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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