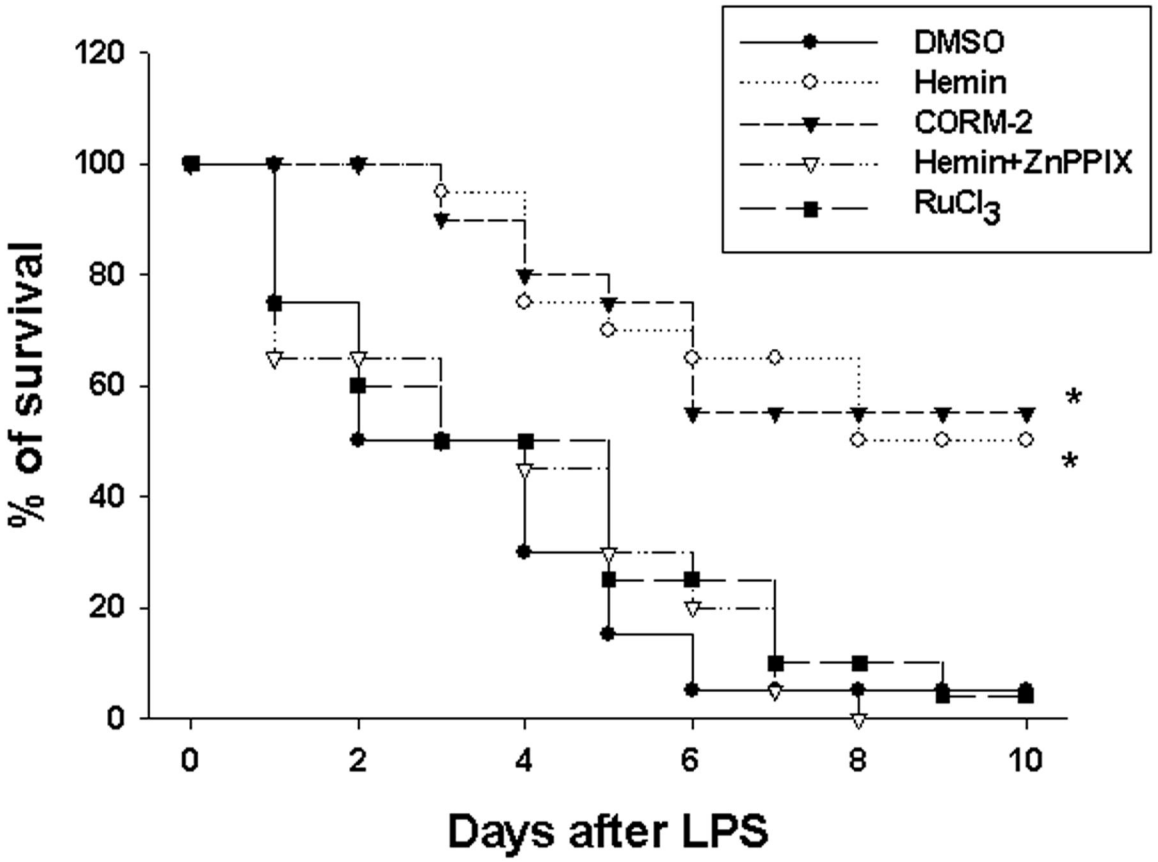

IL-1β pg/ml
300
200
100
0
**
††
††
CLP - + + + + +
Hemin - - + + - -
ZnPPIX - - - + - -
CORM-2 - - - - + -
RuCl3 - - - - - +

Figure 8

A)


DMSO
Hemin
CORM-2
Hemin+ZnPPIX
RuCl3
% of survival
120
100
80
60
40
20
0
0
2
4
6
8
10
Days after LPS
*
*

B)

