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 downregulated 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...
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 C57BL/6 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 H2O2 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 GGT TCT TAA T-3′; ASC: forward 5′–ACT CAT TCG CAG GGT CAC AGA AGT C-3′ and reverse 5′–GCT TCC TCA TTC TGT CTT GGC TGG T-3′; caspase-1: forward 5′–ACT GAC TGG GAC CCT CAA GGT TGG C-3′ and reverse 5′–GCC AAG ACC TGG TGT CAG AGT CTT GAT T-3′; and caspase-1: forward 5′–ACT GCT TGG GCC CAC AGA GGT C-3′ and reverse 5′–CTT CTC GCC AAC TGG GAG TGT C-3′. Each gene expression was normalized to β-actin mRNA and calculated relatively to naïve sham mice using the comparative CT method.
2.10. Western blot analysis

Proteins (80 or 120 μg) from each right lung homogenate were loaded onto 10–12% polyacrylamide gels for electrophoresis. Proteins were then transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dried milk for 2 h at room temperature to reduce nonspecific binding, and washed with PBS containing 0.1% Tween-20 (PBST). Bands were detected using polyclonal antibodies against NLRP3 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase-1 p10 (1:500; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), HO-1 (1:1000, Cell Signaling Technology, Beverly, MA, USA), ASC (1:1000, Cell Signaling Technology, Beverly, MA, USA) and monoclonal antibody against mouse β-actin (Sigma, St. Louis, MO, USA). The binding of all antibodies was detected using an ECL detection system (Thermo Fisher Scientific, Waltham, MA, USA).

2.11. Co-immunoprecipitation and immunoblotting analysis

Lung tissue lysates were immunoprecipitated with anti-ASC antibodies (1:100, Cell Signaling Technology, Beverly, MA, USA). Immunoprecipitated proteins were eluted with 5× sodium dodecyl sulfate (SDS) loading buffer, separated using SDS gels, and transferred onto a polyvinylidene difluoride membrane, on which the western blot was performed.

2.12. Caspase-1 activity assay

Caspase-1 enzymatic activity was tested using a colorimetric assay kit (R&D system, Minneapolis, MN). Lung tissue proteins were extracted with cold protein lysis buffer. Tissue lysate (50 μg) was added to 50 μl of caspase-1 reaction buffer in a 96-well flat bottom microplate. Caspase-1 substrate (WEHD-pNA, 200 mM) was added to each sample, followed by 2 h of incubation at 37 °C. Caspase-1 activity was measured at a wavelength of 405 nm.

2.13. HO-1 activity assay

HO-1 activity was measured by bilirubin generation, as previously described [14,17]. Lung tissues were homogenized on ice in 1.15% (w/v) KCl containing protease inhibitors and centrifuged at 10,000 g for 30 min at 4 °C. The supernatant was further centrifuged with mouse liver cytosol (3 mg), a source of biliverdin reductase, hemin (20 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 μM), and NADPH (0.8 mM) for 1 h at 37 °C in the dark. The produced bilirubin was extracted with chloroform. Optical density was measured at 465–530 nm and enzyme activity was calculated as picomoles of bilirubin formed per minute per milligram of proteins.

2.14. NF-κB transcription factor assay

Nuclear protein extracts were obtained from lung tissues using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA), according to the manufacturer’s instructions. The p65 DNA binding activity was measured using the TransAM NF-κB Kit (Active Motif, Carlsbad, CA, USA).

2.15. Lung tissue malondialdehyde (MDA) and ROS determination

Malondialdehyde (MDA) levels in lung tissues were determined using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer’s instructions. The MDA content was expressed as nanomoles per milligram of proteins. The oxidative fluorescent dye dihydroethidium was used to evaluate in situ production of reactive oxygen species (ROS) from lung tissue. Lung cryosections (10 μm) were placed on microscope slides, and were equilibrated for 30 min at 37 °C in PBS. PBS containing DHE (5 μmol/L) was applied to each tissue section and incubated for 30 min in a light-protected and humidified chamber at 37 °C. Oxidized DHE was detected by fluorescence microscopy (TE2000, Nikon, Tokyo, Japan; excitation 488 nm, emission 610 nm).

