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Metformin relieves acute respiratory distress syndrome by reducing miR-138 expression

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Abstract. – OBJECTIVE: To investigate whether metformin can relieve acute respiratory distress syndrome (ARDS). Its potential mechanism was also explored.

MATERIALS AND METHODS: The ARDS model was established by injecting LPS into mice that received metformin in advance and the mice in the control group. Pulmonary edema was detected by W/D ratios (wet-to-dry weight ratios), and the vascular exudation was reflected by the protein content and cell number of alveolar lavage fluid. Meanwhile, MPO (myeloperoxidase) activity assay was performed to analyze the neutrophil aggregation. The expression of inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-17, were detected by enzyme-linked immunosorbent assay (ELISA). This series of experiments reflected the alleviation effect of metformin on ARDS. To further study the mechanism, we cultured alveolar macrophages (NR8383) *in vitro* and treated them with LPS and metformin. Western blot was used to detect the phosphorylation levels of p38, ERK, NF-kB, and SIRT1 expression level. Bioinformatics method was then used to predict the binding of miR-138 to SIRT1. The mRNA and protein expression of SIRT1 was detected in NR8383 cells transfected with miR-138 inhibitor. The dual luciferase gene reporter assay was used to detect the relative luciferase activities of miR-138 and SIRT1.

RESULTS: Pulmonary edema, vascular exudation, and neutrophil accumulation were observed in the ARDS model mice, and the levels of inflammatory cytokines including TNF-α, IL-1b, IL-6, and IL-17 were significantly increased. After metformin treatment, these pulmonic damage indicators were found to be partially reversed. At the same time, metformin could significantly reduce LPS-induced death. After NR8383 was treated with metformin and LPS, the expression of SIRT1 was higher than that of LPS treatment alone, but the expression of p-p38, p-ERK, and p-NF-κB was significantly decreased. After the addition of metformin in NR8383 after LPS treatment, the expression level of miR-138-5p was significantly decreased, and miR-138-5p was confirmed to target SIRT1 and regulate its expression.

CONCLUSIONS: Metformin could reduce LPS-induced pulmonic injury and increase expression of inflammatory factors. A possible mechanism might be that metformin-induced low expression of mir-138-5p could target SIRT1 to increase its expression and suppress the MAPK pathway, thus alleviating ARDS.

Key Words:

Acute respiratory distress syndrome, Metformin, Lipopolysaccharide (LPS), MAPK, MiR-138.

Introduction

Acute respiratory distress syndrome (ARDS) refers to the acute and progressive hypoxic respiratory failure caused by various extra-pulmonary and external pathogenic factors (such as sepsis, inhalation of harmful gases, severe burns, trauma, etc.)[1,2]. The prevalence of ARDS in Europe and the United States has not increased in the past 10 years, and the mortality rate is still as high as 40%-50%[3]. Therefore, in-depth study of its pathogenesis and exploration of more effective treatment methods are clinically significant.

The pathogenesis of ARDS is not yet clear, and may be related to a variety of factors such as uncontrolled inflammatory response and imbalance of oxidative stress. When the human body is subjected to serious infections, trauma, etc., the formation of systemic inflammatory response syndrome and compensatory anti-inflammatory response syndrome can be induced. When the two are out of balance and the systemic inflammatory response syndrome is dominant, a large amount of inflammatory cells in lungs are ac-

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tivated, and multiple inflammatory factors and mediators are released, which can result in alveolar epithelial cell damage, increased pulmonary capillary permeability, and pulmonary edema[4,5].

Lipopolysaccharide is the main component of the cell wall of Gram-negative bacteria. Under the stimulation of lipopolysaccharide, NF-κB is transferred from the cytoplasm to the nucleus, thereby initiating transcription of genes such as inflammatory cytokines, inflammatory mediators, and chemokines. Endotoxin is currently the most common agent for inducing animal models of acute lung injury. Because the rodent animal is similar to the human body structure, it can be used to well mimic the human inflammatory response, thus mimicking the pathology of acute lung injury. The process has become one of the extensive research methods[6,7].

