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Suppression of NLRP3 and NF-κB signaling pathways by α-Cyperone via activating SIRT1 contributes to attenuation of LPS-induced acute lung injury in mice

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ABSTRACT

α-Cyperone is the volatile oil component in *Cyperus rotundus* L. The previous reports had shown the inhibition of α-Cyperone on NF-κB signaling in LPS-stimulated RAW264.7 cells. However, it is still unclear whether α-Cyperone could suppress inflammatory response in acute lung injury (ALI) induced by LPS. The aim of this study is to investigate the suppression of α-Cyperone on LPS-induced ALI in mice. In this study, we established the LPS-induced ALI model and compared different doses of α-Cyperone with the control group and LPS group. Accordingly, the following indexes would be compared, including lung wet/dry ratio, MPO activity, inflammatory cell number, histopathological changes, levels of inflammatory cytokines, NF-κB and NLRP3 signaling pathways activation. The results demonstrated that α-Cyperone had the effect on reducing the wet/dry ratio and MPO activity. Furthermore, the increase of inflammatory cells and inflammatory cytokines could be inhibited by α-Cyperone. Meanwhile, α-Cyperone could downregulate NF-κB and NLRP3 signaling pathways. Finally, we found α-Cyperone could up-regulate the expression of SIRT1 and SIRT1 inhibitor could reverse the protective effects of α-Cyperone on ALI. In conclusion, α-Cyperone showed the protective effect on LPS-induced ALI in mice by suppressing the NF-κB and NLRP3 signaling pathways, mainly via up-regulating SIRT1. This provides a potential drug for the treatment of LPS-induced ALI.

1. Introduction

Acute lung injury (ALI) is a common respiratory system disease around the world, and its main characteristic is the lung pulmonary edema caused by inflammatory cell infiltration, which induced by pathogenic factors inside and outside [1]. Symptoms of severe edema will turn into respiratory distress syndrome, and as one of the major pathogenic factors, lipopolysaccharide (LPS) can activate the TLR4 signaling pathway [2]. LPS combined with CD-14 in vitro and LPS will collect a variety of protein molecules to activate inflammatory cytokines, leading to excessive inflammatory response in the body [3]. In the process of the body's immune response, LPS can induce alveolar cell pathological changes, alveolar edema and increase number of immune cells, etc. In previous reports, inflammatory cells and cytokines were significantly increased after the lungs were stimulated by LPS [4]. Accordingly, the activity of NF-κB signaling was up-regulated in the excessive concentrations of LPS [5]. By studying the pathological mechanism of LPS on lung, we can explore new drugs or treatment methods against ALI.

α-Cyperone is isolated from the rhizomes of *Cyperus rotundus*, which has been reported to inhibit LPS-induced inflammation in BV-2 cells through suppression of NF-κB signaling pathway [6]. α-Cyperone had the anti-inflammatory effects on LPS-treated rat cardiac microvascular endothelial cells and had an inhibitory effects on COX-2 expression through the negative regulation of NF-κB signaling in RAW 264.7 cells [7]. The focus of this research was to determine whether α-Cyperone has protective effect on LPS-induced ALI in mice. It has been found from the previous studies that α-Cyperone could suppress NF-κB signaling pathway and it might be a key point to investigate the regulatory effect of α-Cyperone on ALI.

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2. Materials and methods

2.1. Reagents

α-Cyperone standard product was purchased from China institute of pharmaceutical biological products (Beijing, China). SIRT1 inhibitor EX-527, Dimethyl sulfoxide (DMSO), and LPS were obtained from Sigma (MO, USA). TNF-α and IL-1β ELISA assay kits were obtained from Biolegend (CA, USA). MPO assay kit was manufactured from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The primary antibodies for SIRT1, NF-κB and NLRP3 signaling pathways, as well as the secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA).