2.16. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). Comparison of continuous variables among the four groups was performed using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test for multiple comparisons. P < 0.05 was considered statistically significant.

3. Results

3.1. Hemin improves the severity of acute lung injury

H&E-stained lung tissue sections obtained from hemin-pretreated mice were compared with the other groups to determine if hemin improves sepsis-induced lung injury. CLP-induced lung injury showed diffuse pathological changes characterized by alveolar congestion and inflammatory cells infiltration into the airspace. However, these changes were inhibited in the hemin group (Fig. 1C). Evaluation of pathological severity of the lung, as performed by independently scoring four parameters, showed a significant decrease in total histopathological score in the hemin group compared with the CLP or ZnP groups (Fig. 1E). In agreement with this pathological analysis, the W/D ratio of lung tissue showed a similar result (Fig. 1F). These results suggest that hemin-pretreated mice were resistant to CLP-induced ALI.

3.2. NLRP3 inflammasome-regulated cytokines are attenuated by hemin

Serum and lung homogenate levels of IL-1β and IL-18 were tested to determine if NLRP3 inflammasome-dependent cytokines were reduced by hemin. CLP induced significant increases in IL-1β and IL-18 levels (Fig. 2). We observed a notable reduction in serum and lung tissue levels of IL-1β and IL-18 in hemin-pretreated mice (Fig. 2). These results indicate that hemin may protect mice against sepsis-induced ALI by inhibiting NLRP3 inflammasome activation.

3.3. CLP induced recruitment of inflammatory cells and elevation of NLRP3 is repressed in the hemin group

Lung tissue sections were immunostained with anti-NLRP3 to locate NLRP3 in the lung and to determine if hemin reduces the positively stained cells. We observed that the NLRP3 protein was mainly expressed in the inflammatory cells (i.e. macrophages, neutrophils). There were fewer positively stained cells in lung tissues of the hemin group compared with the CLP or ZnP group (Fig. 3E). MPO activity, indicating accumulation of neutrophils in the lung, showed a similar result (Fig. 3F). These results suggest that NLRP3 inflammasome activation may accelerate inflammatory cells infiltration in CLP-induced ALI, and that hemin may alleviate the infiltration by NLRP3 inflammasome inhibition.

3.4. NLRP3, ASC, caspase-1 mRNA expression in lung are inhibited by hemin

Relative NLRP3, ASC, and caspase-1 mRNA levels in lung tissues were detected to determine if NLRP3 inflammasome was inhibited by hemin. CLP significantly increased NLRP3, ASC and caspase-1 mRNA expression compared with sham-operated mice. Hemin pretreatment suppressed the up-regulation of these mRNAs, while ZnP pretreatment enhanced these mRNA expressions (Fig. 4). Relative HO-1 mRNA levels were significantly up-regulated by hemin.
compared with the CLP and ZnPP groups. These results show that hemin inhibited NLRP3 inflammasome expression at the transcriptional level and involved the HO-1 pathway.

### 3.5. NLRP3, ASC, caspase-1 protein expression are suppressed in the hemin group

Proteins NLRP3, ASC, and caspase-1 in lung tissue were immunoblotted to further determine if NLRP3 inflammasome expressions were down-regulated by hemin at translational levels. We observed marked up-regulations of NLRP3, ASC and caspase-1 protein levels in CLP-induced lung injury. Hemin pretreatment decreased these protein expressions significantly compared with CLP group or ZnPP group (Fig. 5). HO-1 protein expression was markedly enhanced by hemin compared with the CLP and ZnPP groups. These results show that hemin inhibits NLRP3 inflammasome protein expression, and involved HO-1 induction.

