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β_1-Adrenergic receptor-mediated HO-1 induction, via PI3K and p38 MAPK, by isoproterenol in RAW 264.7 cells leads to inhibition of HMGB1 release in LPS-activated RAW 264.7 cells and increases in survival rate of CLP-induced septic mice

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ABSTRACT

High mobility group box (HMGB)-1 plays an important role in sepsis-associated death in experimental studies. Heme oxygenase-1 (HO-1) inducers were reported to reduce HMGB1 release in experimental sepsis. Previously, we reported on the importance of the β1-adrenergic receptor and protein kinase A pathway in the regulation of HO-1 expression by isoproterenol (ISO) in RAW 264.7 cells. We investigated whether ISO reduces HMGB1 release in LPS-activated RAW 264.7 cells and improves survival rate in septic mice due to HO-1 induction. ISO concentration-dependently increased HO-1 via Nrf-2 translocation and inhibited release of HMGB1 through the β_1-adrenergic receptor (β_1-AR) in LPS-activated RAW 264.7 cells. This conclusion was supported by the finding that dobutamine but not salbutamol increased HO-1 expression in both RAW 264.7 cells. ISO failed to inhibit HMGB1 release when HO-1 expression was suppressed by ZnPPIX, an HO-1 inhibitor in RAW 264.7 cells. ISO significantly inhibited phosphorylation of IκB-α and NF-κB-driven luciferase activity in LPS-activated RAW 264.7 cells. In addition, LY294002, a PI3K inhibitor, and SB203580, a p38 MAPK inhibitor, significantly inhibited not only HO-1 induction but also HMGB1 release by ISO. Importantly, ISO increased HO-1 protein expression in heart and lung tissues, reduced HMGB1 in plasma and increased survival rate in CLP-treated septic mice, which was significantly reversed by co-treatment with ZnPPIX. Taken together, we conclude that inhibition of HMGB1 release during sepsis via β_1-AR-mediated HO-1 induction is a novel mechanism for the beneficial effects of ISO in the treatment of sepsis.

1. Introduction

Sepsis describes a complex clinical syndrome that develops when the initial, appropriate host response to an infection becomes amplified. In Gram-negative bacteria, lipopolysaccharides (LPS) play a dominant role in the process of sepsis. Macrophages are among the first cells in the host to confront microbes and are important effector cells in the body's innate resistance to intracellular microbial pathogens. It has been reported that catecholamines and acetylcholine are potent regulators of peripheral immune functions [1–4]. The catecholamines are among the most important stress hormone regulating macrophage function. Cells in the monocyte/macrophage lineage express G-protein coupled catecholamine receptors on the cell surface and respond to receptor occupation by selective ligands by altering such diverse activities as cytokine production [5–7]. Heme oxygenase (HO) catalyses the rate-limiting step in the oxidative degradation of heme (a potent oxidant) to biliverdin (rapidly converted to bilirubin, an anti-oxidant), iron (sequestered by ferritin), and carbon monoxide (CO, a vasodilatory gas that has anti-inflammatory properties) [8]. A growing body of evidence suggests that high mobility group box 1 (HMGB1) plays a critical role in organ failure in septic animals and in humans [9,10].

HMGB1, a ubiquitous nuclear protein that binds DNA and participates in the maintenance of chromatin structure, is released into the extracellular environment by necrotic cells or by activated leukocytes where it functions as a late-acting cytokine mediator of

Abbreviations: β-AR, beta adrenergic receptor; CLP, cecal ligation and puncture; CO, carbon monoxide; HMGB1, high mobility group box 1; HO-1, heme oxygenase-1; ISO, isoproterenol; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinase; Nrf2, nuclear factor-erythroid 2-related factor 2; PI3K, phosphoinositol-3-kinase.

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lethal organ damage during sepsis [11–13]. While apoptotic cells do not release HMGB1 [14], the excessive accumulation of apoptotic cells during sepsis stimulates HMGB1 release from macrophages [15]. HMGB1 is, therefore, representative of a novel family of inflammatory cytokines composed of intracellular proteins that when present in the extracellular milieu, are recognized by the innate immune system as signals of tissue damage [11,16]. Accordingly, therapeutic strategies to neutralize the activity of HMGB1 or to prevent its release from macrophages are currently under investigation for the treatment of severe sepsis.

Previously we reported that isoproterenol (ISO) induces HO-1 protein by increasing cAMP levels in RAW 264.7 cells via activation of β_1-adrenergic receptors (β_1-AR) [17]. We extended our study to get new insight on the anti-inflammatory role of ISO in sepsis, because ISO has been reported to benefit animals under septic conditions [18,19] and to improve hemodynamics and oxygen derived variables in septic shock patients [20]. Although adenylate cyclase activation and the resulting increase in cAMP levels are regarded as important actions of ISO, no report is available for the modulation of HMGB1 release by this drug during sepsis. Thus, the aim of the present study is to test our hypothesis that ISO reduces HMGB1 release by induction of HO-1 and thereby may be beneficial in septic conditions. Here, we provide evidence that ISO indeed reduced HMGB1 release through the β_1-AR in LPS-activated RAW 264.7 cells and increased survival rate in cecal ligation and puncture (CLP)-induced septic mice by induction of HO-1.

2. Materials and methods

2.1. Materials

ISO was purchased from Sigma–Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin) were purchased from Gibco BRL (Rockville, MD). Anti-HMGB1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-HO-1, anti-Nrf2 and anti-PCNA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin was purchased from Sigma–Aldrich (St. Louis, MO). Horseradish peroxidase labeled goat anti-rabbit IgG and donkey anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SB203580 was purchased from Calbiochem (San Diego, CA) and LY294002 was purchased from Sigma–Aldrich (St. Louis, MO). Enhanced chemiluminescence (ECL) and Western blotting detection reagent were from Amersham (Buckinghamshire, UK). LPS (*Escherichia coli* serotype 0128:B12) was purchased from Sigma–Aldrich (St. Louis, MO). Nrf2 siRNA and control siRNA were purchased from Invitrogen (Calsbad, CA).

2.2. Cell culture and stimulation

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 mM N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated FBS. Cells from passage 4–10 were used for the experiments. RAW 264.7 cells were plated at a density of 1×10^7 cells per 100 mm dish. The cells were rinsed with fresh medium and stimulated with LPS (1 μg/ml) in the presence of different concentration of ISO (50, 100 and 200 μM). ISO was dissolved in sterile distilled water and sterilized via a 0.2 μm filter.

2.3. Western blot analysis

The cytoplasmic/nuclear fractionation was performed using the nuclear/cytosol fractionation kit (Cat # K266-25, BioVision, Mountain view, CA) according to manufacturer's instructions. Lysis buffer contained 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 (HMGB1), whole cell lysates (HO-1, β-actin), and nuclear- and cytosol-lysates (Nrf-2) were subjected to polyacrylamide gel electrophoresis (PAGE); the percent composition of the gels was varied depending on the size of the protein of interest. Cells were lysed in PRO-PREP protein extraction solution. The sample was centrifuged at 13,000 × *g* for 5 min at 4 °C. Protein concentrations were determined by the Bradford method. An equal volume of 2× sample buffer was added to aliquots of the sample supernatant and the mix was boiled for 5 min. Thirty micrograms of protein were loaded per lane and resolved by 10% SDS-PAGE for 1 h 30 min at 30 mA. The separated proteins were transferred to PVDF membranes (Millipore) for 1 h at 100 V with a SD Semi-dry Transfer Cell (Bio-Rad). The membranes were blocked with 5% skim milk in 1× PBS containing 0.05% Tween 20 (PBS-T) for 1 h at room temperature. The membranes were then incubated with antibodies against HO-1, HMGB1, Nrf-2 or β-actin. Proteins were detected with a horseradish peroxidase-coupled secondary antibody by means of the ECL system.