Metformin (metformin), a first-line hypoglycemic agent for the treatment of type 2 diabetes, has been used clinically for many years, with low cost and high safety[8]. While metformin exerts hypoglycemic effects, it also has significant anti-inflammatory effects[9]. The anti-inflammatory protective effect of metformin was confirmed in the fulminant hepatitis model[10], and this effect is partially dependent on MAPK. Therefore, we hypothesized that metformin might also exert anti-inflammatory effects in LPS-induced ARDS. To investigate the potential protective effect and related mechanisms of metformin in LPS-induced ARDS, we used LPS to induce ARDS models and investigated the mechanism from lung injury, inflammation, MAPK, and other aspects.

MicroRNAs (miRNAs), an important class of non-coding small RNAs that regulate gene expression at the post-transcriptional level; they are also involved in the regulation of post-transcriptional gene expression[11]. They can base pair with the mRNA of their target gene to form miRNA-induced silencing complexes (miRISCs), thereby degrading the target gene mRNA or inhibiting its translation. A series of studies have shown that the abnormal expression of miRNA is closely related to inflammatory reactions and plays an important role in a variety of acute and chronic inflammatory diseases. Successive research groups[12,13] have found that miRNAs can act alone or interact with other proteins to enable powerful post-transcriptional regulation of inflammatory factor expression. However, little is known in ARDS about the role of miRNAs in the regulation of the major inflammatory factors.

Materials and Methods

ARDS Model Construction

A total of 32 SPF-grade healthy male BALB/c mice were purchased from the Experimental Animal Center of Chongqing Medical University. Before the animal experiment, the mice were fed with the standard diet for 1 week in a standard environment. All mice were randomly divided into 4 groups of 8 each, which was numbered A, B, C, D. Group A was blank control group; group B was intraperitoneally injected with 5 mg/kg of LPS; group C was orally administrated with 50 mg/kg metformin seven days in advance, and 5 mg/kg of LPS was intraperitoneally injected 90 minutes after the last oral administration; and group D was was treated with metformin alone. This study was approved by the Animal Ethics Committee of Jingjiang People's Hospital Animal Center.

Specimen Collection

In all animal models, the skin was incised through the midline neck incision, the muscles were separated layer by layer, and the right common carotid artery was exposed. Animals were sacrificed by carotid bloodletting, and their thorax was quickly opened to find the right main bronchus for ligation. Subsequently, 3 ml of cold physiological saline was slowly injected in the trachea. And the syringe was repeatedly suctioned 3-4 times with 1-minute intervals. The above steps were completed and the bronchoalveolar lavage fluid (BALF) was collected. In addition, upper lobe of right lung was punched to detect W/D.

W/D Ratio Measurement

The upper lobe of right lung was accurately weighed and placed in a constant temperature oven at 70°C. The dry weight of the lung tissue was determined after baking for 72 h to constant weight. Meanwhile, W/D was calculated to evaluate the extent of edema in the lung tissue.

Analysis of Bronchoalveolar Lavage Fluid

The collected BALF was centrifuged at 1000 rpm at 4°C for 5 min. The supernatant was assayed for protein content using the bicinchoninic acid (BCA) protein concentration assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The cell debris obtained from the supernatant of the BALF fluid was resuspended in red blood cell lysis buffer and counted under a microscope.

Determination of MPO Activity

Using the enzyme-linked immunosorbent assay (ELISA) kit MPO activity (R&D Systems, Minneapolis, MN, USA), the collected lung tissue was diluted with pre-cooled phosphate buffered saline (PBS) at a ratio of 1:10. After homogenate, tissues were centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant was collected according to the ELISA procedure. The MPO activity was detected at a wavelength of 460 nm.

Cell Culture and Processing

Alveolar macrophages were purchased from American Type Culture Collection ATCC (Manassas, VA, USA) and cells were rapidly resuscitated. The cells were resuspended in F12 medium containing 10% fetal bovine serum and placed in a cell incubator at 37°C with 5% CO_2 saturation humidity. To explore the mechanism of the effect of metformin on LPS-induced lung injury, cells were divided into 2 groups: LPS (1 μg/mL) injection group and LPS (1 μg/mL) + metformin treatment group (40 μg/mL). Cells were collected after 12 hours of treatment for subsequent experiments.