2.2. Animals

60 male healthy BABL/c mice, 18–24 g; 9–10 weeks-old, were purchased from Beijing Vital River Laboratory Animal Technology Co. Mice were housed in a laboratory environment with suitable air flow temperature and humidity. Mice could eat and drink freely in the 12-hour light-dark cycle feeding environment. All procedures are in accordance with the Laboratory Management Regulations, and the experimental process is in accordance with Institutional Animal Care Organization of Jilin University.

2.3. LPS-induced ALI model

After feeding for one week under normal conditions, the mice were randomly divided into six groups: control group, LPS group, α-Cyperone groups (10, 20, 40 mg/kg) + LPS groups, and LPS + α-Cyperone + EX-527 (10 mg/kg) group. The mice of α-Cyperone + LPS groups were intraperitoneally injected with α-Cyperone at the doses of 10, 20, 40 mg/kg, respectively. The mice of the control group and LPS group were injected with the same volume PBS. One hour later, the mice of the LPS group and α-Cyperone groups were anesthetized and given LPS by intratracheal instillation (10 μg of LPS in 50 μl PBS). The control mice were received 50 μl PBS by intratracheal instillation. The mice of LPS + α-Cyperone + EX-527 (10 mg/kg) group were intraperitoneally administrated with 10 mg/kg EX-527, and then received α-Cyperone and LPS.

2.4. Lung wet-to-dry weight ratio

The lungs of the mice were removed 7 h after LPS inhalation. The tissues were drained the surface with filter paper after washing with PBS. The wet weight was obtained after weighing. The lung tissues were then placed in an 80-degree incubator for 48 h and weighed to obtain a dry weight. Wet weight divided by dry weight gave a wet-dry ratio to measure the degree of pulmonary edema.

2.5. Collection of BALF and cell counting

The tracheas were separated after the mice were sacrificed, and the trachea sleeves were placed on the mice trachea and the tracheas were washed with 1.5 ml PBS for 3 times. The collected BALF were placed in a 3000 rpm centrifuge tube for 15 min. The precipitated cells were stained with the Kwik-Diff staining set (Thermo, PA, USA) and a cell counter was used to detect the number of cells, macrophages and neutrophils.

2.6. MPO activity

100 mg lung tissue was frozen, ground with liquid nitrogen, and homogenized after LPS injection. MPO activity was measured by using the MPO kits according to the manufacturer's instructions. The absorbance was set at 450 nm.

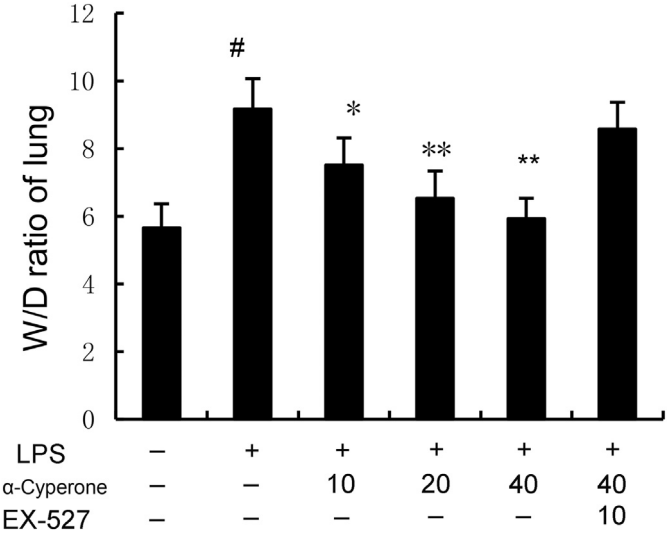

Fig. 1. Effects of α-Cyperone on the lung W/D ratio of LPS-induced ALI mice. The values presented are the means ± SEM of three independent experiments. *#p < 0.01* vs. control group, **p < 0.05* and ***p < 0.01* vs. LPS group.