2.4. Assay for HO enzyme activity

HO enzyme activity was measured by the method previously described [21]. Briefly, microsomes from harvested cells were added to a reaction mixture containing NADPH, rat liver cytosol as a source of biliverdin reductase, and the substrate hemin. The reaction was carried out in the dark for 1 h at 37 °C and terminated by the addition of 1 ml chloroform, and bilirubin extracted was calculated by the difference in absorbance between 464 and 530 nm.

2.5. Nuclear factor-erythroid 2-related factor 2 (Nrf-2) luciferase activity

RAW264.7 cells were plated on 6-well plates at a density of 2×10^5 cells/ml and incubated overnight. Triplicate samples of the luciferase reporter plasmid construct containing ARE promoter (2 μg) and the β-galactosidase expression vector plasmid (10 μl) were co-transfected using transfection reagents (Qiagen, Hilden, Germany). pSV-β-galactosidase was used to correct for the transfection efficiency. After 2 h of treatment, the luciferase activity was determined according to the manufacturer's instructions (Promega, Madison, WI). Briefly, cells were washed with cold PBS and harvested in passive lysis buffer. After centrifugation, 20 μl of the supernatant was used for determining the luciferase activity, which was measured by a luminometer (AutoLumat LB 953, EG&G Berthold, Bad Widbad, Germany).

2.6. Nrf-2 small interfering RNA transfection study

RAW264.7 cells were transfected with 300 nM control siRNA or Nrf2 siRNA using transfection reagents (Qiagen, Valencia, CA) according to the manufacturer's instructions. The cells were incubated for 24 h in serum-free media. The transfected cells were washed with 4 ml of PBS and pretreated with or without ISO, following LPS stimulation and subjected to Western blot analysis.

2.7. HMGB1 analysis

Analysis of HMGB1 was carried out as described previously [22]. In brief, culture medium samples were briefly centrifuged. Equal volumes of the samples were then concentrated 40-fold with

Amicon Ultra-4-10000 NMWL (Millipore, Billerica, MA). Samples were centrifuged at 7500 × *g* for 20 min at 4 °C with the aid of a fixed angle (35°) rotor. Blood was collected by cardiac puncture into a tube containing sodium heparin. After centrifugation, plasma samples were filtered and concentrated by means of Centricon YM-100 and YM-10 (Millipore, Billerica, MA), respectively. The concentrated samples were subjected to SDS-PAGE electrophoresis. Ponceau S staining was used as loading control.

2.8. Survival rate measurement in a mouse model of CLP-induced sepsis

The CLP-induced sepsis model was implemented as described previously [22]. In brief, BALB/c mice (male, 7–8 weeks old, 20–25 g) were anesthetized with ketamine (30 mg/kg) and xylazine (6 mg/kg). Next, a 2-cm midline incision was made to allow exposure of the cecum and adjoining intestine. The cecum was tightly ligated with a 3.0-silk suture at its base, below the ileo-cecal valve, and was punctured through both cecal walls with an 18-gauge needle twice (top and bottom). 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). The protocol was approved in advance by the Animal Research Committee of Gyeongsang National University. Mice were subjected to CLP ($n = 14$) and treated right after surgery with ISO (*i.p.*, 10 mg/kg, $n = 14$) or ZnPPIX (*i.p.*, 10 mg/kg) plus ISO ($n = 14$) with repeated injections 12, 24, and 48 h after the onset of sepsis. Survival was monitored daily for up to 10 days.

2.9. Plasma HMGB1 level and HO-1 expression in CLP-induced septic mice

For measurement of plasma HMGB1 and tissue HO-1 expression, BALB/c mice (male, 7–8 weeks old, 20–25 g) were subjected to CLP additionally ($n = 5$) with or without further treatment with ISO (*i.p.*, 10 mg/kg, $n = 6$,) or ISO plus ZnPPIX (*i.p.*, 10 mg/kg, $n = 6$). Twenty four hours after completion of the CLP operation, blood for HMGB1 analysis was withdrawn by cardiac puncture from animals in each group anesthetized with ketamine (30 mg/kg) and xylazine (6 mg/kg); tissues such as heart, lung, liver and kidney were collected and subjected to Western blot analysis for HO-1 as described above.

2.10. Statistical analysis

Data are expressed as the mean ± SEM along with the number of replicate treatments. Differences between data sets were assessed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls *post hoc* tests. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. ISO induces HO-1 protein expression via Nrf-2 translocation

Previously, we reported that ISO induces HO-1 protein expression through the β_1-AR in RAW 264.7 cells [17]. As shown in Fig. 1a and b, we confirmed that ISO increased HO-1 protein expression in a time- and concentration-dependent manner in RAW 264.7 cells. Fig. 1c shows that ISO significantly and concentration-dependently translocated Nrf-2 from cytosol to nucleus as required to initiate HO-1 induction. Furthermore, ISO failed to induce HO-1 protein expression in Nrf-2 siRNA-

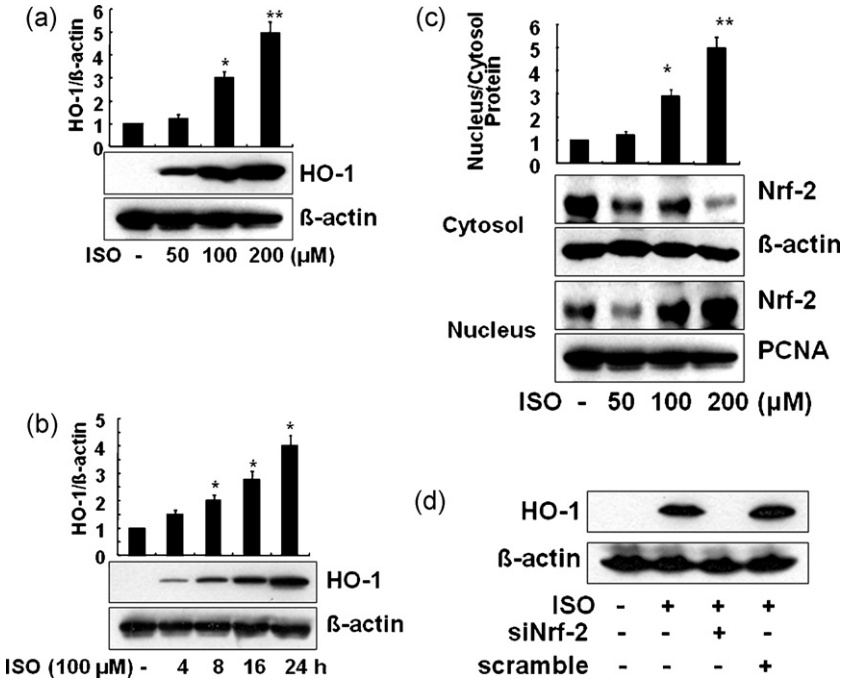

Fig. 1. Isoproterenol induces HO-1 in a concentration and time-dependent manner via Nrf-2 activation. RAW 264.7 cells were treated with isoproterenol (ISO) for 8 h at different concentrations (a). Cells were incubated for the indicated period of time with a fixed dose of ISO (100 μM) (b), and then Western blot analysis was performed. To determine whether Nrf-2 translocation is involved in HO-1 induction by isoproterenol, cytosol and nuclear fraction were separated after 3 h of treatment with the indicated concentrations of isoproterenol. Western blot analysis was performed with anti-Nrf-2 antibody (c). Using Nrf-2 siRNA-transfected cells, HO-1 induction was investigated in the presence and absence of ISO (d). Representative blot shown is from one of the three independent experiments with similar results. Data are expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$; compared to the control.

