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Troglitazone-activated PPARγ inhibits LPS-induced lung alveolar type II epithelial cells injuries via TNF-α

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Abstract Acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) are common syndromes characterized by diffuse, acute injury to the alveolar epithelium and pulmonary vascular endothelial cells, with high mortality rate for there are no effective pharmacological therapies. Peroxisome proliferators-activated receptor γ (PPARγ), a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors, is ubiquitously expressed within the lung. Recent studies have indicated PPARγ can protect lung tissue and alleviate pulmonary inflammatory injury. But no studies examined whether PPARγ agonists can protect the alveolar epithelial cells cultured in vitro. We observed the protective effect of PPARγ in LPS-induced alveolar type II epithelial cells injury. The results showed troglitazone-activated PPARγ could inhibit the production of TNF-α, one of the most important inflammatory factors, and then increased the expression of surfactant-associated protein A (SP-A) and attenuate the apoptosis of alveolar type II epithelial cells. Our results suggest that PPARγ may have a potential therapeutic effect on ALI.

Keywords Alveolar type II epithelial cell · Lipopolysaccharide · Peroxisome proliferator-activated receptor γ · Tumor necrosis factor-α

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Introduction

Acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS), are characterized by diffuse, acute injury to the alveolar epithelium and pulmonary vascular endothelial cells. In clinical practice, ALI presents with significant respiratory distress, refractory hypoxemia and a progressive decline in lung compliance. The pathogenesis of ALI is complex and has not been fully understood. ALI is currently regarded as a pulmonary representation of systemic multi-organ dysfunction. Cytokines are involved in and are thought to play critical roles in modulating ALI [1–5], which usually results in an increase of proinflammatory factors, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6, along with a decrease in anti-inflammatory cytokines including IL-4, IL-10 and IL-13. This increased inflammation and concurrent weakening of the anti-inflammatory system leads to an excessive inflammatory response which is thought to injure the lungs [6]. Injury of the alveolar epithelial cells is an important pathological change of ALI/ARDS. Alveolar type II epithelial cells are alveolar epithelial stem cells which play critical roles in ALI on alveolar walls damage repair [7]. And alveolar type II epithelial cells can synthesize and secrete the surfactant [8]. Reduce the damage of alveolar type II epithelial cells and resume its function are expected to be the effective method to treat ALI.

Peroxisome proliferator-activated receptor (PPAR), a member of the non-steroid hormone receptors of the nuclear receptor superfamily [9], is an evolutionary conserved one in numerous species. Three subtypes of PPAR exist, PPARα, PPARβ and PPARγ, each having a unique pattern of tissue distribution, expression and physiological function [10, 11]. Widely distributed in the body, especially in adipose tissue and immunocytes, PPARγ regulates

a variety of physiological reactions, including lipid metabolism, glucose homeostasis, cell proliferation, differentiation and apoptosis [12, 13]. PPARγ is also found in the pulmonary monocytes, macrophages [14, 15], alveolar and airway epithelial cells [16–22], vascular endothelial cells and smooth muscle cells [23, 24]. Recent studies have suggested that the PPARγ agonist 15-PDJ$_2$ and rosiglitazone could inhibit the excessive production of inflammatory mediators, and alleviate the pulmonary inflammatory injury in the carrageenan and LPS-induced ALI animal model, respectively [25, 26]. Other studies have also shown that PPARγ agonist could protect lung tissues by means of modulation of the activity of nuclear factor-κB (NF-κB) pathway [27].

Although treatment with PPARγ agonists can protect lung tissues and alleviate pulmonary inflammatory injuries, no studies have examined whether PPARγ agonists can protect the alveolar epithelial cells cultured in vitro. In this study, we studied the protective effect of PPARγ in LPS-induced injury on alveolar type II epithelial cells. Our results will further elucidate the biological functions of PPARγ in the lung, and provide the theoretical basis for the application of PPARγ agonist.

Materials and methods

Agents

LPS (Sigma, USA), PPARγ agonist: troglitazone (Sigma, USA), PPARγ antagonist: GW9662 (Sigma, USA).