Detection of Inflammatory Factors

The expression of TNF-α, IL-1β, IL-6, and IL-17 in lung tissue was detected by ELISA. The lung tissue was homogenized and PBS was added to dilute homogenate, which was centrifuged with 12,000 rpm at 4°C for 5 minutes. The procedure was performed according to the ELISA kit instruction. Different kits were used to detect the expression of TNF-α, IL-1β, IL-6, and IL-17 at different wavelengths.

RNA Extraction and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA), chloroform, and isopropanol, and the extracted RNA was measured for concentration and stored at -80°C until use. The complementary Deoxyribose Nucleic Acid (cDNA) was obtained by reverse transcription. Then, 1 μL of cDNA and SYBR Green was used for PCR detection. The primer sequences were: SIRT1 (F: 5'-TCGCAACTATACCCAGAACATAGACA-3', R 5'-TGTTGCAAAGGAACCATGACA-3') GAPDH (F: 5'-ACCCACTCCTCCACCTTTGA-3', R: 5'-CTGTTGCTGTAGCCAAATTCGT-3'); miR-138-5p (F: 5'-GTCGTACCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTAGT-3', R: 5'-GCCCGTGAAATGTTTAGGACCAC-3').

Transfection of Cells

Cells that grew well were taken for transfection. The transfection experiment was divided into two groups: miR-138-5p inhibitor group and negative control group. The sequences were as follows, miR-138-5p inhibitor sequence: (5'-CUAGUGGUCCUAAACAUUUCAC-3'); miR-138-5p inhibitor NC sequence: (5'-CAGUACUUUUGUGUAGUACAA-3').

Luciferase Reporter Vector Construction and Activity Detection

The 3'UTR sequence of SIRT1 was downloaded at the NCBI website to construct the SIRT1 wild-type sequence (SIRT1 WT 3'UTR) and the mutant sequence (SIRT1 MUT 3'UTR). The cells were then plated in 96-well plates, and 50 pmol/L of mir-138-5p mimics or negative controls were co-transfected with the constructed 80 ng SIRTl wild-type or mutant plasmid. After 48 hours of transfection, cells were lysed using a solution from dual luciferase reporter gene assay system to detect fluorescence intensity.

Western Blot Analysis

Cells were collected and then disrupted by sonication. After centrifugation, the supernatant was harvested and boiled for 10 min, then stored at -20°C until use. The protein sample was dissolved directly when used. After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), which was then cut according to the molecular weight. The antigen on the membrane was blocked in blocking solution of 5% skim milk powder for 1h, and then specific primary antibody was added to incubate overnight. After washing the membrane, the secondary antibody was used to incubate protein bands. Finally, exposure was performed to analyze each protein content.

Statistical Analysis

All experiments were repeated 3 times. Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA) was used for analysis. Measured data were expressed as mean ± standard deviation and were compared using the *t*-test. Kaplan-Meier and the log-rank test were used for survival analysis. The difference was statistically significant when $p < 0.05$.

Results

Metformin Reduced LPS-Induced Lung Injury

After the acute respiratory distress syndrome model was established by intraperitoneal injection of LPS (5 mg/kg), we measured the dry/wet weight of the lung tissue. It was found that the specific gravity was significantly higher, which was then partially reversed in the mice model pretreated with metformin (50 mg/kg). However, there was no significant difference between the metformin-treated group and the control group (Figure 1A), suggesting that metformin alleviated LPS-induced pulmonary edema. Subsequently, we collected bronchoalveolar lavage fluid from each group of mice and tested the protein content and cell number. It was found that the BALF protein content and cell number were significantly elevated in the lung injury model, while metformin treatment could inhibit the LPS-induced elevation. Metformin treatment alone was not significantly different from the control (Figure 1B and 1C). This result suggested that metformin relieved vascular leakage caused by LPS. Subsequently, the collected lung tissue was assayed for its MPO activity, and it was found that LPS-induced increase in MPO activity was reversed by metformin, and metformin alone did not affect MPO activity (Figure 1D). This result showed that metformin relieved LPS-induced neutrophil accumulation. To further verify the protective effect of metformin on LPS, we recorded the survival of mice in different treatment groups for 72 hours. No death occurred in mice of the control group and metformin alone treatment group. However, the survival rate of the mice in the metformin-pretreated group was significantly higher than that in the LPS alone treatment group (Figure 1E), indicating that metformin alleviated LPS-induced death. These above results demonstrated that metformin could alleviate LPS-induced lung injury.