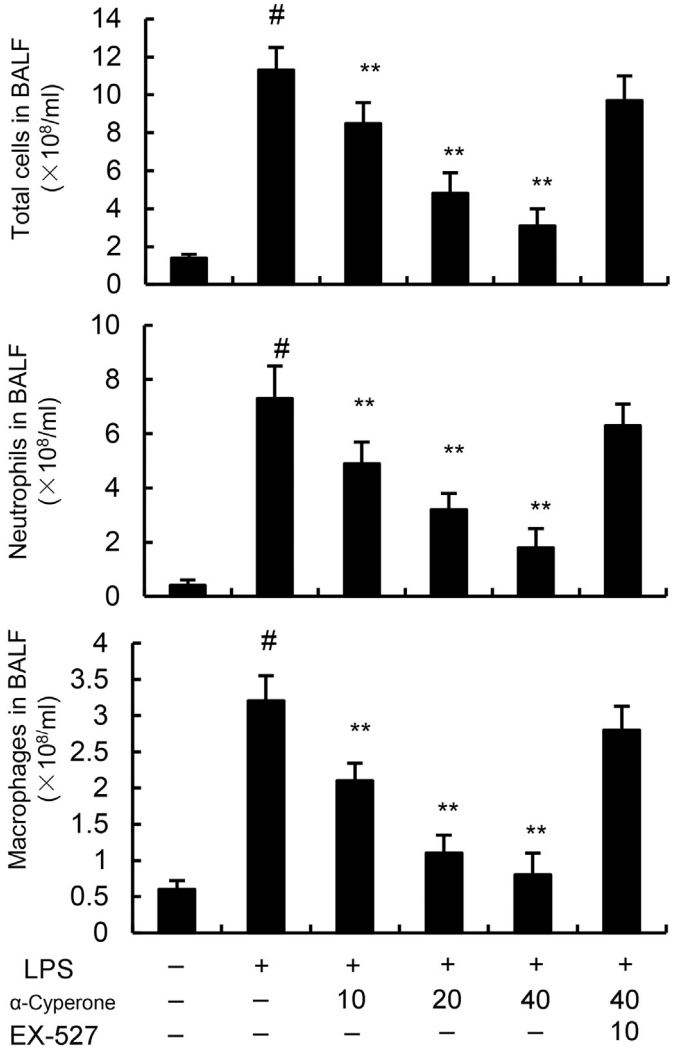

Fig. 2. Effects of α-Cyperone on the number of inflammatory cells in the BALF of LPS-induced ALI mice. The values presented are mean ± SEM of three independent experiments. $p\# < 0.01$ vs. control group, $p^* < 0.05$, $p^{**} < 0.01$ vs. LPS group.

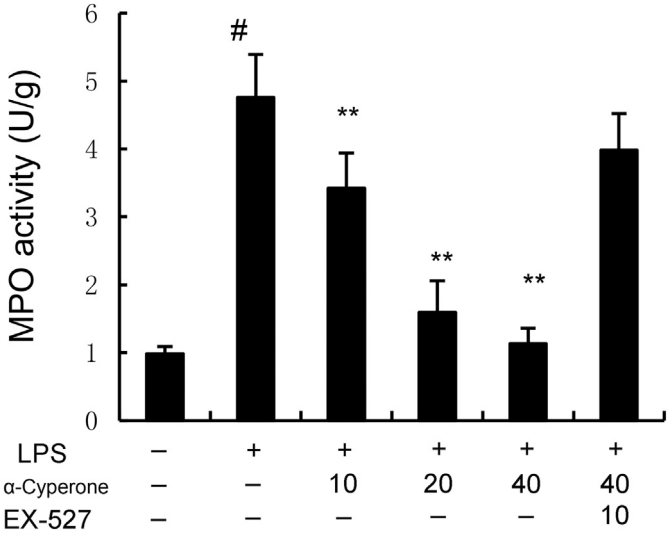

Fig. 3. Effects of α-Cyperone on MPO activity. The values presented are the mean ± SEM of three independent experiments. $p\# < 0.01$ vs. control group, $p^* < 0.05$, $p^{**} < 0.01$ vs. LPS group.

