Research Article

Heme Oxygenase-1 Inhibits the NLRP3 Inflammasome to Protect Against Severe Acute Pancreatitis

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Abstract

Background: Systemic Inflammatory Response Syndrome (SIRS) and IL-1β and IL-18 maturation and production are encouraged by the NLRP3 inflammasome. Severe Acute Pancreatitis (SAP) may be less severe if certain tactics are used to prevent the NLRP3 inflammasome. The rate-limiting enzyme in the breakdown of heme, Heme Oxygenase-1 (HO-1), has anti-inflammatory, antioxidant, and anti-proliferative properties. HO-1 activity profoundly affects the host's ability to forbear infection by reducing tissue damage or affecting resistance and increasing the capacity to pathogen load. We postulated that hemin, a strong HO-1 inducer, could decrease NLRP3 inflammasome activation, which would lessen the severity of SAP and acute lung injury caused by pancreatitis.

Methods: By administering intraperitoneal injections of Caerulein (Cae) and Lipopolysaccharide (LPS) to SD rats, a model of SAP was created. Hemin and zinc protoporphyrin IX (Znpp, a HO-1 inhibitor) pretreatments respectively stimulated and inhibited the HO-1 enzyme.

Results: The SAP model rats' pancreas and lungs suffered considerable pathological damage after Cae and LPS injection, and their serum levels of amylase, lipase, and the cytokines IL-1β and IL-18 also rose. Hemin pretreatment decreased IL-1β and IL-18 release in the serum and prevented pancreatic and pulmonary damage. Hemin dramatically reduced oxidative stress, downregulated the expression of the proteins NLRP3, ASC, and Caspase-1, and elevated the expression of the protein HO-1. On the contrary, there were no discernible changes between the SAP and Znpp groups.

Conclusions: These results show that hemin prevents Cae and LPS-induced lung and pancreatic harm through suppression of the NLRP3 inflammasome. Hemin's impact on the activity of the NLRP3 inflammasome depends critically on HO-1.

Keywords: Severe acute pancreatitis; NLRP3; HO-1; Inflammation

Abbreviation: SIRS: Systemic Inflammatory Response Syndrome; SAP: Severe Acute Pancreatitis; HO-1: Heme Oxygenase-1; Cae: Caeruelin; LPS: Lipopolysaccharide; Znpp: Zinc Protoporphyrin IX; ALI: Acute Lung Injury; ARDS: Acute Respiratory Distress Syndrome; NLRs: Nod-Like Receptors; SD: Sprague-Dawley; CO: Carbon Monoxide; MDA: Malondialdehyde; SOD: Superoxide Dismutase; ANOVA: One-Way Analysis of Variance

Introduction

A pancreatic inflammation that poses a serious risk to life is known as Severe Acute Pancreatitis (SAP). Inflammatory mediators have an essential effect on the evolution of AP from mild to severe and in the occurrence of MODS in SAP. The causes of SAP death are mostly related to MODS, among which Acute Lung Injury (ALI) and ALI-induced Acute Respiratory Distress Syndrome (ARDS) are the most severe and common. During SAP, premature trypsin activation within pancreatic acinar cells causes

pancreatic auto-digestion, leading to a local inflammatory process; these mediators release many relevant pro-inflammatory factors. The increase of capillary permeability and extravascular lung water is a crucial pathophysiological change in ALI/ARDS, and the severe ventilatory blood flow ratio imbalance caused by increases of extravascular lung water is an important reason for refractory hypoxemia and high mortality of ALI/ARDS. The inflammatory cascade brought on by sterile inflammasome activation has been associated with SAP and ALI. Inflammasome NLRP3 is an oligomeric molecular complex activated not only by bacteria and viruses but also by its "danger signals."

The specific intracellular pattern recognition molecules known as Nod-Like Receptors (NLRs) control the host's innate immune response [1]. The aggregation of inflammasomes is facilitated by NLRP3, which functions similarly to other NLR proteins. A NOD-like receptor, adaptor protein ASC [Caspase-1 Activator domain-containing protein (CARD)], and caspase-1 itself make up the macromolecular multi-protein NLRP3 inflammasome. The NLRP3 inflammasome is created when NLRP3 is activated, ASC, and pro-caspase-1 are recruited, and then caspase-1 is activated, which results in the conversion of pro-IL-1β and pro-IL-18 into their activated versions [2].