Cell culture

Alveolar type II epithelial cells were separated and purified as described by Dobbs [28]. Evaluated by alkaline phosphatase (AKP) staining [29], the purity of alveolar type II epithelial cells was over 90%. The cells were cultured in DMEM F12 culture medium supplemented with 10% fetal bovine serum, and incubated in a 37°C incubator with 5% CO_2. After being inoculated to 35 mm culture dishes with a density of 1.0×10^6/ml, the cells were cultured for 3 days before being used in experiments. The cells were divided into four groups: control, LPS, troglitazone–LPS and troglitazone–GW9662–LPS according to the experimental requirements. Control group: Phosphate buffered saline (PBS) stimulated 6 h with dimethyl sulphoxide (DMSO, the vehicle for troglitazone and GW9662) pretreatment for 0.5 h. LPS group: LPS (1 μg/ml) stimulated 6 h with DMSO pretreatment for 0.5 h. Troglitazone–LPS group: LPS (1 μg/ml) stimulated 6 h with troglitazone (10 μmol/l) pretreatment for 0.5 h. Troglitazone–GW9662–LPS group: LPS (1 μg/ml) stimulated 6 h with troglitazone (10 μmol/l) and GW9662 (10 μmol/l) pretreatment for 0.5 h. The doses and the time course of LPS, troglitazone and GW9662 in the experiment were according to the reference and the preliminary experiment [30–35].

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using the Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Total RNA at the dose of 1 μg from each group was used as template to perform a reverse transcriptase reaction. The reaction was performed according to the manual of the kit as follows: room temperature for 10 min, 42°C for 45 min, 95°C for 5 min and finally 4°C for 10 min. The synthesized cDNA could be used for immediate PCR amplification. The primers of TNF-α, SP-A, glyceraldehydes-phosphate dehydrogenase (GAPDH) were designed according to their sequences on GenBank. The 380 bp fragment of TNF-α was amplified with the sense primer, 5′-CTTCTGTCTACTGAACTTCGG-3′, and the antisense primer, 5′-GTGCTTGATCTGTTGTTTCC-3′. The reaction conditions were as follows: 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 55°C for 45 s, and 72°C for 30 s, and lastly at 72°C for 5 min. The 359 bp fragment of SP-A was amplified with the sense primer, 5′-AGATTCTGCAAACAATGGGAGT-3′, and the antisense primer, 5′-CCTATCATTCCATGTCCCATCT-3′. The PCR reaction conditions were as follows: 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 58°C for 45 s, and 72°C for 30 s, and lastly at 72°C for 5 min. The 196 bp fragment of GAPDH was amplified with the sense primer, 5′-CCATGGAGAAGGCTGGGG-3′ and the antisense primer, 5′-CAAAGTTGTCATGGATGACC-3′ using the following PCR conditions: 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 62°C for 45 s, and 72°C for 30 s, and lastly at 72°C for 5 min. The PCR products were separated on 2.0% agarose gel and stained with ethidium bromide. The relative expressions of mRNA were assessed by densitometry using a gel documentation and analysis system (Lambda Bio-20, USA) and normalized to values for the housekeeping gene GAPDH.

Measurement of TNF-α by enzyme linked immunosorbent assay (ELISA)

TNF-α level in the supernatant of cell homogenates was measured by a rat TNF-α ELISA kit (Jingmei Co., China). These assays were performed according to the manufacturer's instructions.

Protein extraction and Western blot

The total protein of alveolar type II epithelial cells was extracted according to the manual of radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% SDS, 1 M PMSF, 10 μg/ml leupeptin, 1 mM aprotinin, 50 Mm Tris–Cl, pH 7.4). And the protein concentrations were quantitated by the Coomassie protein assay (Bradford method). Total protein at the dose of 20 μg from each sample were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked by 5% skimmed milk powder at room temperature for 1 h. Following blocking, the membranes were incubated in mouse anti-β-actin monoclonal antibody (Santa Cruz, USA, 1:500) and rabbit anti-SP-A polyclonal antibody (Santa Cruz, USA, 1:200) at 4°C overnight, respectively. The membranes were then washed and incubated in horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (1:8,000 dilution) or HRP-labeled goat anti-mouse antibody (1:5,000 dilution) for 1 h. Immunoreactive proteins were visualized with enhanced chemiluminescence detection kit (Amersham Biosciences, NJ, USA). The relative amount of proteins were quantified from relative absorbance of the band by image analysis system (G Box Chemi XT16, Syngene, USA) and expressed as mean relative optical density value.

Immunofluorescence

The expression of SP-A in alveolar type II epithelial cells was detected by immunofluorescence. The cell-attached coverslips of alveolar type II epithelial cells from each group were collected, conventionally fixed, penetrated, blocked, washed and then incubated with anti-SP-A antibody (1:50) at 4°C overnight. After being washed, the cells were incubated with FITC-labeled secondary antibody (Amersham, USA, 1:200) at 37°C for 60 min. Images were acquired on a fluorescence microscopy.

