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Ascorbic acid reduces HMGB1 secretion in lipopolysaccharide-activated RAW 264.7 cells and improves survival rate in septic mice by activation of Nrf2/HO-1 signals

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ABSTRACT

High mobility group box 1 (HMGB1) is now recognized as a late mediator of sepsis. We tested hypothesis that ascorbic acid (AscA) induces heme oxygenase (HO)-1 which inhibits HMGB1 release in lipopolysaccharide (LPS)-stimulated cells and increases survival of septic mice. AscA increased HO-1 protein expression in a concentration- and time-dependent manner via Nrf2 activation in RAW 264.7 cells. HO-1 induction by AscA was significantly reduced by Nrf2 siRNA-transfected cells. Mutation of cysteine to serine of keap-1 proteins (C151S, C273S, and C288S) lost the ability of HO-1 induction by AscA, due to failure of translocation of Nrf-2 to nucleus. The PI3 kinase inhibitor, LY294002, inhibited HO-1 induction by AscA. Oxyhemoglobin (HbO_2), LY294002, and ZnPPIX (HO-1 enzyme inhibitor) reversed effect of AscA on HMGB1 release. Most importantly, administration of AscA (200 mg/kg, *i.p.*) significantly increased survival in LPS-induced endotoxemic mice. In cecal ligation and puncture (CLP)-induced septic mice, AscA reduced hepatic injury and serum HMGB1 and plasminogen activator inhibitor (PAI)-1 in a ZnPPIX-sensitive manner. In addition, AscA failed to increase survival in *Nrf2* knockout mice by LPS. Thus, we concluded that high dose of AscA may be useful in the treatment of sepsis, at least, by activation of Nrf2/HO-1 signals.

A recommended section: Inflammation, Immunopharmacology, and Asthma

1. Introduction

Despite recent advances in intensive care treatment and the discovery of antibiotics, sepsis remains associated with a high mortality rate [1,2]. Sepsis is characterized by an overwhelming systemic inflammatory response that can lead to lethal multiple organ failure. The endotoxin lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria, contributing significantly to the structural integrity of the bacteria and protecting the membrane from certain types of chemical attack. Endotoxins and other pathogen components stimulate macrophages to produce pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin-1β (IL-1β). Excessive production of these cytokines can cause a lethal systemic inflammation that produces the most dramatic pathological sequelae of sepsis, including systemic capillary leakage syndrome, tissue injury and fatal organ failure [3,4]. Activated macrophages

Abbreviations: **AscA**, Ascorbic acid; **ALT**, Alanine aminotransferase; **ARE**, Antioxidant response element; **AST**, Aspartate Aminotransferase; **CLP**, Cecal ligation and puncture; **CO**, Carbon monoxide; **ELISA**, Enzyme-linked immunosorbent assay; **HMGB1**, High-mobility Group Box 1; **HO-1**, Heme oxygenase-1; **IL-1β**, interleukin-1β; **Keap1**, Kelch-like ECH-associated protein 1; **LPS**, Lipopolysaccharide; **NF- kB**, Nuclear factor- kB; **Nrf2**, NF-E2-related factor; **PAI-1**, Plasminogen activated inhibitor-1; **PI3K**, Phosphoinositide 3-kinase; **siRNA**, Small interfering RNA; **TNF**, Tumor necrosis factor; **ZnPPIX**, Zinc protoporphyrin IX.

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also release high mobility group box 1 (HMGB1), originally described as an intracellular DNA-binding protein, into the extracellular milieu. Several observations have suggested that HMGB1 is a sufficient and necessary mediator of sepsis [5,6]. Extracellular HMGB1 can trigger a lethal inflammatory process [7] by significantly increasing the release of inflammatory cytokines such as TNF, IL-1β, IL-6, IL-8, and macrophage inflammatory protein-1β [8].