Metformin Relieved LPS-Induced Inflammatory Factors

TNF-a, IL-1β, IL-6, and IL-17 are important inflammatory factors and can reflect the severity of inflammation. Therefore, we measured the levels of TNF-a, IL-1β, IL-6, and IL-17 in lung tis-

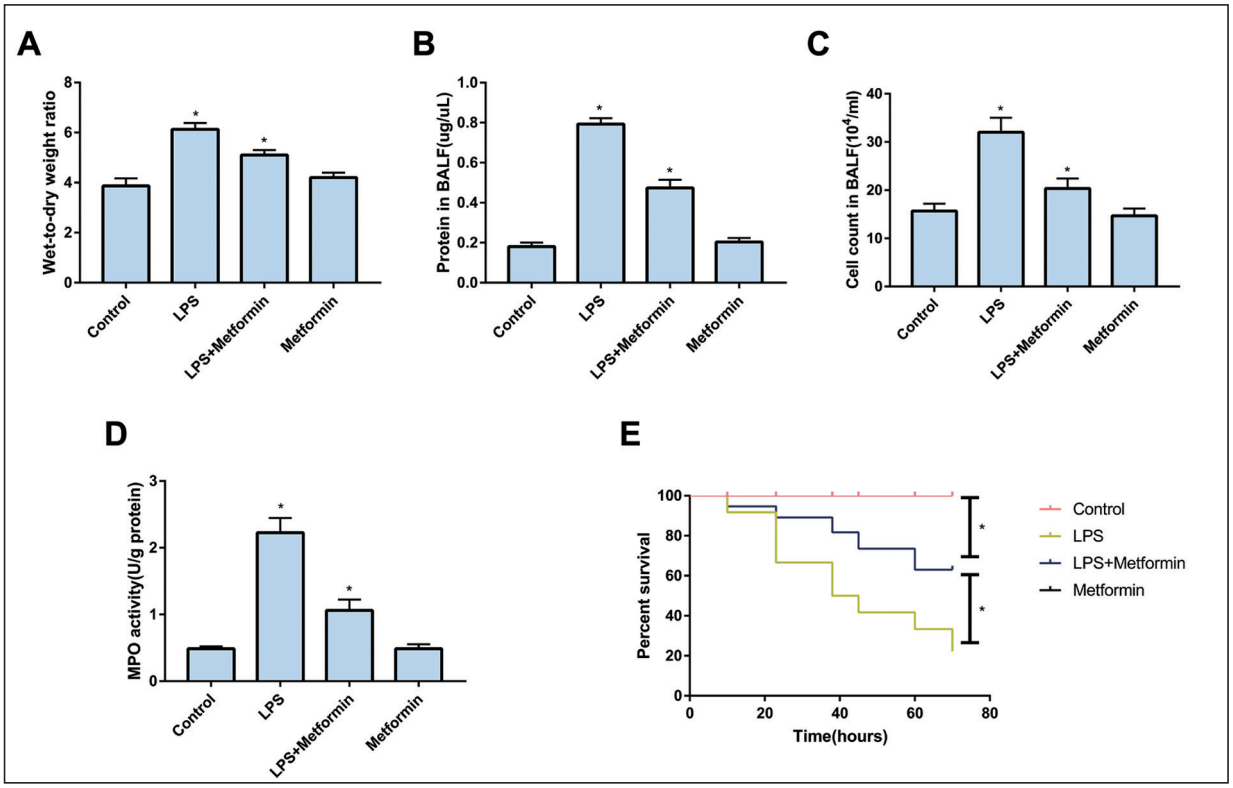

Figure 1. Metformin reduced LPS-induced lung injury. ***A,*** Metformin relieved pulmonary edema caused by LPS. ***B, C,*** Metformin relieved vascular leakage caused by LPS. ***D,*** Metformin relieved LPS-induced neutrophil aggregation. ***E,*** Metformin relieved LPS-induced death.