The inducible isomer of Heme Oxygenase-1 (HO-1) is a rate-limiting enzyme that catalyzes the oxidative breakdown of heme [3]. Its physiological role is to accelerate the production of heme, which releases ferrous iron, biliverdin, and Carbon Monoxide (CO). HO-1 is an essential metabolic enzyme and a vital intermediate between stress response and cellular injury adaptation. In many cellular and preclinical damage models, the transcriptional regulation of HO-1 has been widely related to cytoprotection and the protective inhibition of inflammation [4,5]. HO-1 and heme degradation products may modulate inflammation. CO can stimulate the mitochondrial to produce ROS, promoting the autophagy program, activating HIF-1a, and downregulating the pro-inflammatory transcription factor Egr1. Additionally, recent research suggests that CO can influence how the NLRP3 inflammasome, which controls the production of IL-1β and IL-18, is activated [6].

Two hypotheses were intended to be proved by this investigation. Firstly, by preventing the NLRP3 inflammasome from becoming activated in the Cae and LPS-induced SAP mice, hemin could reduce organ injury and inflammation. Second, it's possible that HO-1 contributes to the NLPR3 inflammasome's activation.

Materials and Methods

Animals

Healthy male Sprague-Dawley rats weighing 260–300g were procured from the Experimental Animal Center of Anhui Medical University. The National Society for Medical Research and Guidelines for Laboratory Animal Care provided the animals with humane treatment. The rats were kept in a temperature-controlled space (25±1°C) with a 12-hour light/dark cycle and unrestricted access to food and drink.

Animal Model of Severe Acute Pancreatitis

Twenty-four male SD rats were divided into four groups at random: control, SAP, Hemin (40g/kg; Sigma Chemical, St. Louis, MO) and Zn-PP (40g/kg; Sigma Chemical, St. Louis, MO) groups. The HO-1 stimulator Hemin was injected intraperitoneally 30 min after SAP induction, and the HO-1 inhibitor Zn-PP was injected intraperitoneally 30 min later. SAP was induced by injecting 50g/kg of cerulein (Sigma-Aldrich) in 0.9% Nacl. LPS (Sigma-Aldrich) was administered intraperitoneally to the animals at a dose of 5 mg/kg following the last injection. 24 hours after the development of SAP, rats were given intraperitoneal sodium pentobarbital (40 g/kg) to induce anesthesia before being put to death. Intracardiac puncture blood samples were centrifuged, and the serum was kept at -80°C for investigation of the levels of amylase, lipase, IL-1β, and IL-18. Western blot and histological analysis of the pancreatic and lung tissues were used to compare the histopathological alterations in the organs between subgroups.

Histological Examination Analysis

Pancreas and lung tissue samples were fixed in 10% formalin solution, embedded in paraffin, and sectioned following hematoxylin and eosin staining for use in light microscopy. For each rat, The sections were evaluated blindly by two observers. Alveolar walls were characterized by diffuse reactions, thickening, and the presence of inflammatory cells (neutrophil and mononuclear) [7]. The scale for the score is 0 to 4; an injury severity scale would seem as follows: Injury levels range from 0 (lack of injury) to 4 (severe or intense injury), where 4 denotes the highest level of injury. The average scores were taken as the final score. Pancreas damage [8] was scored by grading, as shown in Table 1. The average scores were taken as the final score.

Measurements of Cytokines in Serum

To obtain a supernatant, homogenized blood samples from the various groups were centrifuged at 3000 x g for 10min at 4°C. Commercial ELISA kits (R&D Systems, Minneapolis, MN) were used to evaluate the levels of IL-1β and IL-18 in rat blood samples in line with the manufacturer's procedure. In pg/ml, cytokine concentrations were expressed.

Serum Biochemical Assays

Using an automatic biochemical analyzer (UniCel DxC800, Beckman Coulter, CA), the levels of amylase and lipase were determined.

