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Blocking Agent of Heme Oxygenase-1 Zinc Protoporphyria Induces Autophagy and Accelerates Oxidative Damages during Lipopolysaccharide-Induced Lung Injury in Rat

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Abstract

To investigate the effect of blocking agent of heme oxygenase-1 zinc protoporphyrin (Znpp) on lipopolysaccharide (LPS)-induced autophagy in acute lung dysfunction, the rats were divided into control (C), LPS (L), LPS +Hemin (Hemin) and LPS+ZnPP (ZnPP) groups. Treatment with ZnPP induced autophagy and accelerated oxidative damages during lipopolysaccharide treatment in rat lung, LPS+ZnPP increased pathological alterations in lung tissues, the number of ballooned pulmonarycytes, serum tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels, and myeloperoxidase (MPO) and malondialdehyde (MDA) levels in lung tissues ($P < 0.05$) but attenuated by LPS +Hemin treatment. Thus, ZnPP may aggravate LPS-induced acute lung dysfunction in rats, possibly by increasing inflammation and accelerating oxidative damages. LPS+Hemin group prolonged the median survival time and reduced lung dysfunction. Moreover, HO-1 may significantly contribute to lung protection.

Keywords: Zinc protoporphyrin (Znpp); Heme oxygenase-1; Acute lung dysfunction; LPS

Abbreviations:

LPS: Lipopolysaccharide; Znpp: Zinc Protoporphyrin; TNF: Tumor Necrosis Factor; IL: Interleukin; MPO: Myeloperoxidase; MDA: Malondialdehyde; HO: Heme Oxygenase; ELISA: Enzyme-Linked Immunosorbent Assay; NF-κB: Polyclonal Mouse Anti-Nuclear Factor Kappa B; C: Control; TEM: Transmission Electron Microscopy; H&E: Hematoxylin and Eosin

Introduction

The inflammatory cells in pulmonary can be activated by LPS, result in releasing a large amount of inflammatory medium, and further triggering waterfall inflammatory reaction, cause the diffuse lung tissue damage [1]. At present, most of the views consider that the activation of neutrophils, the release of inflammatory factors and inflammatory mediators, the Oxidative stress response, the apoptosis and autophagy of cells et al, play an important role in the pathogenesis of LPS-induced lung injury, and the excessive inflammation in lung is the primary cause of ALI [2,3], the apoptosis and autophagy of cells have a key role the pathogenesis of LPS-induced lung injury [4].

Autophagy is an elimination behavior for the autologous "harmful" or "aging" composition, it prevents toxic accumulation of damaged organelles and misfolded proteins and is responsible for intracellular clearance of misfolded proteins, which may threaten the cell survival, its function plays an important role in the process of numerous diseases. It is an catabolic process where organelles and proteins are isolated by a double-membraned vesicle, which is called autophagosome, then the autophagosome fuses with endosomes to form amphisomes which, in turn, later fuse with lysosomes, at last the entrapped cytosolic contents are recycled and digested [5-12]. As significative physiological reactions in body, the autophagy, in different forms, under different conditions, will form different "dynamic balance" [13], studies showed that when the macrophages are stimulated by LPS, once the level of autophagy is low, would make the body in the pathological state; however, the excessive autophagy will cause cell death and lead to disease [14-18].

Heme oxygenase (HO) is a kind of enzyme which degrades intracellular heme to free biliverdin, carbon monoxide and iron [19]. The HO-1 is a kind of protective endogenous protein, with the function of anti-inflammation, resistance to oxidative stress and resisting cell apoptosis [20-22], its expression can be induced not only by its physiological substrate heme but also by various noxious stimuli or conditions, such as hypoxia, hyperoxia, proinflammatory, nitric oxide, cytokines [23]. HO-1 can be rapidly induced by oxidative challenge and other noxious stimuli [24,25]. Kana Unuma's [26] studies show that the expression of HO-1 can reduce the sensitivity of experimental animals to the lethal dose of LPS and improve the survival rate. Therefore the pathway of HO-1 plays an important role on the protection of the tissue damage caused by LPS [27-29].

This study use the LPS-induced acute lung injury rats as models, to explore the relationship between the ZnPP and the lung tissue damage of rats, as well as autophagy of lung cell, and to determine whether the ZnPP induces autophagy and accelerates oxidative damages or not.