sue. The results showed that the levels of TNF-a (Figure 2A), IL-1β (Figure 2B), IL-6 (Figure 2C), and IL-17 (Figure 2D) in lung tissue were not significantly different between group of metformin-treated alone and normal group, while in LPS model group, their levels were significantly higher than those in the normal group ($p < 0.05$). The above results implied that metformin could inhibit the increase of LPS-induced TNF-a, IL-1β, IL-6, and IL-17 levels ($p < 0.05$).

Metformin Inhibited MAPK Pathway

Since SIRT1 was verified to play an important role in the occurrence and development of inflammatory response through activation of the MAPK pathway, we hypothesized whether MAPK could participate in alleviating the effect of metformin on LPS-induced acute respiratory distress syndrome. Alveolar macrophages (NR8383) were then cultured *in vitro* and used to detect the MAPK/SIRT1 pathway after treatment with LPS (1 ug/mL) or LPS (1 μg/mL) and metformin (40 μg/mL) for 12 h. The results showed that after treatment of metformin combined with LPS, the expression of p-p38, p-ERK, and p-NF-κB was lower than that of LPS treatment alone, whereas the level of SIRT1 was higher (Figure 3).

Metformin Regulated SIRT1 Expression Through miR-138

The upstream miRNAs of SIRT1 were predicted by bioinformatics method and miR-138 was obtained. Meanwhile, metformin was confirmed to be able to reduce miR-138 expression