Measurement of Superoxide Dismutase (SOD) and Malondialdehyde (MDA) Concentrations

According to the manufacturer's instructions, Beyotime Biotech, Inc. of Jiangsu, China, the relevant kits were used to measure the concentrations of MDA and SOD. After homogenizing and centrifuging the pancreas and lung tissues at a speed of 12,000 for 15 minutes, the supernatant was collected for spectrophotometric analysis.

Table 1: Pancreas pathological scoring criteria.

Ederma	Inflammatory cellular infiltration	Vacuolization	Necrosis
0-absent	0=absent	0=absent	0=absent
1=diffuse expansion of interlobar septa	1=around ductal margin	1=periductal, <5%	1=1-4 necrotic cells/HPF
2=diffuse expansion interlobular septa	2=in parenchyma, <50% of lobules	2=focal, 5-20%	2=5-10 necrotic cells/HPF
3=diffuse expansion interacinar septa	3=in parenchyma, 50-75% of lobules	3=diffuse, 21-50%	3=11-15 necrotic cells/HPF
4=diffuse expansion intercellular septa	4=in parenchyma, >75% of lobules	4=severe, >50%	4≥16 necrotic cells/HPF

HPF, high-power field

Western Blot Analysis

Different groups' lung and pancreatic tissues were homogenized in lysis buffer (P0013B, Beyotime, Shanghai, China), and the supernatant containing the extracted protein was then spun at 4°C for 10 minutes. For electrophoresis, proteins were placed onto 10-12% polyacrylamide gels. Proteins were subsequently transferred to polyvinylidene difluoride membranes. NLRP3 (1:500; ab214185, Abcam), HO-1 (1:1000; ab68477, Abcam), ASC (1:1000; sc-514414, Sant Cruz Biotechnology), Caspase-1 (1:1000; 22915-1-AP, Protein Tech), and monoclonal antibody against mouse -actin (1:1000; ab8227, Abcam) were incubated at 4°C after blocking with blocking buffer. The membranes were then treated with HRP-conjugated anti-rabbit IgG antibody (dilution, 1:5000) for an hour following three washes in tris-buffered saline with Tween-20 (0.1%). The membrane was once more washed three times for a total of 10 minutes using a western blotting detection kit with enhanced chemiluminescence. Thermo Fisher Scientific's ECL detection technology was used to find all antibodies.

Statistical Analysis

Utilizing GraphPad Prism 6 (GraphPad Software Inc, San Diego, CA, USA), statistical analysis was carried out. One-way analysis of variance (ANOVA) is used to compare continuous variables between the four groups, and Tukey's post hoc test for multiple comparisons is used afterwards. Statistics were judged significant at P<0.05.

Results

Hemin Reduced the Severity of SAP-Induced Pancreas and Lung Injury

The SAP model was successfully developed, as shown by the significantly elevated blood levels of AMY and LIPA in the SAP group compared to the control group (Figure 1). When compared to the SAP group, the levels of AMY and LIPA in the serum in the hemin pretreatment group were reduced (P<0.05). However, compared to the SAP group, the AMY and LIPA in the ZnPP administered group rose.

While the pancreatic of SAP rats revealed partial bleeding, necrosis, and neutrophil granulocyte infiltration, the pancreas of control rats showed morphologically normal tissue. While Znpp therapy caused more severe pathological pancreas damage, hemin administration alleviated pathological damages in the pancreas brought on by SAP. In contrast to HO-1 inhibition, which dramatically raised the scores, HO-1 stimulation significantly reduced abnormal scores (Figure 2).
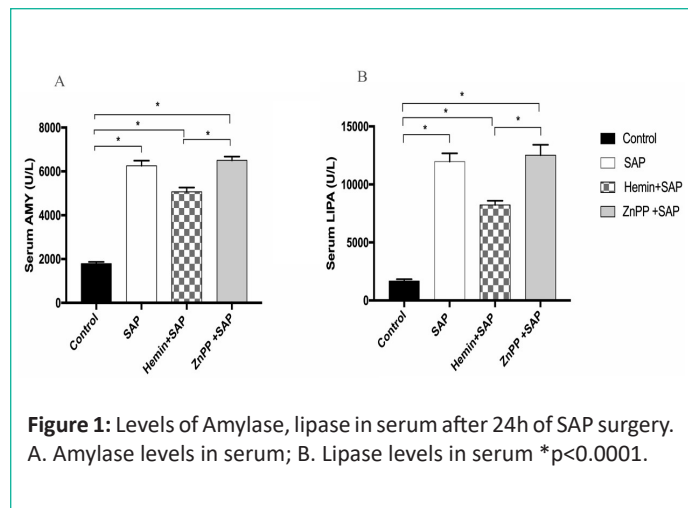