Materials and methods

Reagents

LPS was obtained from Sigma (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α and IL-6 were purchased from Abcam (Cambridge, MA, USA). MPO and MDA detection kits were obtained from the NanJing Jiancheng Bioengineering Institute (China). Polyclonal mouse anti-nuclear factor

kappa B (NF-κB) antibody was obtained from Neomarkers (Fremont, CA, USA). Monoclonal rabbit anti-second mitochondria-derived activator of caspase (Smac) antibody was purchased from Santa Cruz (Santa Cruz, CA, USA).

Animals and treatment

A total of 32 specific pathogen-free (SPF) level male Sprague-Dawley (SD) rats, with a body mass of 250-350 g, were provided by the Laboratory Animal Center at the Affiliated Shengjing Hospital of China Medical University in Shenyang, China. The rats were housed in a controlled environment at 22-25◦C. The animals had free access to food and water. Rats were randomly divided into four groups: c (n=8), L (n=8), Hemin (n=8) and ZnPP (n=8). Rats in the L group were given a single caudal vein injection of 8 mg/kg LPS, and rats in the C group received the same dose of normal saline using the same method. In the Hemin group, rats were given 40 umol/kg Hemin by enterocoelia injection and every 12 h for a continuous 2 d prior to LPS treatment. In the ZnPP group, rats were given 10 umol/kg ZnPP enterocoelia injection 30 min prior to LPS treatment. All rats were anesthetized with intraperitoneal injection of 15% urethane (0.8 ml/kg body weight). Samples were obtained from these rats for examination. The all efforts were made to reduce the amount of animals used and to minimize animal suffering. All animal experiment were approved by the Affiliated Shengjing Hospital of China Medical University.

Determination of lung damage and cytokine concentrations

The degree of lung injury was assessed by the histological activity, Optical microscope analysis and the transmission electron microscopy (TEM) analysis. The serum level of TNF-α and IL-6 were measured by ELISA kits. The level of MPO and MDA in lung tissue was evaluated by detection kits according to the manufacturer's instructions.

Optical microscope analysis

The lung was carefully removed from animals 6 h after the final injection. The paraffin-embedded lung tissue was sectioned into 5 μm slices, then fixed in 4% paraformaldehyde, deparaffinized, and rehydrated at last. The samples were then stained with H&E and then evaluated under a microscope at a 400X magnification.

Transmission electron microscopy (TEM) analysis

Lung tissue was fixed in 2.5% glutaraldehyde, and then post-fixed in 1% osmium tetroxide. After dehydration through a series of ethanol gradients, the samples were embedded in Epon 812 epoxy resin, sectioned and double-stained with uranyl acetate and lead citrate at last. The samples were evaluated using a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan).

Western blot analysis

Total protein samples were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, then the protein samples were transferred to the membranes of polyvinylidene fluoride by wet turn membrane method. After blocking, the membranes were probed using rabbit anti-rat primary antibody. Immune complexes were detected by incubation using horseradish peroxidase (HRP)-conjugated secondary antibody and an ECL detection system.

Statistical analysis

Statistical analyses were accomplished by SPSS software version 17. 0. The data was presented with mean ± standard deviation. The t-test was using to compare the differences between two groups. The P values less than 0.05 were considered with statistically significant.

Results

ZnPP aggravated LPS-induced lung dysfunction

As shown in Figure 1, under the optical microscope, control rats exhibited a normal structure of branches and bronchioles, alveolar walls were made up of alveolar septum and alveolar epithelial cells, the size of alveolar cavity was normal, there were no inflammatory cells could be seen. In L group, Lung damage and the appearance of abnormal lung structure could be seen, pulmonary mesenchyme and alveolar space were diffuse with inflammatory cells, the alveolar septum were getting wide and some of them were even fractured, in addition, the alveoli atrophy and the structures of alveoli were disappeared also could be seen. In Hemin group the pathological changes obviously alleviated. And the damage degree of lung tissue in ZnPP group was higher than the corresponding changes of LPS.

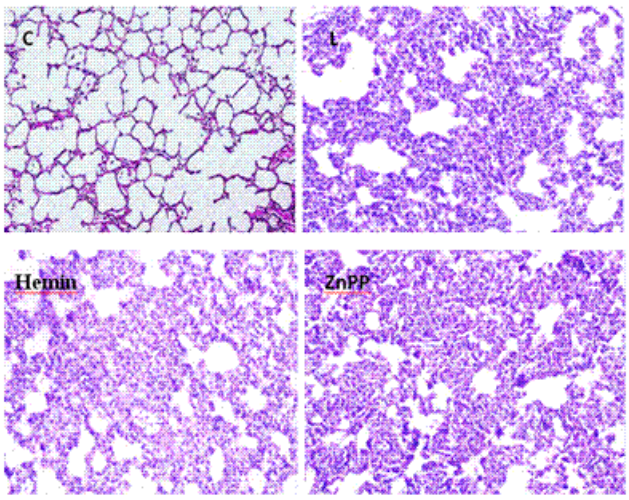

Figure 1: Pathological analysis was performed by H&E staining (400X magnification)