2.7. Histopathological evaluation of the lung tissue

The mice lung tissue was fixed in formaldehyde for 2 days before dehydration and embedded in paraffin. After deparaffinization and dehydration, slides of lung cells were prepared and stained with hematoxylin and eosin. Accordingly, we could observe the changes in the cell wall and cytoplasm under the microscope.

2.8. ELISA

The supernatant in BALF was used to measure the content of inflammatory cytokines. The levels of TNF-α and IL-1β were detected by ELISA kits and operated according to the manufacturer's instructions. Absorbance was set at 450 nm to measure OD number.

2.9. Western blotting

Lung tissues were taken to measure the activity of NF-κB and NLRP3 signaling pathways. The samples were assayed the protein concentrations by the BCA Kit. Protein were separated by 12% SDS-PAGE gels and transferred onto PVDF membranes. The samples were then transferred to PVDF membranes and blocked in 5% skim milk for 2 h.

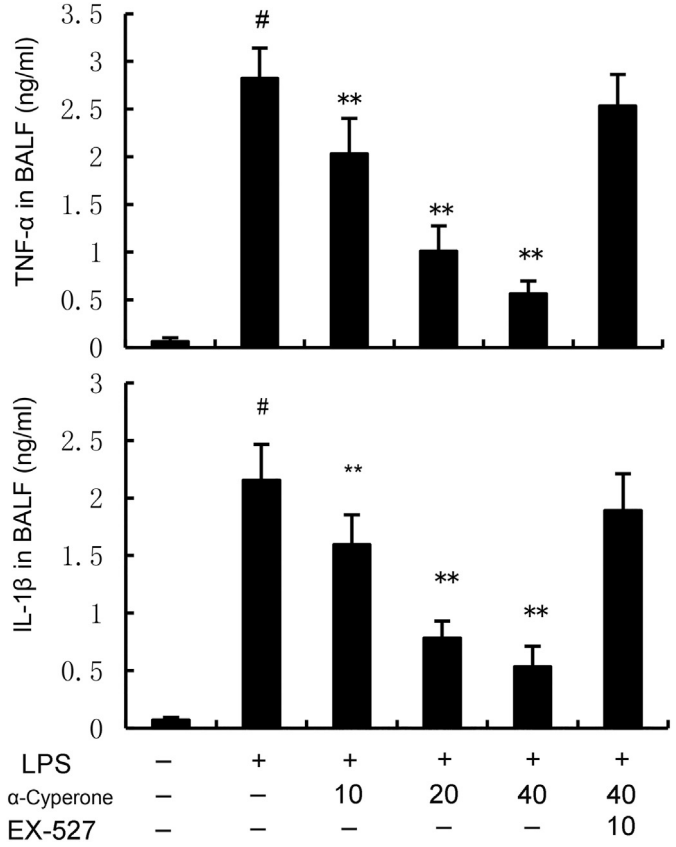

Fig. 5. Effects of α-Cyperone on TNF-α and IL-1ß production in the BALF of LPS-induced ALI mice. The values presented are mean ± SEM of three independent experiments. $p\# < 0.01$ vs. control group, $p^* < 0.05$, $p^{**} < 0.01$ vs. LPS group.

Subsequently, the membranes were incubated with primary antibody at 4 °C overnight. After three times of washing with TBST, the membranes were incubated with secondary antibodies at normal temperature for 1 h. Then membranes were washed with TBST for another three times and incubated with ECL kits to visualize.

2.10. Statistical analyses

The experimental data were described in the form of means ± S.E.M. Differences between any two groups were analyzed by

