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Hemin inhibits NLRP3 inflammasome activation in sepsis-induced acute lung injury, involving heme oxygenase-1

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ABSTRACT

NLRP3 inflammasome activation contributes to acute lung injury (ALI), accelerating caspase-1 maturation, and resulting in IL-1β and IL-18 over-production. Heme oxygenase-1 (HO-1) plays a protective role in ALI. This study investigated the effect of hemin (a potent HO-1 inducer) on NLRP3 inflammasome in sepsis-induced ALI. The sepsis model of cecal ligation and puncture (CLP) was used in C57BL6 mice. In vivo induction and suppression of HO-1 were performed by pretreatment with hemin and zinc protoporphyrin IX (ZnPP, a HO-1 competitive inhibitor) respectively. CLP triggered significant pulmonary damage, neutrophil infiltration, increased levels of IL-1β and IL-18, and edema formation in the lung. Hemin pretreatment exerted inhibitory effect on lung injury and attenuated IL-1β and IL-18 secretion in serum and lung tissue. In lung tissues, hemin down-regulated mRNA and protein levels of NLRP3, ASC and caspase-1. Moreover, hemin reduced malondialdehyde and reactive oxygen species production, and inhibited NF-κB and NLRP3 inflammasome activity. Meanwhile, hemin significantly increased HO-1 mRNA and protein expression and HO-1 enzymatic activity. In contrast, no significant differences were observed between the CLP and ZnPP groups. Our study suggests that hemin-inhibited NLRP3 inflammasome activation involved HO-1, reducing IL-1β and IL-18 secretion and limiting the inflammatory response.

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1. Introduction

Sepsis is a condition resulting in a harmful systemic inflammatory response to an infection. Sepsis is a major cause of morbidity and mortality despite extensive research efforts and improvements in care [1]. Sepsis may lead to end-organ dysfunction, and septic patients are particularly at risk of developing acute lung injury (ALI) [2]. ALI is a common and severe pulmonary disease, and affects 10–15% of patients hospitalized in ICU. The most severe form of ALI, acute respiratory distress syndrome (ARDS), still has a high mortality despite progresses in ICU care. Sepsis remains the leading cause of ALI/ARDS [3].

The nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3) inflammasome is a multiprotein complex that regulates the maturation of pro-inflammatory cytokines IL-1β and IL-18. It consists of NOD-like receptor, NLRP3, the adaptor protein ASC [apoptosis-associated speck-like protein containing caspase-1 activator domain (CARD)] and caspase-1. Upon exogenous and endogenous stimuli, the NLRP3 inflammasome forms through activation of NLRP3 and recruitment of ASC and pro-caspase-1, resulting in caspase-1 activation and subsequently processing pro-IL-1β and pro-IL-18 into their active forms. The NLRP3 inflammasome can be activated by various stimulating factors such as bacteria, virus, fungi, and components of dying cells [4]. Dysregulated NLRP3 inflammasome activation participates in the pathogenesis of sepsis and ALI [5,6]. The inflammasome-associated cytokines belong to the IL-1 cytokine family, and propagate the acute inflammatory response.

Heme oxygenase-1 (HO-1) is an inducible and rate-limiting enzyme in heme degradation, catalyzing heme cleavage to form carbon monoxide (CO), ferrous iron and biliverdin [7]. HO-1 can be induced by a variety of stimuli such as heat shock, cytokines, nitric oxide, endotoxin, and hyperoxia, all of which are produced in sepsis. Biliverdin is subsequently converted into bilirubin with significant anti-oxidant and anti-inflammatory properties. CO has a number of biological activities, especially anti-inflammatory features. HO-1 inhibits pulmonary inflammation and has beneficial effects on ALI and sepsis [8,9]. Hemin is a well-known HO-1 inducer, inhibiting oxidative stress-induced tissue

Abbreviations: HO-1, heme oxygenase-1; NLRP3, nucleotide-binding domain (NOD)-like receptor protein 3; ZnPP, zinc protoporphyrin IX; ASC, apoptosis-associated speck-like protein containing caspase-1 activator domain (CARD); CLP, cecal ligation and perforation; MDA, malondialdehyde; ROS, reactive oxygen species.

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damage [10]. On the other hand, zinc protoporphyrin IX (ZnPP) is a HO-1 inhibitor, and can inhibit the enzymatic activity of HO-1 [11].

Although the up-regulation of HO-1 and its role in the inflammatory response have been extensively studied, the association between HO-1 and the NLRP3 inflammasome remains unclear. Therefore, the aim of the present study was to investigate whether hemin inhibits NLRP3 inflammasome activation in sepsis-induced ALI and involves HO-1 pathway.

2. Materials and methods

2.1. Animals

Male C57BL6 mice (body weight 20–22 g, 6–8 weeks old, purchased from Beijing HFK Bioscience CO., Ltd., Beijing, China), were housed in a light- and temperature-controlled room (21–23 °C; 12 h cycle), with free access to food and water. All experimental procedures were approved by the ethical committee of the Harbin Medical College (Heilongjiang, China), and conducted in accordance with all state regulations.

2.2. Animal model of sepsis

Under sodium pentobarbital (50 mg/kg) anesthesia, sepsis was induced through cecal ligation and perforation (CLP), as previously described, with minor modifications [12]. Briefly, the abdomen was incised and the cecum was exposed. The distal half of the cecum was ligated, and then punctured through-and-through once using a 22G needle after the cecal content was gently pushed toward the distal cecum. A small amount of feces was extruded from both the mesenteric and antimesenteric penetration holes to ensure patency. Then, the cecum was repositioned into the abdominal cavity, and the incision was closed with 6.0 surgical sutures.