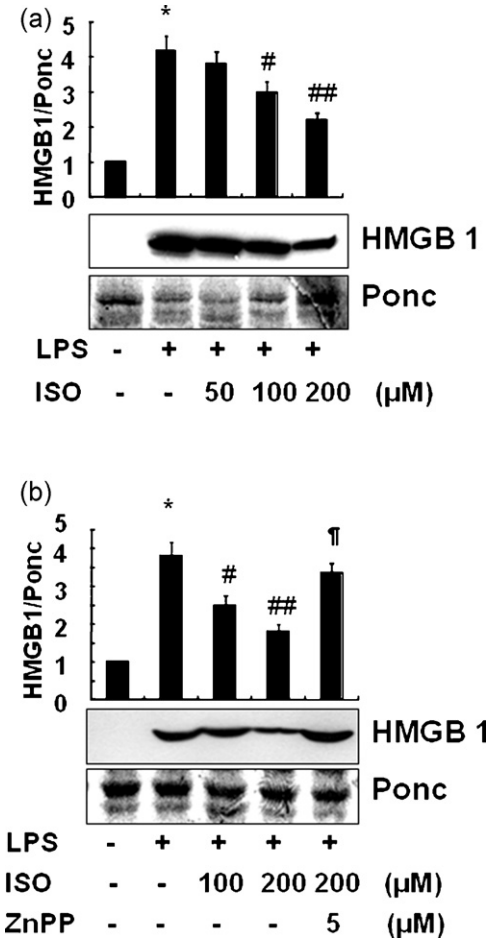

Fig. 2. Isoproterenol reduces HMGB1 release in LPS-activated RAW 264.7 cells by HO-1 induction. RAW 264.7 cells were pretreated with the indicated concentrations of isoproterenol (ISO) 1 h prior to addition of 1 μg/ml LPS. After a 16-h incubation period, detection of HMGB1 released into the medium was performed by Western blot analysis (a). Ponceau S (ponc) band was used as a loading control. To understand whether the reduced HMGB1 release is due to increased HO-1 activity in the presence of ISO, ZnPPIX (5 μM), an HO-1 inhibitor, was coadministered with ISO and HMGB1 detected after 16 h of incubation with LPS (b). Representative blot shown is from one of the three independent experiments with similar results. Data are given as mean ± SEM of three independent experiments. $^{*}p < 0.05$, *vs.* control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to LPS; $^{\P}p < 0.05$, compared to isoproterenol (200 μM).

transfected cells (Fig. 1d), indicating that Nrf-2 is an important regulator of HO-1induction by ISO.

3.2. ISO reduces HMGB1 release in LPS-activated RAW 264.7 cells due to HO-1 induction

Because HO-1 induction has been reported to prevent the release of HMGB1 in endotoxin-activated macrophages *in vitro* and septic animals *in vivo* [22], we addressed whether ISO reduces HMGB1 release in LPS-activated RAW 264.7 cells. As shown in Fig. 2a, LPS increased HMGB1 release in the media after 16 h in RAW 264.7 cells, whereas pretreatment with ISO (50–200 μM) significantly and concentration-dependently reduced the release of HMGB1. To verify that this reduction is associated with HO-1 activity, ZnPPIX, an HO-1 inhibitor, was used. ZnPPIX reversed the reduction in HMGB1 release caused by ISO (Fig. 2b).

3.3. Involvement of PI3Kinase and p38 MAPK pathways in the action of ISO

Next, we asked what signals are involved in HO-1 induction by ISO. Fig. 3a and b shows that the PI3K inhibitor (LY294002) and the p38 MAPK inhibitor (SB203580) significantly reduced HO-1 protein expression induced by ISO in RAW 264.7 cells. Thus, we addressed whether these kinase inhibitors block ISO action on HMGB1 release due to inhibition of HO-1 induction. As expected, the reduced HMGB1 release by ISO was significantly reversed by the presence of LY294002 and SB2030580 in LPS-activated RAW 264.7 cells (Fig. 3c and d). Moreover, the increased Nrf-2 luciferase activity produced by ISO was significantly inhibited by LY294002 and SB203580 (Fig. 3e), confirming that these kinases play key roles in the induction of HO-1 by ISO.

3.4. Adrenergic β_1- but not β_2-receptor is responsible for induction of HO-1

Although we reported that the β_1-AR is responsible for HO-1 induction in RAW264.7 cells [17], β_2-AR was also reported to induce HO-1 in vascular smooth muscle cells [23]. We therefore wanted to confirm our previous finding that the β_1-AR is responsible for HO-1 induction. As shown in Fig. 4a, we confirmed that LPS induced HO-1 expression in macrophages [24], whereas addition of ISO further increased HO-1 expression, which was significantly reduced by propranolol, a non selective β-AR antagonist and metoprolol, a selective β_1-AR antagonist. In contrast, butoxamine, a β_2-AR antagonist, did not reduce HO-1 expression induced by ISO. Next, we reexamined the assumption that induction of HO-1 relies solely on β_1-AR activation. As shown in Fig. 4b, the reduced HMGB1 release caused by ISO was reversed by propranolol and metoprolol but not butoxamine. To further confirm this result, HO-1 expression was investigated using dobutamine, a β_1-selective AR agonist, and salbutamol, a β_2-selective AR agonist. Dobutamine, in a concentration-dependent manner, increased HO-1 expression in RAW 264.7 cells, but salbutamol did not (Fig. 4c). The same results were obtained in THP-1, a human macrophage cell lines (data not shown), suggesting that activation of the β_1-AR induces HO-1 in macrophages of both animal and human origin. We also demonstrated that LPS-increased HO-1 expression and activity were further significantly increased by ISO (Fig. 4d and e).

3.5. ISO concentration-dependently inhibits phosphorylation of IκB and NF-κB activity

Because activation of NF-κB is critical for induction of inflammatory cytokines such as TNF-α, IL-1β, NO, and release of HMGB1 in macrophages [25], we examined the possibility that ISO inhibits NF-κB activity. As shown in Fig. 5a and b, ISO concentration-dependently inhibited phosphorylation of IκB-α and NF-κB-driven luciferase activity in LPS-activated macrophages, respectively, and the latter was reversed by the presence of ZnPPIX.