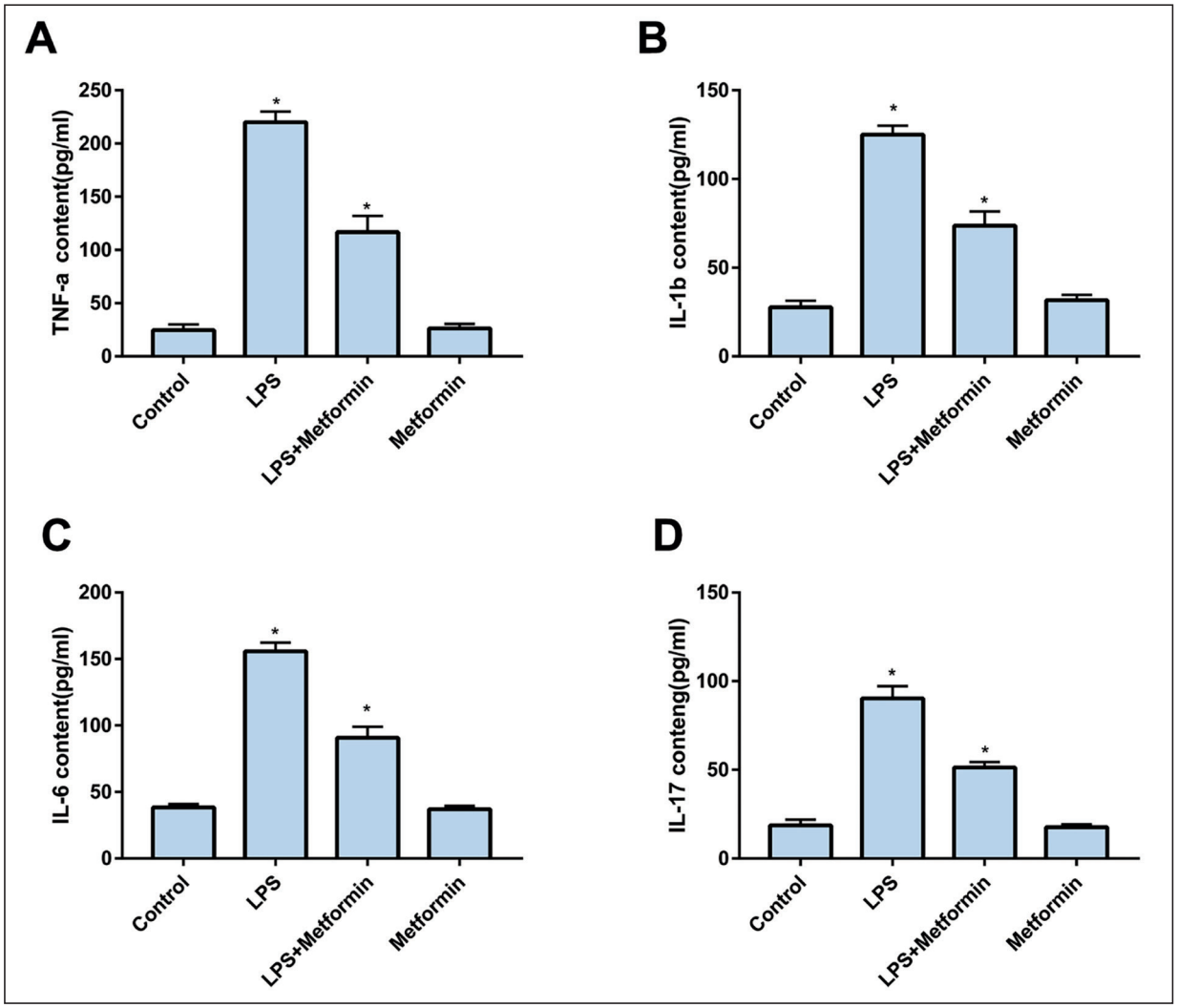

Figure 2. Metformin alleviated LPS-induced inflammatory factors. Metformin was able to relieve the secretion of LPS-induced inflammatory cytokines including TNF-α (***A***), IL-1β (***B***), IL-6 (***C***), and IL-17 (***D***) in the lungs.

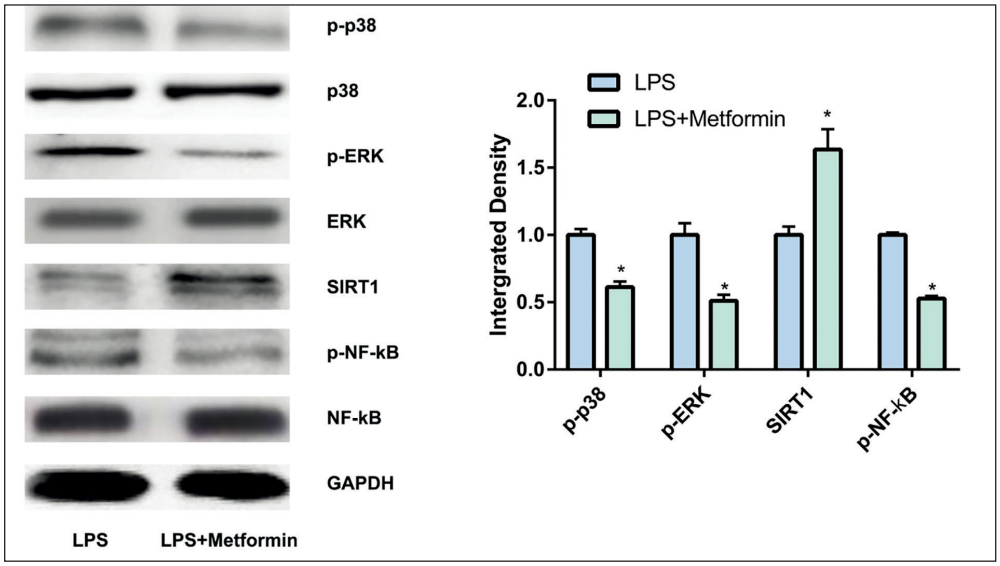

Figure 3. Metformin inhibited MAPK pathway. After 12 h treatment with LPS (1 μg/mL) or LPS (1 μg/mL) + metformin (40 μg/mL), MAPK pathway was detected *in vitro* cultured alveolar macrophage cell line, NR8383. Metformin significantly inhibited the phosphorylation of p38, ERK, and NF-kB in the MAPK pathway, but increased SIRT1 expression.