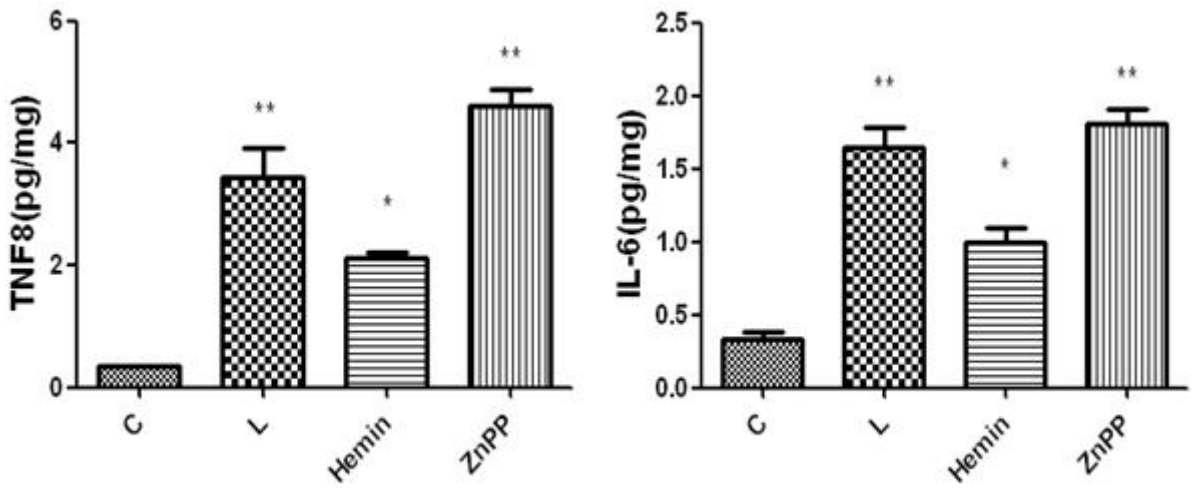

Figure 2: Both L and ZnPP group were significantly higher than group C, **, $P<0.01$. Hemin group was obviously higher than group C, lower than group L, *, $P<0.01$

ZnPP increased LPS-induced cytokine production

LPS could significantly promote serum cytokine generation, which included TNF-α and IL-6, compared to the control. However, the ZnPP statistically increased the serum level of both TNF-α and IL-6 in animals that received LPS. And the Hemin reduced the serum level of that (Figure 2).

ZnPP accelerated oxidative damages

As MDA and MPO are biomarkers for oxidative stress, we examined the level of MDA and MPO in lung tissue in rats after different treatment. As expected, LPS stimulating significantly upregulated MDA and MPO expression ($P<0.05$ compared to control), whereas ZnPP increased the level of MDA and MPO compared to LPS treatment alone (Figure 3).

ZnPP induced autophagy during LPS treatment in rat lung

In the electron microscopy, in control group, the volume of alveolar cell of type II was normal, cuboidal and full of microvilli on its surface. The cells' nucleus could be seen obviously, it was circular and with little heterochromatin. In cytoplasm, some osmiophilic multilamellar body could be seen, the layer was clear. In L and ZnPP group, the nuclear membrane of alveolar cell of type II was fuzzy and edema, the chromatin within nucleus concentrated to the edge and the mitochondrial cristae decreased. We also observed the autophagosome in these two groups. The ZnPP group was more significant. In Hemin group, the pathologic change level of alveolar cell of type II and the number of autophagosome were descending compared to the LPS group (Figure 4).

Variation of HO-1 expression

The outcomes of western blot showed that, compared with C group, the HO-1 expression of the other three groups were all increasing, the highest of which was Hemin group, L group second, the least was ZnPP group (Figure 5).