3.6. Administration of ISO significantly increases survival rate in CLP-induced septic mice and increases HO-1expression in lung and heart tissues in LPS-injected mice

To investigate the *in vitro* relevance of the *in vivo* results, we measured survival rates in CLP-induced septic mice. As shown in Fig. 6a and b, ISO clearly improved survival rates, reduced plasma HMGB1, and increased HO-1 expression in heart as well as lung tissues, all of which were significantly reversed by the presence of ZnPPIX.

4. Discussion

This study clearly demonstrates a novel anti-inflammatory action of ISO that it can inhibit HMGB1 release by induction of HO-

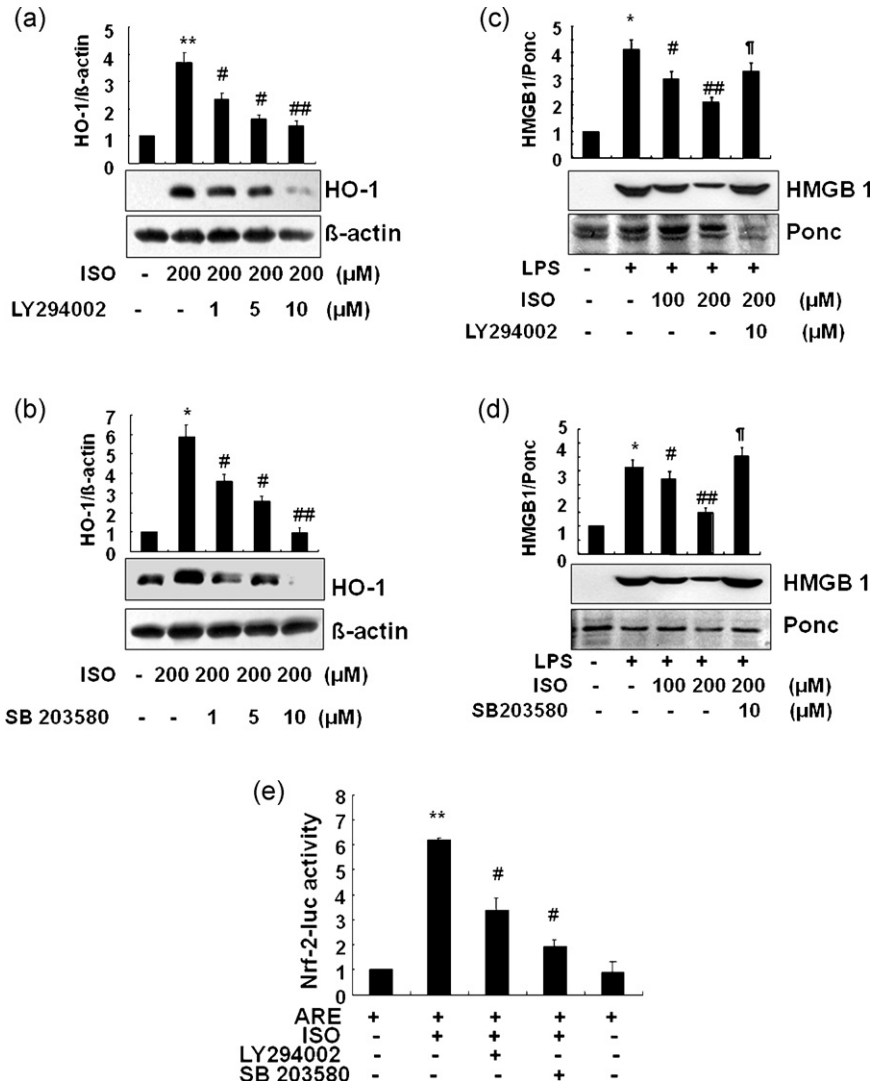

Fig. 3. Isoproterenol induces HO-1 expression via a PI3K- and p38 MAPK-dependent pathway. RAW 264.7 cells were pretreated 30 min prior to addition of isoproterenol (ISO, 200 μM) with different concentrations of LY294002, a PI3K inhibitor (a) or SB203580, a p38 MAPK inhibitor (b) to identify the signaling pathway involved in HO-1 induction. HO-1 protein was detected after 8 h of incubation with ISO. To confirm that these signaling molecules are involved in inhibition of HMGB1 release (c and d) due to HO-1 induction, RAW 264.7 cells were pretreated with the indicated concentrations of ISO 1 h prior to addition of 1 μg/ml LPS. LY294002 (10 μM) or SB 203580 (10 μM) was administered 30 min prior to treatment with ISO. Cells were incubated for 16 h after LPS addition for the purpose of detecting HMGB1. To further confirm that ISO-induced HO-1 expression is related to Nrf-2, which is also sensitive to PI3K and p38 MAPK, Nrf-2 luciferase activity was measured in the presence or absence of LY294002 or SB 203580 (e). Cells transfected with the Nrf-2 luciferase gene were incubated for 1 h with ISO (200 μM) with or without the inhibitors (each at 10 μM). Intensity is represented as fold increase in activity. Representative blot shown is from one of the three independent experiments with similar results. Data are given as mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to corresponding control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to corresponding controls, $^{¶}p < 0.05$, compared to isoproterenol (200 μM) group.

1 expression through the β_1-AR in LPS-activated macrophages and increased survival rate in CLP-induced septic mice. It has been reported that β-AR stimulation by ISO improved hepatosplanchnic perfusion and enhanced tissue oxygen extraction in *E. coli* endotoxin-injected endotoxemic animals [26] and also improved hemodynamics and oxygen derived variables in septic shock patients [20]. In addition, ISO regulated TNF-α, IL-10, IL-6 and NO production and improved vascular reactivity in LPS-treated rodents [18,19]. These reports indicate that anti-inflammatory action of ISO can be beneficial in sepsis with no clear underlying molecular mechanism of action. Recently, HO-1 and its metabolites including CO or biliverdin emerged as valuable drug candidates for treatment of sepsis [22,27]. Because we reported previously that ISO induces HO-1 protein expression in RAW 264.7 cells through the β_1-AR [17], the anti-inflammatory action of ISO including inhibition of iNOS, TNF-α, and HMGB1 expression can be explained, at least in part, by HO-1 induction. Thus, we extended our study to the mechanism of ISO in the induction of HO-1 protein expression in RAW 264.7 cells and, then, asked does ISO really have a beneficial effect in the CLP-induced sepsis model?