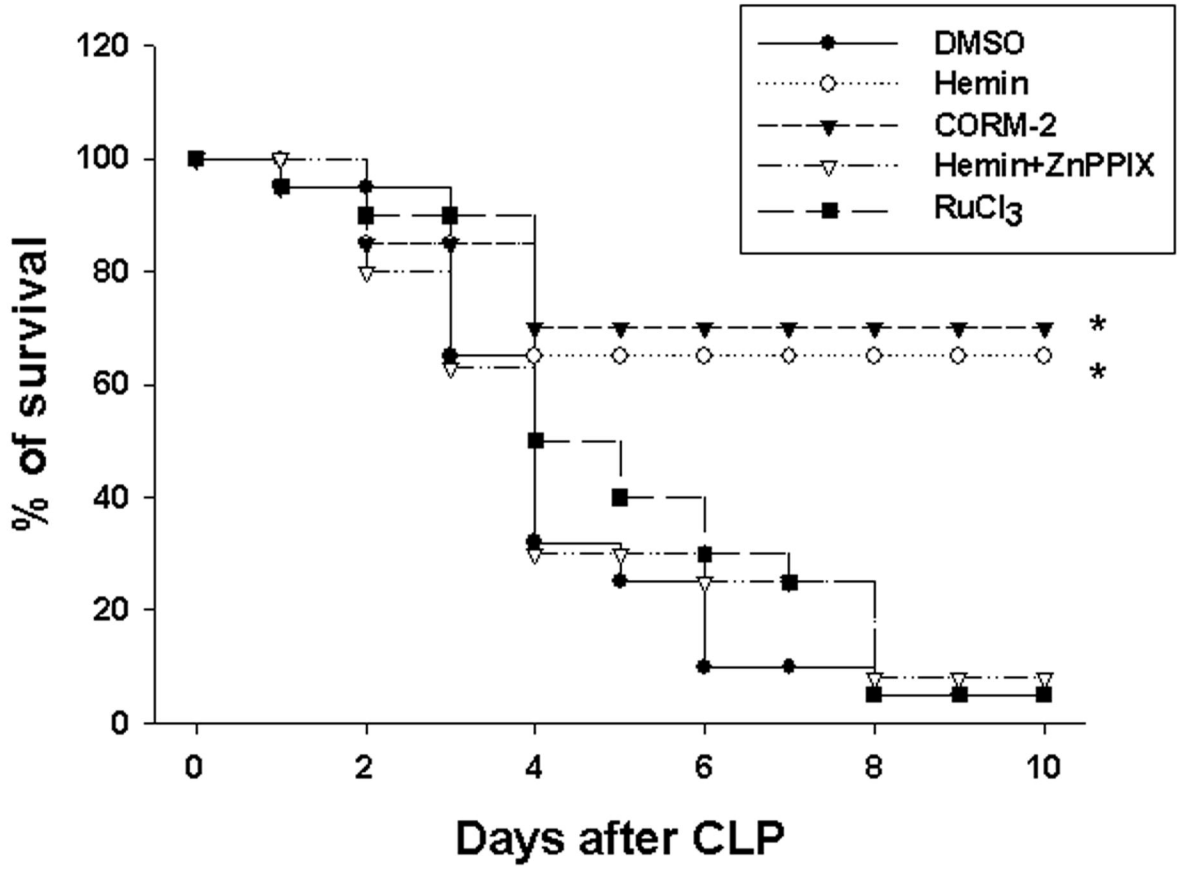
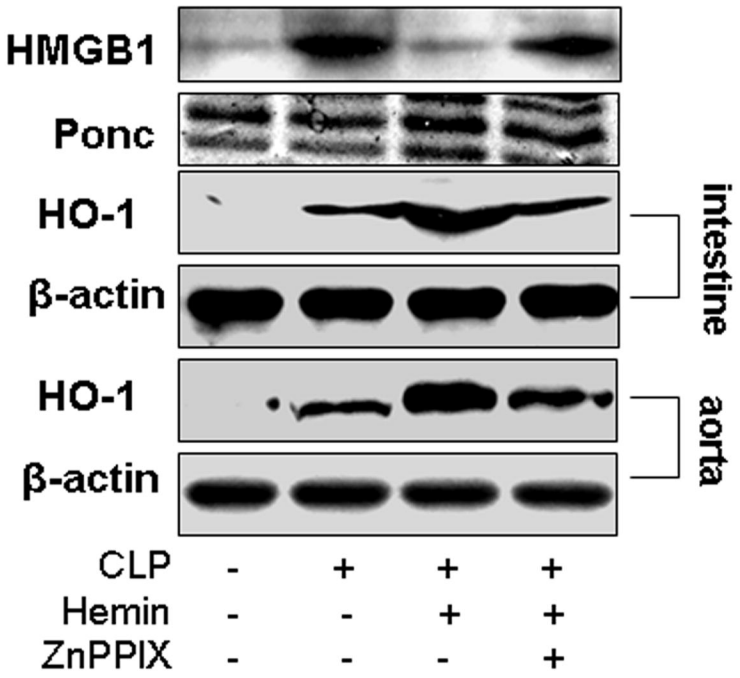

DMSO
Hemin
CORM-2
Hemin+ZnPPIX
RuCl3
% of survival
120
100
80
60
40
20
0
0
2
4
6
8
10
Days after CLP
*
*

Figure 9

A)