(Figure 4A). Further research on the binding relationship between miR-138 and SIRT1 was carried on, and the result indicated that the SIRT1 gene expression was significantly increased after miR-138 was inhibited (Figure 4B) by adding miR-138 inhibitor to NR8383 (Figure 4C). Subsequent detection of changes in protein levels demonstrated that low expression of miR-138 significantly enhanced SIRT1 protein level (Figure 4D). To further verify whether miR-138 can bind to SIRT1, the SIRT1 wild-type sequence (SIRT1-WT 3'UTR) and the mutant sequence (SIRT1-MUT 3'UTR) were constructed. After transfecting miR-138 in NR8383 cells, the lu-

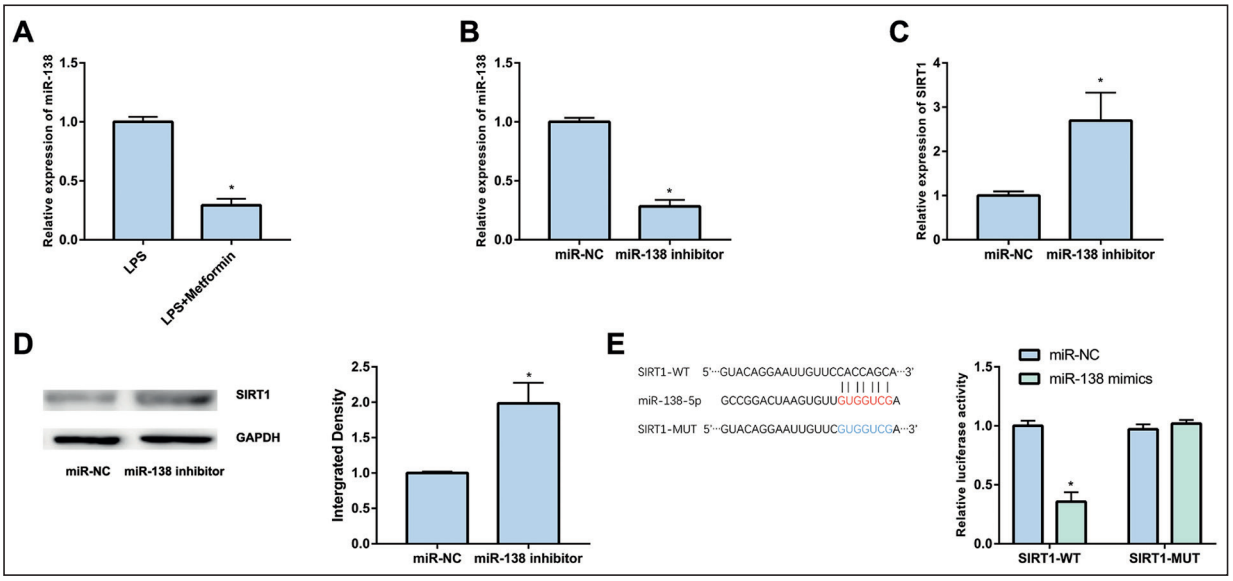

Figure 4. Metformin regulated SIRT1 expression through miR-138. ***A,*** Metformin reduced miR-138 expression. ***B,*** Transfection of miR-138 inhibitor in cells reduced miR-138 expression. ***C,*** Lowering miR-138 expression increased SIRT1 RNA level. ***D,*** Lowering miR-138 increased SIRT1 protein level. ***E,*** A reporter gene plasmid for SIRT1 was constructed. The results suggested that miR-138 could bind directly to SIRT1.

ciferase of SIRT1-WT 3'UTR group decreased, while that of SIRT1-MUT 3'UTR showed no significant difference (Figure 4E), indicating that SIRT1 could combine with miR-138. These above results showed that miR-138 might exert its effect by inhibiting SIRT1 expression.