As our understanding of the complexity and dynamic pathophysiological process of sepsis increases, more effective therapeutic approaches for the treatment of sepsis will become necessary. One of the potential molecular targets in the treatment of sepsis is HMGB1, a ubiquitous nuclear protein, which has been found to act as a "late" inflammatory cytokine that contributes to the pathophysiological progression of sepsis and other inflammatory disorders [10]. Moreover, attention to HMGB1 is increasing dramatically nowadays as its importance in the field of inflammation is uncovered. That is, HMGB1 plays a critical role not only in infection but also in sterile inflammation, e.g., ischemia–reperfusion injury [28]. Therefore, we focused on whether ISO reduces

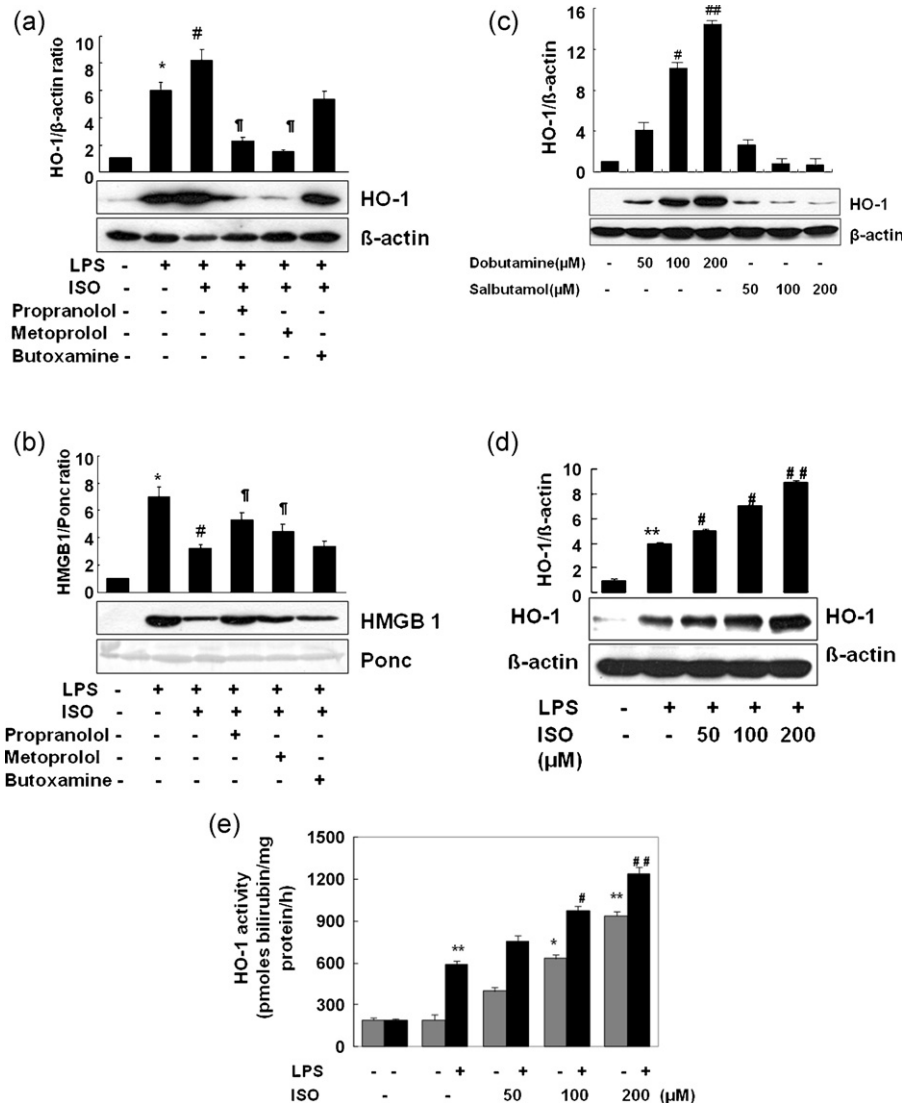

Fig. 4. β_1-Adrenoceptor activation induces HO-1 expression and activity in macrophage cells. RAW 264.7 cells were treated with isoproterenol (ISO, 100 μM), ISO + propranolol (100 μM), a non-selective β-AR antagonist, ISO + metoprolol (100 μM), a selective β_1-AR antagonist, or ISO + butoxamine (100 μM), a selective β_2-AR antagonist 30 min prior to addition of LPS (1 μg/ml). After incubation for 8 h (HO-1, a) or 16 h (HMGB1, b), cells were harvested and subjected to Western blot analysis as described in Section 2. To confirm that HO-1 induction by isoproterenol is mediated by the β_1-AR, different concentrations of dobutamine, a β_1-selective AR agonist, and salbutamol, a β_2-selective AR agonist, were administered for 8 h to RAW 264.7 cells (c). Cells were incubated with LPS (1 μg/ml) or LPS + ISO (50, 100, and 200 μM) for 8 h and HO-1 expression and activity were measured as described in Section 2. Representative blot shown is from one of the three independent experiments with similar results. Data are given as mean ± SEM of three independent experiments. *, **, $p < 0.05$, $p < 0.01$, compared to control, respectively; $^{\#}p < 0.05$, compared to LPS, $^{¶}p < 0.05$, compared to isoproterenol (200 μM).

HMGB1 release in LPS-activated RAW 264.7 cells. Indeed, ISO in a concentration-dependent and HO-1 sensitive manner reduced HMGB1 release in LPS-stimulated RAW 264.7 cells *in vitro*. Because we demonstrated previously that inducers of HO-1 can reduce HMGB1 release in LPS-stimulated RAW 264.7 cells [22], we addressed this issue by pretreatment with the HO-1 enzyme inhibitor, ZnPPIX. As expected, the reduced HMGB1 release caused by ISO was significantly inhibited by the HO-1 inhibitor, indicating that HO-1 activity is involved in this effect. This conclusion was further supported by the findings of other investigators [27], who observed that circulating levels of HMGB1 were higher in HO-$1^{-/-}$ mice than HO-$1^{+/+}$ mice. Furthermore, HO-$1^{-/-}$ mice given HMGB1 neutralizing antibody showed improvement in survival compared with littermates receiving control antibody. These reports strongly suggest of the potential therapeutic effect of HO-1 in the treatment of sepsis [22,27]. More recently, we reported that nicotine, a cholinergic agonist, induced HO-1 via α7nAChR in RAW 264.7 cells which reduced HMGB1 in LPS-activated macrophages and increased survival rate in CLP-induced septic mice [29], indicating that a possible molecular target of the cholinergic anti-inflammatory pathway activated by nicotine may be the HO-1 protein. In addition, we also reported that CO reduces HMGB1 release through regulation of the IFN-β/JAK2/STAT-1/iNOS/NO signaling pathway in macrophages in response to LPS [30], indicating that HO-1/CO acts as a negative functional regulator of HMGB1 in sepsis. Thus, further investigation will be needed to address which metabolites of HO-1 reduce HMGB1 release by ISO in sepsis.