### 3.6. Interaction between NLRP3 and ASC was inhibited by hemin

To determine whether the interaction of NLRP3 with ASC was affected by hemin, lung tissue lysate was subjected to immunoprecipitation with ASC. The amount of NLRP3 associated with ASC was increased by CLP treatment. This increase was inhibited by hemin, while it was not enhanced by ZnPP (Fig. 6A). These results show that the activity of NLRP3 inflammasome may be suppressed by hemin.

### 3.7. Caspase-1 activity was inhibited by hemin

To further determine whether NLRP3 inflammasome activity was inhibited by hemin, caspase-1 enzymatic activity was measured in lung tissues. Caspase-1 activity was higher in the CLP group, but hemin pretreatment inhibited CLP-enhanced caspase-1 activity.

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**Fig. 1.** Effects of hemin on septic lung injury in mice. Representative H&E sections of lung (×200). Tissue morphology was analyzed 12 h after cecal ligation and puncture (CLP) induction. Sham-operated animals served as negative controls (sham) (A). Mice were pretreated with vehicle (normal saline) (CLP) (B), hemin (hemin + CLP) (C) or ZnPP (ZnPP + CLP) (D) 12 h before CLP induction. Semi-quantitative lung injury analysis was evaluated in H&E-stained lung tissue sections. Total histopathological score was expressed as summed scores of four independent parameters: alveolar congestion, hemorrhage, aggregation of neutrophil or leukocyte infiltration, and thickness of the alveolar wall (E). The W/D ratio of lung is also shown (F). Results are presented as mean ± standard deviation (SD) (n = 7 in each group). *P < 0.05 vs. the sham group; #P < 0.05 vs. the CLP group.
ZnPP did not increase caspase-1 activity in the CLP group (Fig. 6B). These results show that NLRP3 inflammasome activation induced by CLP may be inhibited by hemin.

3.8. HO-1 enzyme activity is inhibited by ZnPP

We observed that HO-1 was slightly up-regulated by ZnPP pretreatment at the transcriptional and translational levels (Figs. 4A, 5). HO-1 activity assay was performed to determine if HO-1 activity was successfully inhibited by ZnPP. We found that administration of hemin increased HO-1 enzyme activity in mice lung tissues, and that HO-1 enzyme activity was significantly decreased using ZnPP pretreatment (Fig. 7A).

3.9. NF-κB activity was inhibited by hemin

NF-κB p65 transcriptional factor was assayed to determine whether NF-κB activity was inhibited by hemin. NF-κB activity was significantly increased compared with the sham group. However, hemin pretreatment inhibited CLP-induced NF-κB activity in the lung. ZnPP did not increase NF-κB activity compared with the CLP group (Fig. 7B). These results show that hemin inhibited the NLRP3 inflammasome expression possibly by down-regulating NF-κB activity.

3.10. MDA and ROS were reduced by hemin

Determination of lung MDA and ROS levels revealed that hemin pretreatment could reduce MDA and ROS levels in lung tissues compared with the CLP group, but that ZnPP did not increase their levels compared with the CLP group (Fig. 7C, D). These results show that hemin inhibited the activity of NLRP3 inflammasome, possibly by down-regulating ROS levels.

4. Discussion

Our study shows that hemin played important functional roles in the protection against sepsis-induced ALI by NLRP3 inflammasome regulation. After a septic insult, hemin pretreatment inhibited NLRP3 inflammasome activation, inflammatory cells infiltration in the lung, and significantly down-regulated lung injury scores. Hemin is believed to be an inducer of HO-1, and our results show that the expression and enzymatic activity of HO-1 were induced by hemin. We may then conclude that the effect of hemin is partly mediated via HO-1 induction, in accordance with our previous studies [8,14], in which pretreatment of HO-1 with hemin was associated with alleviated sepsis-induced lung injury. However, administration of ZnPP and hemin abolished the protective effects of HO-1 [13].