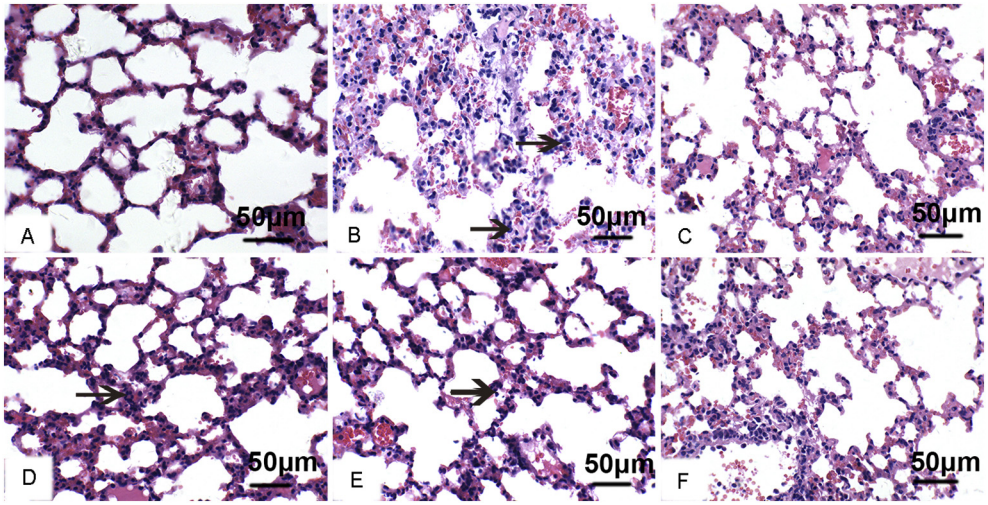

Fig. 4. Effects of α-Cyperone on histopathological changes in lung tissues in LPS-induced ALI mice. Representative histological changes of lung obtained from mice of different groups. A: Control group, B: LPS group, C: LPS + α-Cyperone (10 mg/kg) group, D: LPS + α-Cyperone (20 mg/kg) group, E: LPS + α-Cyperone (40 mg/kg) group, F: LPS + α-Cyperone (40 mg/kg) + EX-527 group (Hematoxylin and eosin staining, magnification 200×).