Understanding the signaling pathways involved in HO-1 induction by ISO may give us information helpful for the development of new therapeutic agents for treatment of sepsis. Because HO-1 induction was reported to depend on PI3K and p38 MAPK in many cells [31–33], we asked whether these signaling molecules are critical for HO-1 induction by ISO. We found that HO-1 induction by ISO was also significantly and dose-dependently reduced by LY294002 and SB203580. In agreement with these findings, reduction of HMGB1 release by ISO in LPS-activated RAW

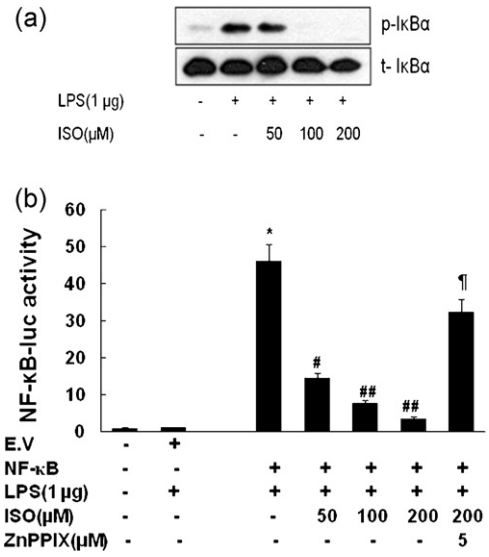

Fig. 5. Inhibition of inflammatory cytokines is related to NF-κB activity. The involvement of NF-κB activity in the anti-inflammatory action of isoproterenol (ISO), phosphorylation of IκBα (a) and NF-κB luciferase activity (b) were measured. Cells were incubated after 1 h of incubation with LPS (1 μg/ml) in the absence or presence of different concentrations of ISO or ISO (200 μM) + ZnPPIX. Then Western blot analysis was performed using phosphor-IκBα and IκBα antibodies and NF-κB-driven luciferase activity was measured in cells transfected with NF-κB as described in Section 2. Activity was presented as fold increase. Representative blot shown is from one of the three independent experiments with similar results. Data are given as mean ± SEM of three independent experiments. *, $p < 0.05$, compared to control, #, ## $p < 0.05$ and $p < 0.01$, compared to LPS, respectively. ¶, $p < 0.05$, compared to isoproterenol (200 μM).

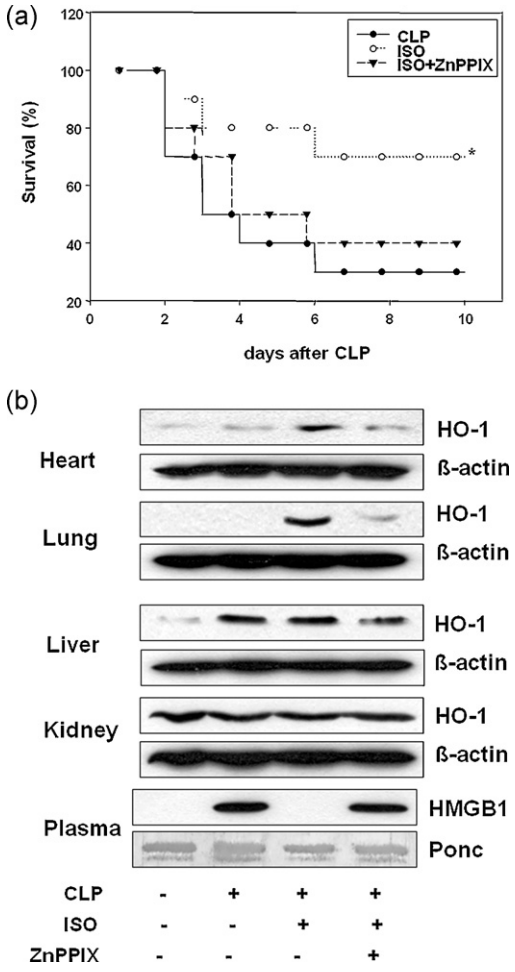

Fig. 6. Isoproterenol improves survival rate and reduces serum HMGB1 in CLP-induced septic mice in an HO-1 sensitive manner. Health male Balb/c mice were injected with isoproterenol (ISO, 10 mg/kg, *i.p.* $n = 14$) 1 h before CLP ($n = 14$). Survival was monitored daily for 10 days (a). ISO significantly increased survival rate ($p < 0.038$). In separate experiments, mice were treated with saline ($n = 2$), CLP ($n = 6$), CLP + ISO ($n = 6$), CLP + ISO + ZnPPIX ($n = 6$) and 24 h later tissue (heart, lung, liver, and kidney) HO-1 protein levels and serum HMGB1 levels were measured by Western blot methods (b). *, $p < 0.05$ compared to sham control.

264.7 cells was significantly reversed by inhibitors of PI3K or p38MAPK. As Nrf2, a basic leucine zipper transcription factor that regulates redox balance and stress response, plays an important role in the induction of HO-1 in many cells including RAW 264.7 cells, we found that the increased Nfr-2 luciferase activity induced by ISO was significantly reduced by LY294002 and SB203580, again highlighting the fact that HO-1 induction by ISO involves the PI3K/p38MAPK/Nrf-2 signaling pathway (Fig. 7). It should be mentioned that the protein kinase A pathway is also important for HO-1 induction by ISO in RAW 264.7 cells [17,23]. Of note, pharmacological inhibition and genetic deletion of p38 MAPK has been reported to induce HO-1 gene expression in various cells including RAW 264.7 cells [34,35], which shows how complex signal(s) regulate(s) inflammatory responses by a host of different stimuli. Because activation of NF-κB is critical for induction of inflammatory cytokines such as TNF-α, IL-1β, NO, and release of HMGB1 in macrophages [25], we investigated whether ISO inhibits NF-κB. In agreement with others [36], we demonstrated that ISO concentration-dependently inhibited phosphorylation of IκBα and reduced NF-κB-driven luciferase activity in LPS-activated RAW 264.7 cells. In addition, ISO-induced inhibition of NF-κB was significantly reversed by ZnPPIX, supporting further evidence that HO-1 activity plays an important role in anti-inflammatory action of ISO in macrophages. Thus, β1-AR/PI3K/p38MAPK/Nrf2/HO-1/NF-κB signaling is responsible for reduction of HMGB1 release by ISO in LPS-activated RAW 264.7 cells. We did not measure such cytokines as TNF-α and IL-10 etc., in the present study, because these cytokines and NO are reported to be reduced by ISO in LPS-activated macrophages [18,19].

Another interesting finding is that the β_1-AR but not the β_2-AR subtype is solely responsible for HO-1 induction in RAW 264.7 cells. There are many reports on β-AR-mediated modulation of inflammatory effects. However, far fewer studies have focused on the β-AR subtype responsible for HO-1 induction. We confirmed that HO-1 induction by ISO was significantly inhibited by propranolol and metoprolol but not butoxamine [17]. When challenged with dobutamine, a selective β_1-AR agonist and salbutamol, a selective β_2-AR agonist, we still found that HO-1 induction in macrophages was mediated through the β_1- but not the β_2-AR receptor. It seems likely that both receptor subtypes are involved in HO-1 induction depending on the specific tissue involved. For example, the β_1-AR is the responsible receptor in RAW 264.7 cells [17], hepatocytes [37], and THP-1 cells (data not shown); whereas the β_2-AR is the receptor involved in vascular smooth muscle cells [23]. Although stimulation of the β_2-AR by salbutamol had an anti-inflammatory action on LPS-activated macrophages, it did not induce HO-1 [38] supporting the concept that activation of the β_1-AR in macrophages accounts for induction of HO-1. Because ISO is a non-selective β-AR agonist, its anti-inflammatory action can be manifested in a HO-1-dependent manner via the β_1-AR and in a HO-1-independent fashion via the β_2-AR in macrophages.