In the lung, alveolar macrophages are the most abundant innate immune cells, which usually generate excessive quantities of IL-1β in ARDS patients [18]. HO-1 expression in macrophages mediates potent anti-inflammatory effects, possibly by restraining them from inducing lung injury, and by regulating their role in the inflammatory response [19]. IL-1β induces surface abnormalities [20], and alveolar epithelial and vascular endothelial permeability [21], resulting in alveolar edema which is a key component of lung injury. IL-6 levels in sepsis are also sensitive to IL-1β, and infusion of IL-1β in humans led to increased IL-6 plasma levels in a dose-dependent manner [22]. Inhibition of the IL-1 pathway improved ALI scores in animal models [23], but most ARDS patients did not get much benefit from IL-1 receptor antagonist treatment [24]. It may be effective to treat sepsis or ARDS patients by inhibiting more than one pro-inflammatory pathway. Lung tissue injury can be alleviated by genetic deletion or chemical blockade of IL-18 in ALI. Elevated IL-18 concentrations are related to morbidity and mortality in patients with ARDS [6,25]. NLRP3 inflammasome-dependent cytokines IL-33 and HMGB1 also play important roles in sepsis-induced ALI. IL-33 belongs to the IL-1
cytokine family, which also contains IL-1β and IL-18, and NLRP3 inflammasome activation catalyzes its maturation and release. IL-33 increases inflammatory response in the lung [26]. High mobility group box 1 (HMGB1) is a late mediator of lethal systemic inflammation in sepsis. It has been demonstrated that NLRP3 and ASC/caspase-1/IL-1β signaling promotes HMGB1 induction and release [27,28]. NLRP3 inflammasome activation in macrophages influences the secretion of these pro-inflammatory mediators, as well as alveolar permeability [29].

Our study shows that sepsis-induced IL-1β and IL-18 elevation in serum and lung tissues were markedly suppressed by hemin. It has been reported that HO-1 can down-regulate IL-33 and HMGB1 to protect against lung tissue injury [9,26]. These results suggest that the anti-inflammatory effects of hemin may result from its ability to repress NLRP3 inflammasome activation through HO-1, in turn inhibiting caspase-1 maturation and secretion of IL-1β and IL-18. NLRP3 inflammasome inhibition or deletion in ALI may be an effective strategy to decrease caspase-1 activation and improve sepsis-induced ALI [15].

Neutrophil infiltration is a critical characteristic of sepsis-induced ALI and has detrimental effects on lungs during sepsis. However, neutrophil accumulation in the lung can be inhibited by hemin, as shown in the present study. In terms of the relationship between NLRP3 inflammasome and neutrophil recruitment, it has been reported that neutrophil recruitment to thermally-injured liver surface can be blunted by NLRP3 or ASC gene knockout [30]. NLRP3-deficient mice are also resistant to hyperoxia-induced ALI because of decreased neutrophil infiltration [15]. Hemin may inhibit neutrophils recruitment in ALI through NLRP3 inflammasome inhibition.

To investigate the effect of hemin on NLRP3 inflammasome, we observed that hemin down-regulated the components of NLRP3 inflammasome expression in lung tissue. NLRP3 inflammasome is activated in a two-step manner. In the first step, TLR4 agonist induces NLRP3, pro-caspase-1, pro-IL-1β and pro-IL-18 expressions through the NF-κB pathway [31]. Sepsis is usually caused by Gram-negative bacteria infection and involves inflammatory cytokines mediated by endotoxins such as LPS. LPS is the most common TLR4 agonist in polymicrobial sepsis. After LPS binding to TLR4, monocytes and macrophages in the lung are activated, and release inflammatory cytokines. LPS activates NF-κB through both MyD88-dependent and -independent pathways, resulting in the induction of NLRP3 protein expression. It has been demonstrated that NF-κB plays an important role in NLRP3 in LPS-induced NLRP3 expression and its associated pro-mediators [31].
HO-1 and its catalyzed product CO have been reported to negatively regulate TLR signaling pathway, to inhibit NF-κB activation in various cells and tissues [32]. In the present study, we observed that NF-κB activity in ALI was decreased by hemin. Therefore, hemin may inhibit NLRP3 inflammasome expression by involving the HO-1 pathway.