Sham-operated mice underwent the same procedure, including opening of the peritoneum and exposing the cecum, but without ligation and needle perforation of the cecum.

After surgery, mice were resuscitated by injecting 1 mL of physiologic saline solution subcutaneously. Mice were then returned to their cages and provided food and water ad libitum. Animals were reanesthetized 12 h after CLP induction. The upper part of the left lung was used for lung wet/dry (W/D) weight ratio estimation. The lower part of left lung was fixed in 4% paraformaldehyde for histology and immunohistochemistry. The right lung was snap-frozen in liquid nitrogen, and stored at −80 °C for enzyme-linked immunosorbent assay (ELISA), myeloperoxidase (MPO) activity, real-time reverse transcription-polymerase chain reaction (real-time RT-PCR), and western blot analysis.

2.3. Experimental protocols

Hemin (Sigma, St. Louis, MO, USA, 28 mg/kg), a HO-1 inducer, and ZnPP (Sigma, St Louis, MO, USA, 28 mg/kg), a HO-1 inhibitor, were prepared under subdued light by dissolving the compound in 1 mL of 0.1 N NaOH, adjusting the pH to 7.4 with 1 N HCl, and diluting the solution to the final volume with normal saline (NS) [13]. Mice received intraperitoneal administration with hemin or ZnPP 12 h before CLP induction, and an equal volume of NS was administered to the control animals intraperitoneally. The dose and injection time of ZnPP and hemin treatment we used in the present study were based on previous reports and our preliminary studies [8,14]. Mice were randomly divided into four groups: vehicle-treated sham; vehicle + CLP; hemin + CLP; and ZnPP + CLP. All mice were sacrificed 12 h after CLP. No animal died before being sacrificed.

2.4. Lung wet/dry weight ratio

Lung edema was estimated by determining lung W/D weight ratios. The fresh upper part of the left lung was weighed and dried in an oven at 80 °C for at least 24 h, then weighed again when it was dry, to calculate the lung W/D weight ratio.

2.5. Histology

Left lung paraffin sections (5 μm) were stained with hematoxylin–eosin (H&E) and evaluated using an optical microscope (Olympus Optical, Tokyo, Japan). Pathological severity response of the lung was determined using a previously described semiquantitative scoring system [15], which includes alveolar congestion, hemorrhage, aggregation of neutrophil or leukocyte infiltration, and thickness of the alveolar wall, each graded on a zero (absent) to four points (extensive) scale.

2.6. Measurements of cytokines in serum and lung tissue

Serum and lung homogenate levels of cytokines IL-1β and IL-18 were analyzed using commercially available ELISA kits (R&D Systems, Abingdon, Oxon, UK), according to the manufacturer's instructions. Blood was coagulated for 1 h at room temperature and the serum was separated by centrifugation at 4000 rpm for 10 min in a tabletop centrifuge. Tissues (10 mg) from the right lung were homogenized in PBS containing proteinase inhibitor, and centrifuged at 12,000 rpm for 30 min in a refrigerated tabletop centrifuge to obtain protein extracts.

2.7. MPO activity

In brief, a part of the frozen right lung tissue was thawed and homogenized in 1 mL of 0.5% hexadecyltrimethylammonium bromide. The sample was then freeze-thawed, and the MPO activity of the supernatant was measured as previously described [16]. The enzyme activity was assayed by measuring absorbance changes in the redox reaction of H_2O_2 by spectrophotometry at 450 nm. Results were expressed as MPO units per gram of tissue.

2.8. Immunohistochemistry

Lung tissue sections (5 μm) were stained with rabbit antibodies against NLRP3 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) overnight at 4 °C and incubated with alkaline phosphatase (AP)-conjugated goat anti-rabbit Ig-G (Biosynthesis Biotechnology Co., Ltd., Beijing, China) for 1 h at room temperature. The AP activity was revealed in brown using DAB (Beijing Biosynthesis Biotechnology Co., Ltd.).

2.9. Real-time RT-PCR analysis

Total RNA from a portion of the right lung was extracted using the UltrapureRNA Kit (CoWin Biotech, Beijing, China) and reverse transcribed into first-strand cDNA synthesis using the HiFi-MMLV cDNA Kit (CoWin Biotech). Ultra SYBR Mixture (with ROX) for SYBR Green (CoWin Biotech) and Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) were used for real-time PCR. The following primers were used: NLRP3: forward 5′-TCT GAC CTC TGT GCT CAA AAC CAA C-3′ and reverse 5′-TGA GGT GAG GCT GCA GTT GTC TAA T-3′; ASC: forward 5′-ACT CAT TGC CAG GGT CAC AGA AGT G-3′ and reverse 5′-GCT TCC TCA TCT TGT CTT GGC TGG T-3′; caspase-1: forward:5′-ACT GAC TGG GAC CCT CAA GTT TTG C-3′ and reverse 5′-GGC AAG ACG TGT ACG AGT GGT TGT A-3′; and HO-1: forward 5′-CGA ATG AAC ACT CTG GAG ATG ACA C-3′ and reverse 5′-CCT CTG ACG AAG TGA CGC CAT CTG T-3′. Each gene expression was normalized to β-actin mRNA and calculated relatively to naïve sham mice using the comparative CT method.

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