Finally, we wanted to know if administration of ISO really rescues animals from lethality under septic conditions. As expected, improvement in survival was demonstrated by administration of ISO when followed for 10 days in mice subjected to CLP. This improvement can be explained, at least in part, by HO-1

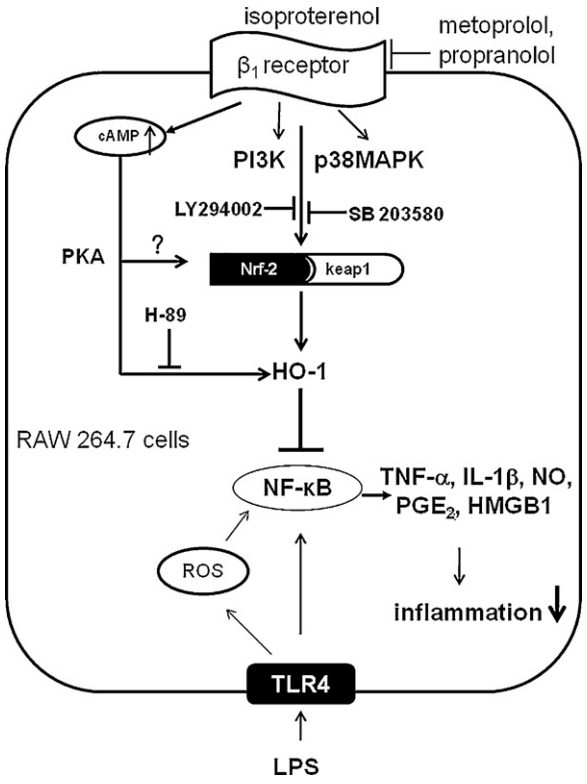

Fig. 7. Model for the anti-inflammatory action of isoproterenol through HO-1 induction in RAW 264.7 cells. Activation of the β_1-AR by isoproterenol leads to an increase in cAMP, which then activates protein kinase A (PKA) (Sun et al. [17]), and also stimulates PI3K and p38MAPK activity. Activated PI3K and p38MAPK make it possible for Nrf2 to move to nucleus, where binding of Nrf2 to the ARE promoter site, leads to the upregulation of HO-1 gene expression. Therefore, the non-selective β-AR blocker, propranolol, or the β_1-selective AR blocker, metoprolol inhibits but the β_1-selective AR agonist dobutamine, activates HO-1 induction. Likewise, the PI3K inhibitor, LY294002, or p38MAPK inhibitor, SB203580, inhibits HO-1 induction. It is not yet known whether cAMP activates PI3K, p38 MAPK, or Nrf2 translocation to induce HO-1 in RAW 264.7 cells. However, H89, a PKA inhibitor blocked ISO-induced HO-1 upregulation. The increased HO-1 expression induced by ISO inhibits NF-κB activity, which can be activated by LPS through TLR4; thus production of pro-inflammatory cytokines dependent on NF-κB, such as TNF-α, IL-1β, NO and HMGB1 can be reduced. It should be noted, however, that LPS also induces HO-1 by production of ROS via TLR4, but it also activates NF-κB which could tilt the balance toward to pro-inflammation.

induction as evidenced by the following: (1) the increase in survival was significantly inhibited by the presence of ZnPPIX, an HO-1 inhibitor, (2) the lung and heart tissues of CLP-mice had significantly increased HO-1 protein expression in the presence of ISO, which was antagonized by ZnPPIX, and (3) the plasma HMGB1 level was significantly reduced by ISO in CLP-mice, which was again reversed by ZnPPIX. These findings further confirm and support recent reports that HO-1 inducers improve survival of septic animals by reducing HMGB1 levels [22,27]. We cannot explain at the present time why liver and kidney were less sensitive than heart and lung tissues to the induction of HO-1 by ISO in CLP-induced septic mice. It is interesting to note that ISO increased expression of biliverdin reductase in isolated myocytes and intact perfused hearts, which, in turn, increased the cellular content of HO-2 protein, another defense protein [37]. This protein serves to limit cardiac apoptosis, although its induction does not eliminate apoptosis entirely [39]. Furthermore, ISO increased CD14 expression and live *E. coli* phagocytosis in murine bone marrow macrophages [40], which may result from the increase in cAMP and PKA signaling [41]. It has been reported that macrophages may exhibit accelerated LPS internalization and detoxification through increased surface CD14 expression [42] or synthesis and release of higher levels of soluble CD14 at inflammatory foci, thus limiting the biological toxicity of LPS. These findings suggest the potential impact of ISO on innate immune functions. An unanswered question is whether plasma catecholamines such as norepinephrine and epinephrine that are increased more than 10-fold during sepsis via sympathetic activation of the adrenal medulla and catecholamine release from nerve terminals, downregulate β-AR's by phosphorylation and internalization, reducing the density of receptors on the cell surface. Thus, the beneficial effects of ISO on human septic patients remain to be established, in particular, regarding to clinical concentration of ISO. Because no study was conducted so far as to whether clinical concentration of ISO induces HO-1 in humans.

In summary, we have set forth the reasons why ISO should be beneficial for the treatment of sepsis. Although much evidence suggests that β-AR stimulation should improve during sepsis, no report was available on the effects of ISO on HMGB1 release in endotoxin-activated cells *in vitro* or in an animal model of sepsis. This is the first report showing that ISO inhibits release of HMGB1, a necessary and sufficient late mediator of severe sepsis, by induction of HO-1. Therefore, we conclude that inhibition of HMGB1 release due to HO-1 induction is a novel mechanism of action for ISO that could prove useful in the treatment of sepsis.

Conflict of interest

There is no report to conflict of interest.

Acknowledgments

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References

[1] Felten DL, Ackerman KD, Wiegand SJ, Felten SY. Noradrenergic sympathetic innervation of the spleen: I. Nerve fibers associate with lymphocytes and macrophages in specific compartments of the splenic white pulp. J Neurosci Res 1987;18:28–36.
[2] Madden KS, Felten SY, Felten DL, Hardy CA, Livnat S. Sympathetic nervous system modulation of the immune system. II. Induction of lymphocyte proliferation and migration in vivo by chemical sympathectomy. J Neuroimmunol 1994;49:67–75.
[3] Sheridan JF, Dobbs C, Brown D, Zwilling B. Psychoneuroimmunology: stress effects on pathogenesis and immunity during infection. Clin Microbiol Rev 1994;7:200–12.
[4] Rinner I, Schauenstein K. Detection of choline-acetyltransferase activity in lymphocytes. J Neurosci Res 1993;35:188–91.
[5] Spengler RN, Chensue SW, Giacherio DA, Blenk N, Kunkel SL. Endogenous norepinephrine regulates tumor necrosis factor-α production from macrophages in vitro. J Immunol 1994;152:3024–31.
[6] Severn A, Rapson NT, Hunter CA, Liew FY. Regulation of tumor necrosis factor production by adrenaline and β-adrenergic agonists. J Immunol 1992;148:3441–5.
[7] Vander Poll T, Coyle SM, Barbosa K, Braxton CC, Lowry SF. Epinephrine inhibits tumor necrosis factorα and potentiates interleukin 10 production during human endotoxemia. J Clin Invest 1996;97:713–9.
[8] Braggins PE, Trakshel GM, Kutty RK, Maines MD. Characterization of two hem oxygenase isoforms in rat spleen: comparison with the hematin-induced and constitutive isoforms of the liver. Biochem Biophys Res Commun 1986;15:528–33.
[9] Takamiya R, Hung CC, Hall SR, Fukunaga K, Nagaishi T, Maeno T, et al. High mobility group box 1 contributes to lethality of endotoxemia in heme oxygenase-1 deficient mice. Am J Respir Cell Mol Biol 2009;41:129–35.
[10] Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999;285:248–51.
[11] Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 2005;5:331–42.
[12] Mantell LL, Parrish WR, Ulloa L. HMGB1 as a therapeutic target for infectious an inflammatory disorders. Shock 2006;25:4–11.
[13] Parrish WR, Ulloa L. High-mobility group box-1 isoforms as potential therapeutic targets in sepsis. Methods Mol Biol 2007;361:145–62.
[14] Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002;418:191–5.