Heme oxygenase (HO)-1 is one of the rate-limiting enzymes in heme catabolism that catalyzes the stereospecific degradation of heme to biliverdin, carbon monoxide (CO), and iron [9]. Most stimuli increase HO-1 transcription via the activation of the Kelch-like ECH-associated protein-1 (Keap1)/nuclear factor (erythroid-derived 2) like 2 (Nrf2) signaling pathway [9]. The redox status of Keap1 is critically important for the ubiquitination of Nrf2 [10]. Among reactive cysteine residues in the Keap1 protein, Cysteine273 and 288 are required for Keap1-dependent ubiquitination of Nrf2 [10], and Cysteine151 was identified as an essential residue for the inhibition of the Keap1-dependent degradation of Nrf2 [11]. A growing body of evidence supports the notion that ascorbic acid (AscA) is protective in the setting of sepsis syndrome [12]. AscA regulates the function of macrophages in animals with endotoxic shock and has been shown to improve survival of mice with sepsis [13]. AscA is a water-soluble antioxidant circulating in plasma. While its role as an endogenous antioxidant is well recognized, Fisher et al. [14] reported that AscA beneficially influences multiple pathways associated with sepsis. Interestingly, recent study indicates that intravenous of AscA (200 mg/kg) infusion was safe to critically ill patients with severe sepsis and gives positively impact the extent of multiple organ failure [15], opening important therapeutic perspectives of AscA in the management of sepsis. Although parenteral administration of high dose of AscA suggests beneficial effect on mitigation of pathophysiyological signs of sepsis in animals and humans [14–16], however, no study was conducted whether AscA reduces HMGB1 release, a critical molecule in late phase of sepsis, in septic conditions *in vitro* and *in vivo*. Thus the aim of the present study was to investigate whether AscA reduces HMGB1 release from LPS-activated macrophage cells, which results in increasing survival and reducing circulating HMGB1 in septic mice *in vivo*. In addition, we explored the inhibitory mechanism of HMGB1 release is related with induction of HO-1 by AscA.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin were purchased from Gibco BRL (Rockville, MD). Anti-p-IκB antibodies were supplied by Cell Signaling Technology (Beverly, MA). Anti-HMGB1 was purchased from Abcam (Cambridge, MA), and both anti-β-actin and anti-HO-1 and anti-Nrf2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) and Western blotting detection reagents were from Amersham (Buckinghamshire, UK). LY294002 was purchased from Calbiochem (San Diego, CA). All other chemicals including LPS (*Escherichia coli* 0111:B4), cobalt protoporphyrin IX, tricarbonyldichlororuthenium (II) dimmer ($[Ru(CO)_3Cl_2]_2$) (CORM-2), and AscA were purchased from Sigma–Aldrich (St. Louis, MO). Bovine hemoglobin (Hb) was purchased from Sigma–Aldrich (St. Louis, MO). Enzyme-linked immunosorbent assay (ELISA) kit for HMGB1 was purchased from Cloud Clone Corp (Houston, TX) and Mouse PAI-1 activity ELISA kit was purchased from Molecular Innovations (Novi, MI).

2.2. Cell culture

RAW264.7 cells were purchased from ATTC (Manassas, VA) and cultured as previously described [17]. In brief, the cells were grown in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 mg/ml), L-glutamine (4.5 mg/ml), glucose (4.5 mg/ml). Cells were maintained at 37 °C under 5% CO_2 in a humidified atmosphere.

2.3. Cell stimulation

RAW264.7 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 or absence of different concentrations of AscA (10, 100 and 300 μM). Levels of HMGB1 were determined 16 h after stimulation. Oxyhemoglobin (HbO_2) was prepared by reducing bovine hemoglobin with sodium hydrosulfite followed by gel filtration with a pre-packed disposable column (PD-10, Pharmacia, Uppsala, Sweden) that was equilibrated with 50 mM Tris–HCl at pH 7.4. The concentration of HbO_2 was determined using a Perkin-Elmer Lambda 5 spectrophotometer at 576 nm [18].

2.4. Western blot analysis

After completion of the experiments, cells were homogenized and centrifuged. After determining protein concentration, each sample was boiled in loading buffer and loaded onto a gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel. Following electrophoresis, proteins were transferred to an immobilon-polyvinylidine fluoride membrane. Membranes were blocked with TBS-T containing 5% (w/v) skim milk powder for 1 h at room temperature. The membrane was incubated with primary antibodies against HO-1, HMGB1 (dilution of 1:500) or β-actin at a dilution of 1:1000 overnight at 4 °C. Proteins were detected with horseradish peroxidase conjugated secondary antibodies at dilutions of 1:5000 in TBS-T containing 5% skim milk powder for 1 h at room temperature and were visualized by enhanced chemiluminescence.

2.5. Small interference RNA

The HO-1 small interference RNA (siRNA) was purchased from Invitrogen (Carlsbad, CA). The sequence of mouse HO-1 siRNA (5 prime to 3 prime) was UUACAUGGCAUAAAUUCCCACUGCC. The siRNA was transfected into RAW264.7 cells according to the manufacturer's protocol using the transfection reagent Super-Fect® from QIAGEN. The cells were incubated with 100 nM HO-1 siRNA for 24 h in serum- and antibiotics-free media. Cells were then incubated for 12 h in media containing antibiotics and FBS, and cells were washed and pretreated with or without AscA followed by LPS stimulation.

2.6. Plasmid constructions

For constructing pcDNA-mNrf2, pcDNA-mKeap1 and pcDNA-mKeap1 mutants (mKeap1C151S, mKeap1C273S, mKeap1C288S, and mKeap1C151S-C273S-C288S), the full-length coding sequence of mouse Keap1 and mouse Nrf2 was inserted into the T&A Cloning Vector (RBC, Chung Ho, Taipei). In the mKeap1 subcloned vector, the Cys151Ser, Cys277Ser, Cys288Ser and Cys151-273-288Ser mutations were generated using the QuikChange II Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the following pair of primers. C151S: 5′-TCC GTG GGC GAG AAG **TCT** GTC CTG CAC GTG ATG-3′ and 5′-CAT CAC GTG CAG GAC **AGA** CTT CTC GCC CAC GGA-3′, C273S: 5′-CTG CGG GCC GTG CGC **TCC** CAT GCG CTC ACG CCG'