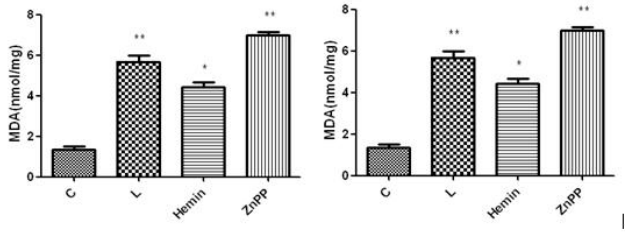

Figure 3: Both L and ZnPP group were significantly higher than group C, **, $P<0.01$. Hemin group was obviously higher than group C, lower than group L, *, $P<0.01$

Discussion

In this experiment, we successfully obtained the model of LPS-induced acute lung injury, we observed, after LPS intravenous injection 6 h, the serum level of TNF-α and IL-6 increased, TEM and optical microscope showed severe lung injury; the autophagosome could be seen in the TEM. The study demonstrated that, except the C group, among the three groups of LPS-induced acute lung injury, the ZnPP group was the most severe, the LPS group took the second place, and the Hemin group was the lightest.

According to the result, except the C group, in ZnPP group, the degree of lung injury in optical microscope; the serum level of TNF-α and IL-6; the level of MDA (the content of MDA was considered as whether the body or organs in oxidation/anti-oxidation state or not [30]) and MPO in lung tissue in the animals that received LPS; the number of autophagosome in the TEM and the dose of HO-1 expression, were all higher than the Hemin group and the LPS group. AT the same time, every indicator of Hemin group was the lowest.

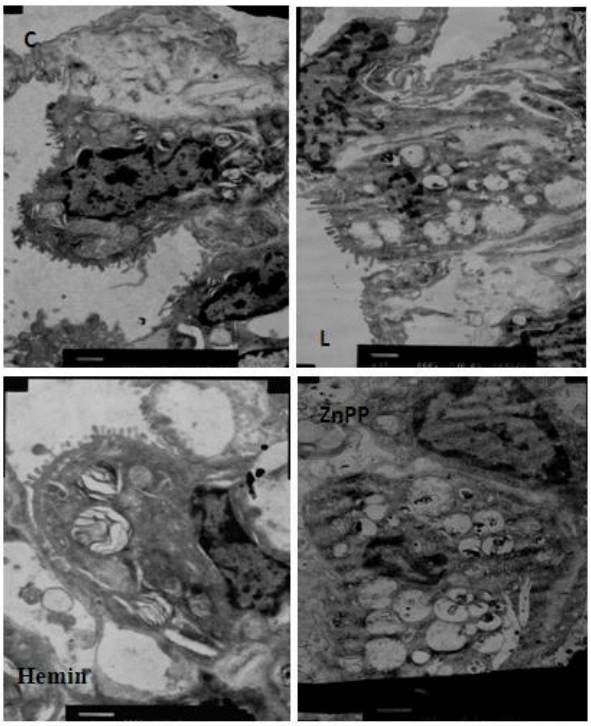

Figure 4: The ultrastructure of ling was observed under transmission electron microscopy (TEM). (8000× magnification)

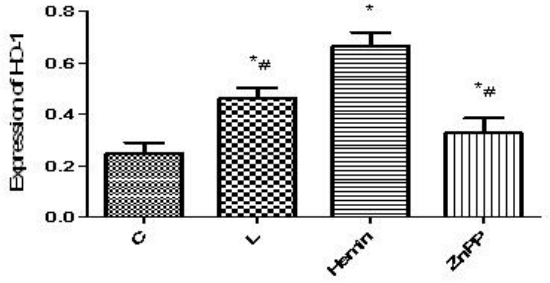

Figure 5: Compared with group C, the other group, *, $P<0.05$; L and ZnPP group were obiviously lower than Hemin group, #, $P<0.05$

Through the comprehensive comparison, our present study demonstrated that ZnPP could further aggravate the process of LPS-induced lung injury, the mechanism may be deduced that ZnPP restrain HO-1 expression, which lead to serum level of TNF-α, IL-6, and the level of MDA and MPO in lung tissues increase, then further activate autophagy. At last, the autophagy is converted into suicide cell autophagy, resulting in excessive cell death, further causing disease [13]. We can also demonstrates that Hemin could further alleviate the process of endotoxin induced lung injury, which mechanism may be deduced that Hemin promote HO-1 expression, leading to serum level of TNF-α, IL-6, the level of MDA and MPO in tissus decline, resulting in reducing the activation of severe autophagy, then alleviating the lung injury.

Conclusion

1. ZnPP can induce excessive autophagy and oxidative stress, aggravating lung injury in the process of LPS induced lung injury.

2. Hemin can inhibit release of inflammatory cytokines, resist oxidative stress to reduce lung injury during the process of LPS induced lung injury.

3. HO-1 has a protective effect on acute lung injury by adjusting the level of autophagy.

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