[15] Qin S, Wang H, Yuan R, Li H, Ochani M, Ochani K, et al. Role of HMGB1 in apoptosis-mediated sepsis lethality. J Exp Med 2006;203:1637–42.
[16] Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, et al. The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. Immunol Rev 2007;220:60–81.
[17] Sun J, Kim SJ, Park MK, Kim HJ, Tsoy I, Kang YJ, et al. Selective activation of adrenergic beta1 receptors induces heme oxygenase 1 production in RAW264.7 cells. FEBS Lett 2005;579:5494–500.
[18] Haskó G, Németh ZH, Szabó C, Zsilla G, Salzman AL, Vizi ES. Isoproterenol inhibits Il-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages. Brain Res Bull 1998;45:183–7.
[19] Szabó C, Haskó G, Zingarelli B, Németh ZH, Salzman AL, Kvetan V, et al. Isoproterenol regulates tumour necrosis factor, interleukin-10, interleukin-6 and nitric oxide production and protects against the development of vascular hyporeactivity in endotoxaemia. Immunology 1997;90:95–100.
[20] Leone M, Boyadjiev I, Boulos E, Antonini F, Visintini P, Albanèse J, et al. A reappraisal of isoproterenol in goal-directed therapy of septic shock. Shock 2006;26:353–7.
[21] Kutty RK, Maines MD. Oxidation of heme c derivatives by purified heme oxygenase: evidence for the presence of one molecular species of heme oxygenase in the rat liver. J Biol Chem 1982;257:9944–52.
[22] Tsoyi K, Lee TY, Lee YS, Kim HJ, Seo HG, Lee JH, et al. 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. Mol Pharmacol 2009;76:173–82.
[23] Kim JE, Kang YJ, Lee KY, Choi HC. Isoproterenol inhibits angiotensin II-stimulated proliferation and reactive oxygen species production in vascular smooth muscle cells through heme oxygenase-1. Biol Pharm Bull 2009;32:1047–52.
[24] Chung SW, Chen YH, Yet SF, Layne MD, Perrella MA. Endotoxin-induced down regulation of Elk-3 facilitates heme oxygenase-1 induction in macrophages. J Immunol 2006;176:2414–20.
[25] Wang H, Liao H, Ochani M, Justiniani M, Lin X, Yang L, et al. Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. Nat Med 2004;10:1216–21.
[26] Zhang H, De Jongh R, De Backer D, Cherkaoui S, Vray B, Vincent JL. Effects of alpha- and beta-adrenergic stimulation on hepatosplanchnic perfusion and oxygen extraction in endotoxic shock. Crit Care Med 2001;29:581–8.
[27] Takamiya R, Hung CC, Hall SR, Fukunaga K, Nagaishi T, Maeno T, et al. High mobility group box 1 contributes to lethality of endotoxemia in heme oxygenase-1-deficient mice. Am J Respir Cell Mol Biol 2009;41:129–35.
[28] Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. Annu Rev Immunol 2011;29:139–62.
[29] Tsoyi K, Jang HJ, Kim JW, Chang HK, Lee YS, Pae HO, et al. Stimulation of alpha7 nicotinic acetylcholine receptor by nicotine attenuates inflammatory response in macrophages and improves survival in experimental model of sepsis through heme oxygenase-1 induction. Antioxid Redox Signal 2011;14:2057–70.
[30] Tsoyi K, Nizamutdinova IT, Jang HJ, Mun L, Kim HJ, Seo HG, et al. Carbon monoxide from CORM-2 reduces HMGB1 release through regulation of IFN-β/JAK2/STAT1/INOS/NO signaling but not COX-2 in TLR-activated macrophages. Shock 2010;34:608–14.
[31] Paine A, Eiz-Vesper B, Blasczyk R, Immenschuh S. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. Biochem Pharmacol 2010;80:1895–903.
[32] Park MK, Kang YJ, Ha YM, Jeong JJ, Kim HJ, Seo HG, et al. EP2 receptor activation by prostaglandin E2 leads to induction of HO-1 via PKA and PI3K pathways in C6 cells. Biochem Biophys Res Commun 2009;379:1043–7.
[33] Maines MD. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 1997;37:517–54.
[34] Yu R, Mandlekar S, Lei W, Fahl WE, Tan TH, Kong AN. p38 Mitogen-activated protein kinase negatively regulates the induction of phase II drug-metabolizing enzymes that detoxify carcinogens. J Biol Chem 2000;275:2322–7.
[35] Naidu S, Vijayan V, Santoso S, Kietzmann T, Immenschuh S. Inhibition and genetic deficiency of p38 MAPK up-regulates heme oxygenase-1 gene expression via Nrf2. J Immunol 2009;182:7048–57.
[36] Farmer P, Pugin J. beta-adrenergic agonists exert their anti-inflammatory effects in monocytic cells through the IkappaB/NF-kappaB pathway. Am J Physiol Lung Cell Mol Physiol 2000;279:675–82.
[37] Rensing H, Bauer I, Kubulus D, Wolf B, Winning J, Ziegeler S, et al. Heme oxygenase-1 gene expression in pericentral hepatocytes through beta1-adrenoceptor stimulation. Shock 2004;21:376–87.
[38] Tanaka S, Tsutsui M, Kishida T, Souma S, Kuroda J, Yoshida T. Salbutamol inhibits lipopolysaccharide-induced inflammatory responses in rat peritoneal macrophages. J Toxicol Sci 2010;35:327–34.
[39] Ding B, Gibbs PE, Brookes PS, Maines MD. The coordinated increased expression of biliverdin reductase and heme oxygenase-2 promotes cardiomyocyte survival: a reductase based peptide counters β-adrenergic receptor ligand-mediated cardiac dysfunction. FASEB J 2011;25:301–13.
[40] Muthu K, He LK, Szilagyi A, Strotmon P, Gamelli RL, Shankar R. β-Adrenergic stimulation increases macrophage CD14 expression and *E. coli* phagocytosis through PKA signaling mechanisms. J Leukoc Biol 2010;88:715–24.
[41] Liu S, Morris Jr SM, Nie S, Shapiro RA, Billiar TR. cAMP induces CD14 expression in murine macrophages via increased transcription. J Leukoc Biol 2000;67:894–901.
[42] Gegner JA, Ulevitch RJ, Tobias PS. Lipopolysaccharide (LPS) signal transduction and clearance. Dual roles for LPS binding protein and membrane CD14. J Biol Chem 1995;270:5320–5.