Figure 1: Levels of Amylase, lipase in serum after 24h of SAP surgery. A. Amylase levels in serum; B. Lipase levels in serum *p<0.0001.
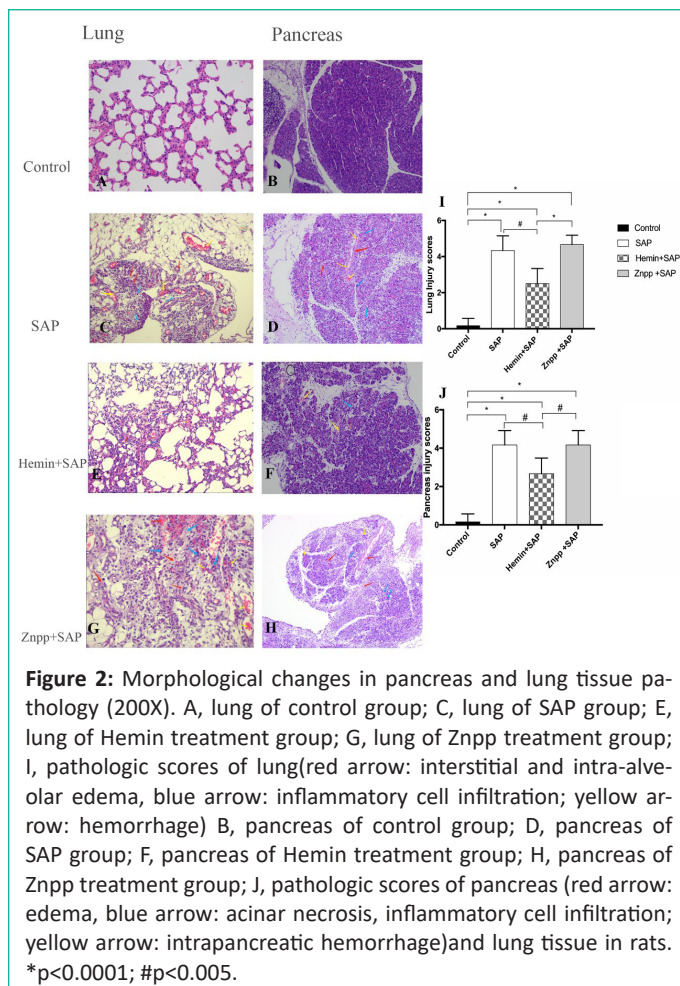