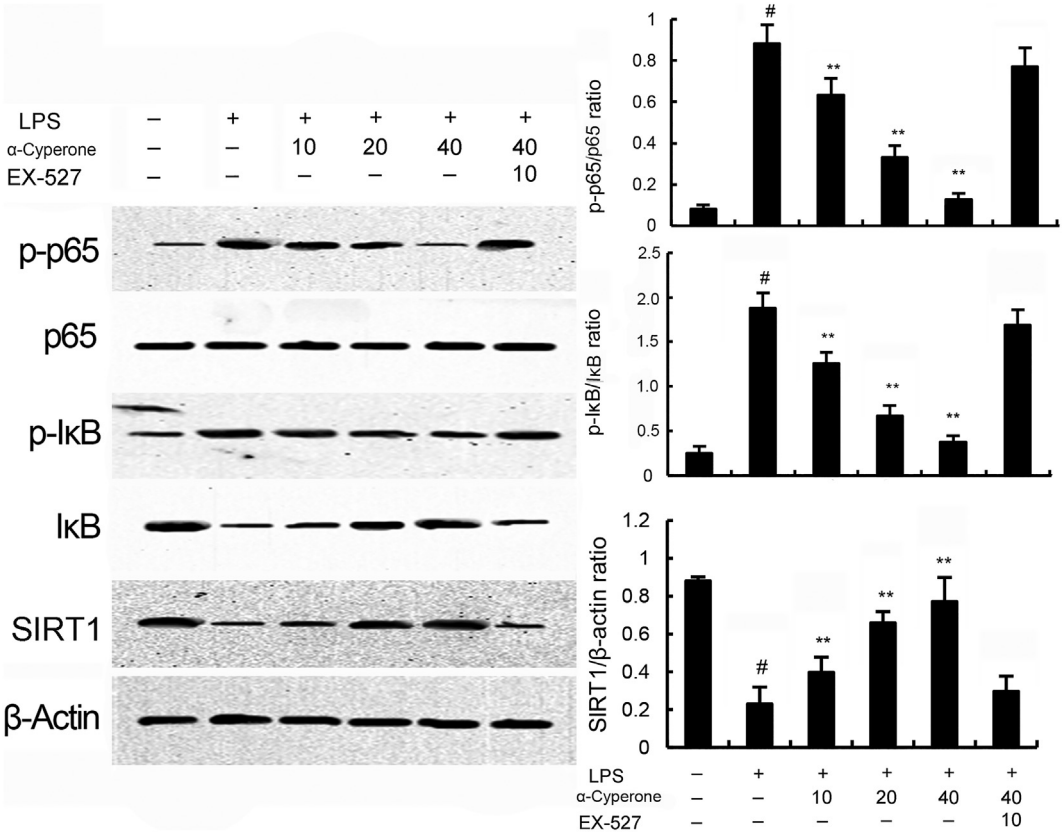

Fig. 6. Effects of α-Cyperone on LPS-induced NF-κB activation and SIRT1 expression. The values presented are the means ± SEM of three independent experiments. $p\# < 0.01$ vs. control group, $p^* < 0.05$, $p^{**} < 0.01$ vs. LPS group.

ANOVA and Students' *t*-test. Setting the value of p as $p < 0.05$ means significant difference, while $p < 0.01$ means extremely significant difference.

3. Results

3.1. Effect of α-Cyperone on the wet-dry ratio in LPS-induced ALI mice

In order to determine pulmonary edema, the wet-to-dry ratio of the lung was measured by the wet weight dividing the dry weight. The results demonstrated the wet-to-dry ratio of the lungs was significantly increased after LPS treatment, while in the pretreatment of α-Cyperone groups, the wet-dry ratio was obviously reduced (Fig. 1). Furthermore, SIRT1 inhibitor EX-527 reversed the inhibition of α-Cyperone on lung wet-to-dry ratio (Fig. 1).

3.2. Effect of α-Cyperone on the number of inflammatory cells in LPS-induced ALI mice

The degree of inflammatory infection can be determined by counting the number of inflammatory cells. As can be seen from the results, after LPS treatment, the numbers of inflammatory cells in LPS group was significantly higher than that in the control group. While, the numbers of inflammatory cells in α-Cyperone-pretreated groups were significantly lower than that in LPS group (Fig. 2). Furthermore, SIRT1 inhibitor EX-527 reversed the inhibition of α-Cyperone on inflammatory cell production (Fig. 2).

3.3. Effect of α-Cyperone on the MPO activity in LPS-induced ALI mice

To detect the accumulation of neutrophils, MPO activity was measured using the MPO kit. The results showed LPS can stimulate the increase of MPO activity when compared with the control group, while α-Cyperone can significantly inhibit the increase. As shown in Fig. 3, the higher the dose of α-Cyperone, the more obvious the inhibition of MPO. Furthermore, SIRT1 inhibitor EX-527 reversed the inhibition of α-Cyperone on MPO activity (Fig. 3).

3.4. Effect of A on the histopathological changes in LPS-induced ALI mice

After H&E staining, the histopathological changes of the lung tissues were observed. As shown in Fig. 4, LPS caused a destructive effect on lung tissues, including alveolar wall thickening, interstitial edema, and inflammatory cells infiltration. Nevertheless, in the α-Cyperone pretreatment groups, the degree of histopathological changes in the lung tissues were relieved. Furthermore, SIRT1 inhibitor EX-527 reversed the inhibition of α-Cyperone on lung histopathological changes (Fig. 4).

3.5. Effect of α-Cyperone on the cytokine production in LPS-induced ALI mice

The contents of inflammatory cytokines were measured by ELISA kits. Compared with the control group, the productions of TNF-α and IL-1β were evidently increased in the LPS group. Nevertheless, in α-Cyperone + LPS groups, the productions of inflammatory cytokines were dramatically reduced. Moreover, the levels of inflammatory cytokines decreased in a dose-dependent manner with the α-Cyperone doses (Fig. 5). Furthermore, SIRT1 inhibitor EX-527 reversed the inhibition of α-Cyperone on inflammatory cytokine production (Fig. 5).