Discussion

ARDS is a diffuse damage of capillary endothelial cells caused by extrapulmonary factors, which can lead to impaired cell barriers, pulmonary edema, and atelectasis. Its clinical mortality is high, but the occurrence and development process is complex and the pathogenesis has not yet been fully elucidated. Studies[14-16] have shown that the occurrence and development of ARDS are related to inflammatory responses, aquaporins, imbalance of coagulation/fibrinolysis systems, and apoptosis of normal lung cells (AM, alveolar epithelial cells, etc.), among which, the inflammatory response may be the "central link" of its pathogenesis.

Gram-negative bacteria are clinically common[17] that can produce LPS. LPS is the most important initiator of septic shock. By binding to the receptors on the membrane of mononuclear macrophages, LPS transmits extracellular stimulation signals to intracellular, causing cells to activate to produce a large number of cytokines and inflammatory mediators[18], which can finally exert damaging biological effects. Therefore, in this experiment, we used LPS intraperitoneal injection to build ARDS model, which is an ideal and commonly used model that is consistent with the clinical pathogenesis of ARDS. After 8 hours of observation, mice in the model group developed pulmonary edema, blood vessel exudation, and neutrophil aggregation, confirming that the model we established in this study was qualified. Subsequently, we detected the expression of related inflammatory factors and found that the levels of TNF-α, IL-1b, IL-6, and IL-17 in the ARDS model were significantly increased.

Metformin is a commonly used oral hypoglycemic agent and has a history of more than 50 years. It is widely used in clinic because of its low price and low side effects. Its hypoglycemic mechanism may be closely related to the reduction of oxidative stress level as well as the release of inflammatory factors[19]. Many scholars have found that metformin has an active therapeutic effect in various lung diseases, among which, metformin was found in asthma mice to reduce airway inflammation and airway remodeling by inhibiting oxidative stress[20]. Another study[21] found that metformin can reduce the release of inflammatory factors, thereby inhibiting the development of inflammation. In addition, this medicine can reduce the number of inflammatory cells in pulmonary lavage fluid in mice with pulmonary fibrosis, inhibit the production of TGF-β and collagen, and reduce the deposition of collagen around the bronchi, thereby inhibiting the occurrence and development of pulmonary fibrosis[22]. In this study, metformin reversed LPS-induced pulmonary edema, vascular leakage, and neutrophil accumulation, and the content of TNF-α, IL-1b, IL-6, and IL-17 was reduced in the ARDS model.

Sirtuin1 (Sirt1), a homolog of mammalian Sir2, is a NAD+-dependent class III histone deacetylase. Sirt1 has been shown to be involved in many pathophysiological processes and plays a key role in rheumatoid arthritis and acute lung inflammation[23]. MAPK signaling pathway is involved in a variety of cell proliferation, differentiation, and apoptosis regulation[24]. P38 MAPK is the most important member of the MAPK family pathway that regulates inflammatory responses. Activated p38 MAPK promotes the production of inflammatory cytokines, leading to uncontrolled inflammatory responses and ARDS in the lung[25]. The p38 MAPK pathway not only promotes the production of many inflammatory factors, but also participates in the activation of NF-κB and aggravates lung injury. The results of this study showed that after LPS injection with metformin treatment, the expression of SIRT1 was higher than that of LPS treatment alone, while the expression of p-p38, p-ERK and p-NF-κB was significantly decreased.

As a member of non-coding RNA, miRNAs can regulate the cell differentiation and proliferation through multiple signaling pathways, and are closely related to many normal or abnormal pathophysiological processes. A large number of studies have shown that a variety of miRNAs (miR-142-3p, miR-155, miR-429, etc.)[26] are closely related to the occurrence and progress of ARDS. Our work showed that the addition of metformin after LPS treatment significantly decreased the expression of miR-138-5p. In addition, miR-138-5p was verified to bind to SIRT1, suggesting that miR-138-5p may be involved in the relief of ARDS by metformin through SIRT1.

Conclusions

This study demonstrated that metformin could reduce LPS-induced lung injury and increased expression of inflammatory factors. A possible mechanism might be that metformin-induced low expression of miR-138-5p targeted SIRT1 to increase its expression and suppress the MAPK pathway to relieve ARDS.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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