Figure 2: Morphological changes in pancreas and lung tissue pathology (200X). A, lung of control group; C, lung of SAP group; E, lung of Hemin treatment group; G, lung of Znpp treatment group; I, pathologic scores of lung(red arrow: interstitial and intra-alveolar edema, blue arrow: inflammatory cell infiltration; yellow arrow: hemorrhage) B, pancreas of control group; D, pancreas of SAP group; F, pancreas of Hemin treatment group; H, pancreas of Znpp treatment group; J, pathologic scores of pancreas (red arrow: edema, blue arrow: acinar necrosis, inflammatory cell infiltration; yellow arrow: intrapancreatic hemorrhage)and lung tissue in rats. *p<0.0001; #p<0.005.
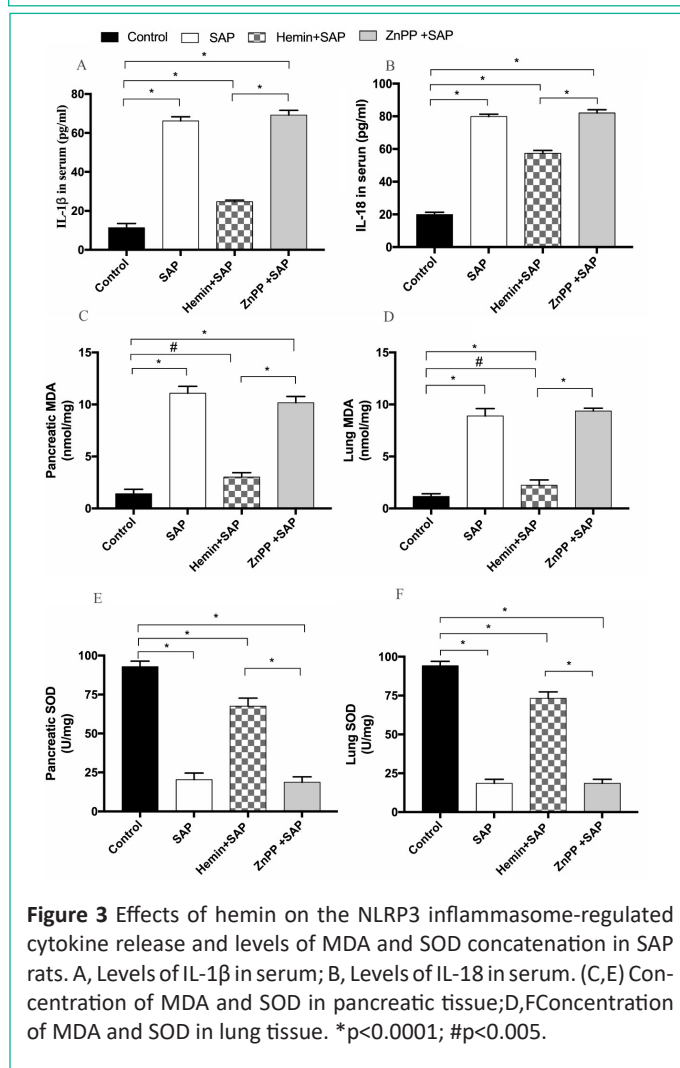

Figure 3 Effects of hemin on the NLRP3 inflammasome-regulated cytokine release and levels of MDA and SOD concatenation in SAP rats. A, Levels of IL-1β in serum; B, Levels of IL-18 in serum. (C,E) Concentration of MDA and SOD in pancreatic tissue;D,FConcentration of MDA and SOD in lung tissue. *p<0.0001; #p<0.005.
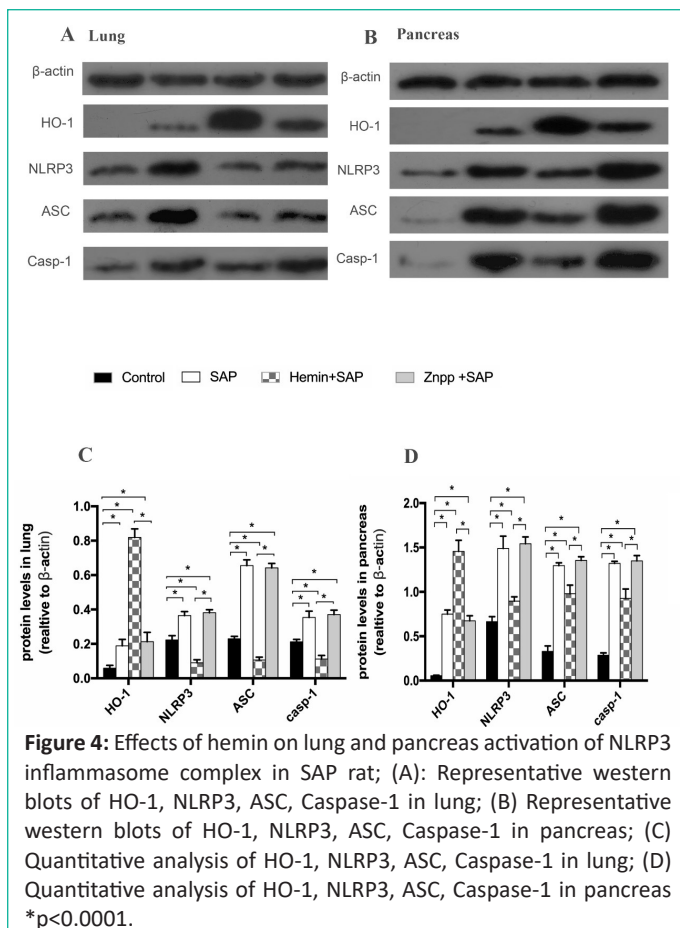
Figure 4: Effects of hemin on lung and pancreas activation of NLRP3 inflammasome complex in SAP rat; (A): Representative western blots of HO-1, NLRP3, ASC, Caspase-1 in lung; (B) Representative western blots of HO-1, NLRP3, ASC, Caspase-1 in pancreas; (C) Quantitative analysis of HO-1, NLRP3, ASC, Caspase-1 in lung; (D) Quantitative analysis of HO-1, NLRP3, ASC, Caspase-1 in pancreas *p<0.0001.