3.6. Effect of α-Cyperone on the NF-κB activation in LPS-induced ALI mice

NF-κB plays a crucial role in regulating the expression of inflammatory cytokines. Western blotting was used to verify whether α-

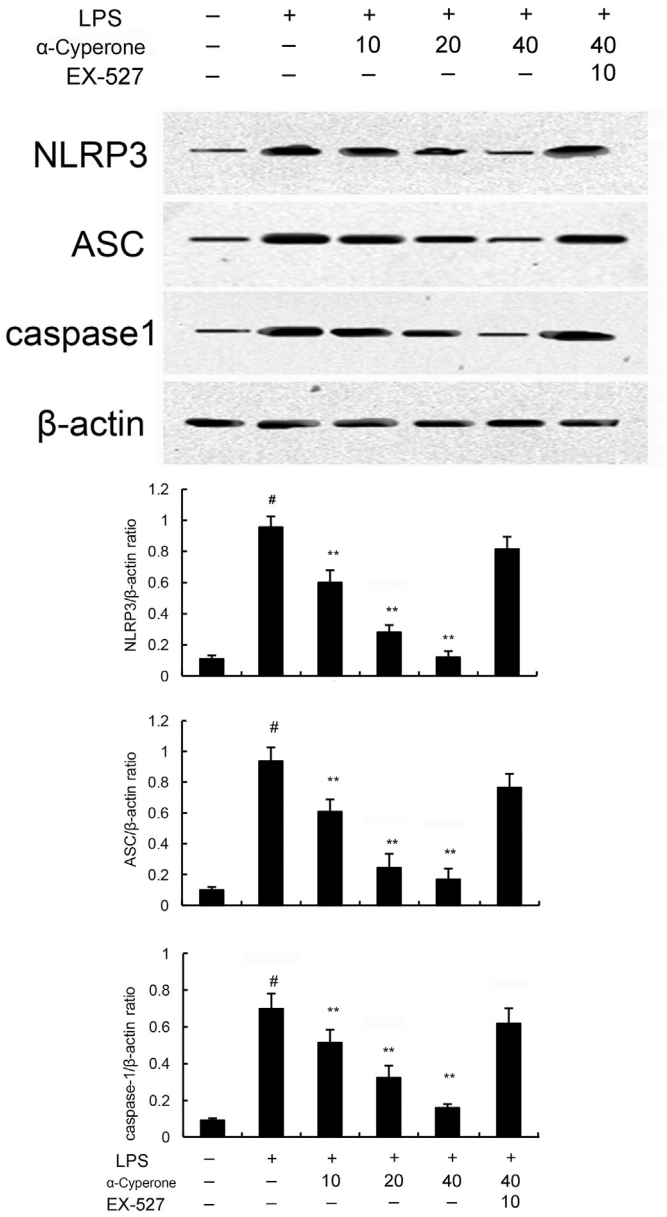

Fig. 7. Effects of α-Cyperone on NLRP3 activation. The values presented are the means ± SEM of three independent experiments. $p\# < 0.01$ vs. control group, $p^* < 0.05$, $p^{**} < 0.01$ vs. LPS group.

Cyperone could inhibit the expression of NF-κB signaling pathway. Compared with the control group, after LPS stimulation, the activity of NF-κB was enhanced. However, in pretreatment of α-Cyperone groups, with the increase of α-Cyperone dose, the activity of NF-κB was decreased (Fig. 6). Furthermore, SIRT1 inhibitor EX-527 reversed the inhibition of α-Cyperone on NF-κB activation (Fig. 6).

3.7. Effect of α-Cyperone on the NLRP3 activation in LPS-induced ALI mice

Western blotting was used to verify whether α-Cyperone could inhibit the activation of NLRP3 signaling pathway. Compared with the control group, after LPS stimulation, the expression of NLRP3, ASC, and caspase-1 were enhanced. However, in pretreatment of α-Cyperone groups, with the increase of α-Cyperone dose, the expression of NLRP3, ASC, and caspase-1 were decreased (Fig. 7). Furthermore, SIRT1 inhibitor EX-527 reversed the inhibition of α-Cyperone on lung NLRP3 activation (Fig. 7).