HMGB1
Ponc
HO-1
β-actin
intestine
HO-1
β-actin
aorta
CLP - + + +
Hemin - - + +
ZnPPIX - - - +

B)

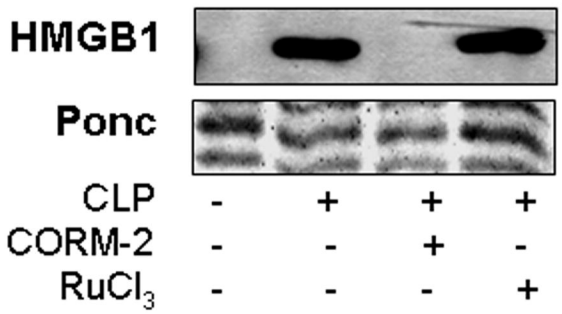
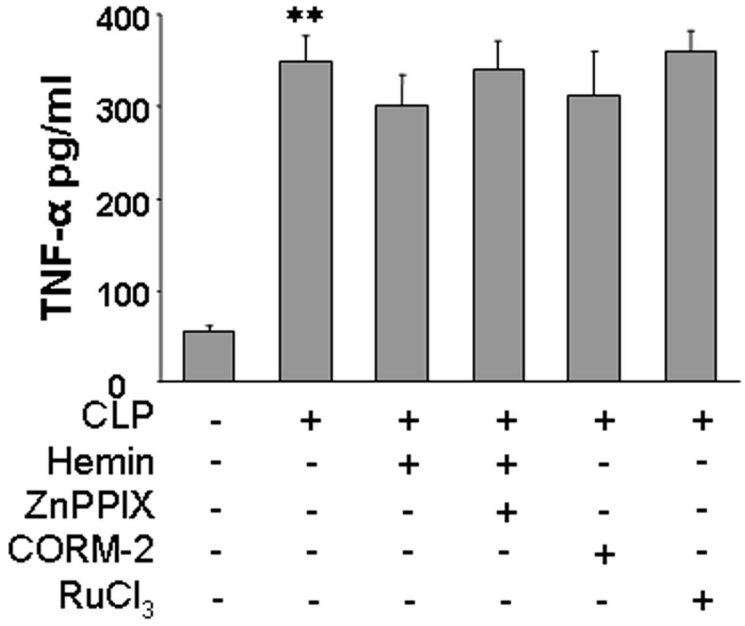

HMGB1
Ponc
CLP - + + +
CORM-2 - - + -
RuCl3 - - - +

Figure 10

C)


TNF-α pg/ml
400
300
200
100
0
**
CLP - + + + + +
Hemin - - + + - -
ZnPPIX - - - + - -
CORM-2 - - - - + -
RuCl3 - - - - - +

D)

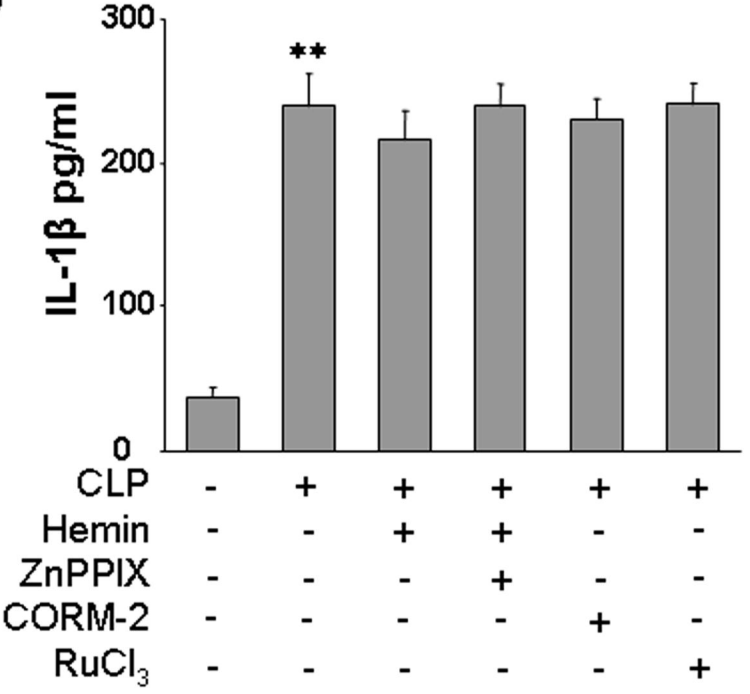

IL-1β pg/ml
300
200
100
0
**
CLP - + + + + +
Hemin - - + + - -
ZnPPIX - - - + - -
CORM-2 - - - - + -
RuCl3 - - - - - +

Figure 10