The control group's lung tissue sections stained with H&E revealed normal morphology and a little infiltration of inflammatory cells. The alveolar ridge did not appear to widen. However, the alveolar wall broadened substantially and was diffusely infiltrated with inflammatory cells in the SAP group. In the group receiving hemin therapy, there were less inflammatory cells present and the alveolar wall was slightly wider. Like the pancreas tissue, the ZnPP treatment caused more severe pathological lung injury (Figure 2).

Hemin Attenuated NLRP3 Inflammasome-Regulated Cytokines and Decreased Oxidative Stress Responses

To find out whether hemin lowered NLRP3 inflammasome-dependent cytokines, IL-1β and IL-18 in the serum were evaluated (Figure 3). Figure 3 demonstrates that the SAP group had significantly higher levels of IL-1β and IL-18 than the control group (p<0.05). In the group that had been pretreated with hemin, there was a significant drop in serum levels of IL-1β and IL-18 (p<0.05). However, the ZnPP group had considerably higher levels of IL-1β and IL-18 (p<0.05). In comparison to the control group, the concentration of MAD in pancreatic and lung tissues was higher in the SAP group, and it significantly decreased in the hemin pretreatment group. Znpp, however, raised the levels of MAD in lung and pancreatic tissues. SOD levels were noticeably decreased in the pancreatic and lung tissues of the SAP group compared to the control group. Hemin pre-treatment raised SOD levels in comparison to the SAP group, however SOD levels drastically fell in the Znpp-treated group.

Hemin Decreased NLRP3 Inflammasome in the SAP Model

When compared to the control group, the NLRP3, ASC, and caspase-1 levels in the lung and pancreas tissue dramatically increased in the SAP group (Figure 4). When compared to the SAP group, the mRNA expression of NLRP3, ACS, and caspase-1 con-

siderably decreased. In contrast to the group pretreated with hemin, the ZnPP pretreatment improved the mRNA expression. Hemin significantly up-regulated HO-1 mRNA levels as compared to SAP and ZnPP.

Discussion

In this study, a rat model of SAP was created using Cae and LPS, which caused injury to the pancreas and lung as well as an inflammatory response. In SAP rats, hemin was essential in protecting against ALI and pancreas injury. Hemin pretreatment lessened the severity of SAP. Treatment with hemin significantly decreased amylase levels in the serum and histopathological alterations in the SAP rats. Hemin pretreatment greatly reduced the rise in MDA levels and increased SOD activity in the tissues of the pancreas and lungs. Additionally, hemin treatment inhibited NLRP3 inflammasome activation. These results suggested that hemin was critical in attenuating the severity of SAP by NLRP3 inflammasome regulation.

The endogenous cytoprotective enzyme Hemeoxygenase (HO-1) is a protein that responds to oxidative stress. An vital endogenous defense mechanism made up of HO-1 and its induction, which has a range of biological functions including anti-inflammatory, antioxidant, and cytoprotective effects. According to reports, HO-1 can reduce the harmful effects of oxidative stress and hence preserve organs [9,10], in part by reducing inflammation brought on by heme metabolites. Thus, HO-1 protects the pancreas and lungs from oxidative stress and inflammation-induced injury.