3.8. Effect of α-Cyperone on SIRT1 expression in LPS-induced ALI mice

Western blotting was used to verify whether α-Cyperone could increase the expression of SIRT1. Compared with the blank group, after LPS stimulation, the expression of SIRT1 was decreased. However, in pretreatment-α-Cyperone groups, with the increase of α-Cyperone dose, the expression of SIRT1 was increased (Fig. 6).

4. Discussion

In this study, we explored the protective effect of α-Cyperone on LPS-induced ALI mice. In the experiment, the LPS-induced ALI model was established, and then the regulatory effects of α-Cyperone on pulmonary edema, inflammatory cell number, MPO activity, pathological changes, inflammatory cytokines production, NLRP3 and NF-κB signaling pathway were tested. This study we found α-Cyperone had protective effects against LPS-induced ALI in mice.

In the current study, as expected, α-Cyperone showed protective effects against LPS-induced ALI mice. First of all, α-Cyperone indeed alleviates the symptoms of pulmonary edema. When LPS stimulates alveolar cells, it causes tissue fluid to flow out and accumulate in the lungs, causing pulmonary edema [8]. In the LPS group, the wet-dry ratio of the lung was significantly increased, while the wet-dry ratio was lower in the pretreatment groups of α-Cyperone. Secondly, α-Cyperone reduced the number of LPS-induced inflammatory cells, because LPS can promote the aggregation of inflammatory cells in lung tissue [9]. However, the numbers of neutrophils and macrophages in the α-Cyperone pretreatment groups were significantly lower than that in the LPS group. In addition, α-Cyperone can reduce the activity of MPO stimulated by LPS. It is well known that the aggregation of inflammatory cells can trigger the increase of MPO secretion, but α-Cyperone can significantly inhibit the activity of MPO compared with LPS alone. Moreover, α-Cyperone can prevent the damage of LPS to lung cells and protect lung tissue. It can be seen from the results that LPS caused more severe histopathological changes in lung tissues. Furthermore, α-Cyperone can down-regulate the production of inflammatory cytokines. Inflammatory cytokines are various cytokines involved in the inflammatory response, which are complementary to the inflammatory response in the immune process [10]. Inflammation can recruit inflammatory cells, and inflammatory cytokines can trigger more inflammatory responses [11].

α-Cyperone is a kind of component of Chinese traditional medicine, has the function of regulating inflammatory response. It has been reported in the past that α-Cyperone can inhibit the inflammation induced by LPS in BV-2 cells and the expression of COX-2 in RAW 264.7 cells by regulating the NF-κB signaling pathway. In this study, it is necessary to examine whether α-Cyperone can protect LPS-induced ALI mice by inhibiting the activity of NF-κB. The signaling pathway of NF-κB plays an important regulatory role in the expression of inflammatory cytokines [12,13]. LPS stimulated phosphorylation of IκB and transferred of NF-κB into the nucleus to initiate gene transcription [14,15]. In the present study, as expected, α-Cyperone evidently inhibited the expression of the NF-κB signaling pathway in LPS-induced ALI in mice. NLRP3 is another signaling pathway that regulates the inflammatory response [16,17]. This study we found α-Cyperone evidently suppressed the expression of NLRP3, ASC, and caspase-1, which suggested α-Cyperone could blocked NLRP3 signaling pathway. Furthermore, we found α-Cyperone significantly increased the expression of SIRT1 and SIRT1 inhibitor significantly reversed the protective effects of α-Cyperone, suggesting α-Cyperone protected mice against LPS-induced ALI through activating SIRT1.

In conclusion, α-Cyperone effectively protected the ALI mice induced by LPS. The results demonstrated that α-Cyperone suppressed the expression of NF-κB and NLRP3 activation via up-regulating SIRT1 to regulate the levels of inflammatory cytokines. In the future, further study on α-Cyperone should be carried out clinically, so as to provide

new treatment for ALI.

Declaration of competing interest

All authors declare that they have no conflict of interest.

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