After this priming phase, NLRP3 inflammasome can be activated by reactive oxygen species (ROS), mitochondrial DNA (mtDNA) or other stimuli [33,34]. A recent study demonstrated that increased ROS promoted NLRP3 expression at the transcriptional level, but not NLRP3 activation [35]. To further evaluate the influence of hemin on NLRP3 inflammasome activation, we observed that hemin repressed the binding of NLRP3 with ASC and caspase-1 activity. Meanwhile, hemin reduced MDA and ROS production in lung tissue. Mitochondrial damage is well recognized in sepsis [36]. Overproduction of ROS and mtDNA released from damaged mitochondria both participate in the pathogenesis of sepsis [37]. HO-1 is an important antioxidant enzyme that modulates intracellular ROS levels. This effect may depend on its derived biliverdin and bilirubin that are considered to be physiologically important defenses against ROS [38]. It has also been reported to protect mitochondria from damage and to facilitate mitochondria biogenesis [39], significantly suppressing ROS generation and oxidative stress [32,40]. Hemin may inhibit NLRP3 inflammasome activation involving HO-1 pathway.

![Image of Fig. 4](image-url)

**Fig. 4.** Effects of hemin on NLRP3, ASC and caspase-1 mRNA expression in lung tissues in the CLP-induced ALI mice model. HO-1 (A), NLRP3 (B), ASC (C) and caspase-1 (D) mRNA expressions were determined by real-time RT-PCR. β-Actin was used as an internal control. Results are presented as mean ± standard deviation (SD) (n = 7 in each group). *P < 0.05 vs. the sham group; #P < 0.05 vs. the CLP group.

![Image of Fig. 5](image-url)

**Fig. 5.** Effects of hemin on NLRP3, ASC and caspase-1 protein expression in the mice model of CLP-induced ALI. HO-1, NLRP3, ASC and caspase-1 p10 protein expressions were determined by western blot. Protein expression was normalized to β-actin. Results are presented as mean ± standard deviation (SD) (n = 3–5 in each group). *P < 0.05 vs. the sham group; #P < 0.05 vs. the CLP group.
This study used a CLP-induced ALI model to investigate how hemin affects NLRP3 in inflammasome through HO-1. Despite the strong negative correlation between HO-1 and NLRP3 in inflammasome in lung tissue, a direct causal interaction could not be confirmed because of the limitations of the present in vivo study. The influence of hemin on the formation of active NLRP3 inflammasome complex in inflammatory cells could not be directly observed. ZnPP also failed to significantly exacerbate lung injury and inflammatory response, which may be due to its incomplete or competitive inhibition. Our study shows that HO-1 protein expression and HO-1 enzymatic activity were slightly elevated in the CLP group. Thus, it may be very difficult to aggravate CLP-induced lung injury and inflammation for a competitive enzymatic inhibitor. ZnPP may reverse the effect of hemin on HO-1 at a higher level of enzymatic activity [13]. We did not use HO-1 knockout mice or an in vitro knockout model to directly study the impact of HO-1 on NLRP3 inflammasome, and there is still a possibility that hemin affects the NLRP3 inflammasome through other independent effects. Nevertheless, our study yielded important new insights about the anti-inflammatory mechanisms of hemin in sepsis-induced ALI.

In summary, hemin protects against sepsis-induced ALI through NLRP3 inflammasome inhibition, reducing IL-1β and IL-18 secretion and restricting the inflammatory response. HO-1 is involved in the effect of hemin on NLRP3 inflammasome. However, the mechanisms of interaction between HO-1 and NLRP3 inflammasome remain to be clarified. A better understanding of the interrelated processes involved in ALI might reveal new therapeutic strategies to prevent and/or treat this life-threatening condition.

Conflict of interest

The authors declare that they have no conflict of interest.

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References