Hemin markedly reduced the levels of IL-1β and IL-18 in the serum in the pancreas or lungs of SAP rats caused by Cae or LPS. Linked to SAP, and their increase correlates positively with its severity, IL-1β and IL-18 are closely [11,12]. A significant reduction in pancreatic pathology, inflammation, and severity of SAP-ALI has been demonstrated by blocking IL-1β and IL-18 [13,14]. Recently, many studies have shown that the pathogenesis and progress of pancreatitis are associated with inflammation and excessive immune response [15,16]. NLRP3 inflammasome, a macromolecular complex, is an inducer of the immune response, which has the function of identifying and targeting a variety of pathogens. An inflammatory response is brought on by the activation of the NLRP3 inflammasome, which causes pre-protease-1 to become caspase-1 and catalyzes the maturation of pre-IL-1β and pre-IL-18.

By western blotting analysis, we examined the NLPR3 inflammasome and HO-1 pathway in the lung and pancreas for correlations. Hemin significantly reduced the expression of NLPR3 inflammasome-associated proteins, including as NLRP3, caspase-1, and ASC, which in turn reduced the production of pro-inflammatory cytokines. A significant amount of the NLRP3 inflammasome is activated during AP, and this inflammasome's components are necessary for complete pancreatic damage. The severity of pancreatitis can be decreased by NLRP3's negative regulation. The maturation and release of IL-1β and further reduction of the inflammatory cascade were observed in NLRP-3 knockdown animals or NLRP3 inhibitor INF-39 treated mice in an experimental model of LPS-induced AP in mice [17]. Our results support prior research' findings that increased HO-1 may prevent the NLRP3 inflammasome from becoming activated [18,19].

Caerulein has been proven to stimulate the maximum secretion of pancreatic amylase and lipase [20]. The excessive se-

cretion of amylase and lipase leads to SAP and SAP-associated ALI. Both the pancreas and lung tissues in SAP rats found many inflammatory cells infiltrated. Neutrophils have been shown to enhance the production of ROS. The recruitment of inflammatory cells and tissue damage are mediated by ROS, which participate in the inflammatory cascade. ROS and oxidative stress contribute to the damage to pancreatic acinar cells that results from SAP [21,22]. Moreover, ROS affects the action of antioxidant enzymes and oxidative stress, which activates the NLRP3 and plays a crucial role in proinflammatory responses [23,24]. NLRP3 activation originates from various stimuli, including the generation of ROS. They are the first intermediate reaction products during inflammasome activation and are responsible for releasing inflammatory factors during the immunological reaction. Previous research discovered that the transcription of antioxidative stress proteins, such as HO-1, is increased when the level of ROS is elevated [25]. The effects of HO-1 induction on inflammation and ROS inhibition were also discovered by researchers [26-28]. An essential antioxidant enzyme called HO-1 controls intracellular ROS levels. Accordingly, Hemin may inhibit NLRP3 inflammasome activation by suppressing ROS production. The Cae and LPS-induced SAP paradigm was used in this investigation to investigate how hemin influences the NLRP3 inflammasome via HO-1. Due to the present study's limitations, a direct causal link between HO-1 and NLRP3 inflammasomes in lung and pancreatic tissue could not be established. The effect of HO-1 on the NLPR3 inflammasome was not specifically investigated using the HO-1 knockout animal model, and it is still possible that hemin affects the NLPR3 inflammasome via other independent processes. Furthermore, the mechanism by which hemin treatment attenuates organic injury of SAP remains to be investigated in further studies.

Conclusion

In summary, hemin prevents lung and pancreatic damage brought on by Cae and LPS by inhibiting the NLRP3 inflammasome. Uncertainty surrounds HO-1 and NLRP3 inflammasome, and HO-1 plays a role in how hemin affects NLRP3 inflammasome. However, by lowering the inflammatory response and organ damage, the mechanisms of interaction between the induction of HO-1 in SAP may offer a brand-new and potent treatment for SAP.

Author Statements

Ethics Approval and Consent to Participate

Not Applicable. This article contains no studies with human participants or animals performed by authors.

Availability of Data and Material

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this paper.

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Author's Contributions

YS and WG: Conceptualization; Funding acquisition; YS and JQ: Methodology, Investigation, Wring Original Draft writing manuscript, TY, and MY: Software, Resources, Data Curation, Visualization; CY: Formal analysis, Investigation, WG: Writing-reviewing and Editing, Supervision. The author(s) read and approved the final manuscript.

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