Flow cytometry by FITC-conjugated annexin V and PI staining

Alveolar type II epithelial cells from each group were digested with 0.25% trypsin, and then were stained with annexin V-FITC and propidium iodide according to the kit manufacturer's instructions (Longji Co., China). The percentages of apoptosis and necrosis of cells were analyzed by flow cytometry (Becton-Dickinson, USA).

Statistical analysis

All experiments were performed in triplicate and were repeated at least three times. All data were expressed as mean ± SD. Statistical difference was determined by one-way ANOVA or the nonparametric test (Kruskal–Wallis H) among multiple groups using SPSS 13.0 software (SPSS Inc., USA). $P < 0.05$ was considered significant.

Results

Troglitazone significantly reduced TNF-α transcription and secretion (Fig. 1)

After stimulated with LPS, mRNA synthesis and protein secretion of the inflammatory factor TNF-α significantly

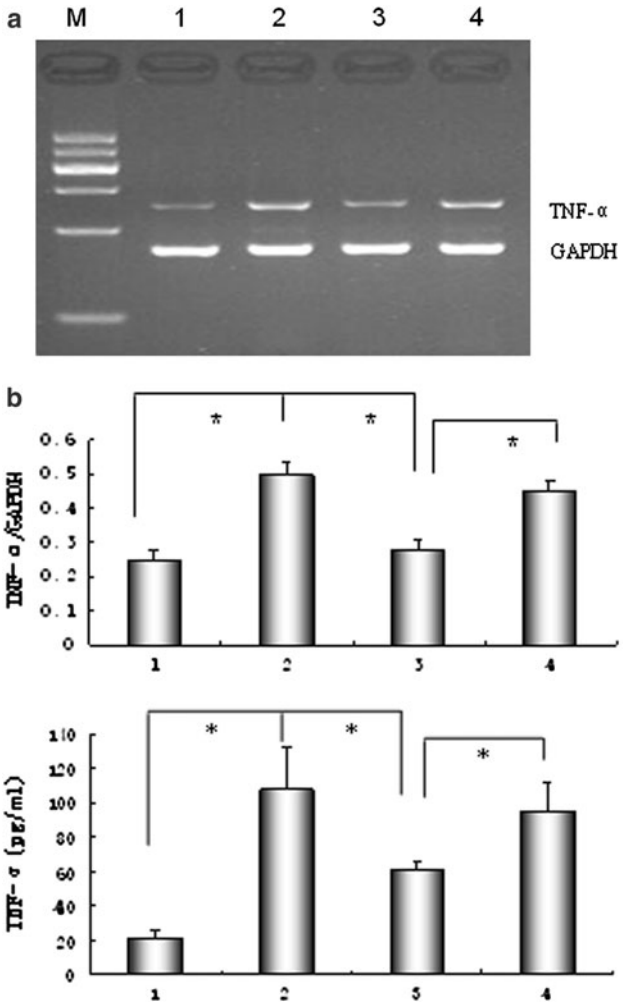

Fig. 1 Troglitazone significantly reduced TNF-α transcription and secretion. **a** RT-PCR showed that treatment with LPS increased TNF-α mRNA transcription in alveolar type II epithelial cells ($P < 0.05$). Intervention with troglitazone reduced the transcription of TNF-α compared to the LPS group ($P < 0.05$). GW9662 blocked the effect of troglitazone. **b** ELISA showed that LPS significantly increased the secretion of inflammatory factor TNF-α ($P < 0.05$), while treatment with troglitazone significantly reduced the expression of TNF-α to the LPS group ($P < 0.05$). GW9662 blocked the effect of troglitazone. *1* control, *2* LPS, *3* troglitazone–LPS, *4* troglitazone–GW9662–LPS. *$P < 0.05$

increased in alveolar type II epithelial cells cultured in vitro ($P < 0.05$). Treatment with troglitazone significantly decreased both the transcription and secretion of TNF-α as compared with those in the LPS group ($P < 0.05$). GW9662 blocked the effect of troglitazone.

Activation of PPARγ by troglitazone increased the SP-A expression in alveolar type II epithelial cells (Figs. 2, 3)

After treatment with troglitazone, both the mRNA transcription and protein expression of SP-A increased significantly ($P < 0.05$), as compared with those in the LPS group. GW9662 blocked the effect of troglitazone on SP-A expression.

Activation of PPARγ by troglitazone inhibited LPS-induced alveolar type II epithelial cells apoptosis (Fig. 4)

The flow cytometry results showed that the rates of apoptosis and necrosis in rat alveolar type II epithelial cells increased significantly after stimulated with LPS

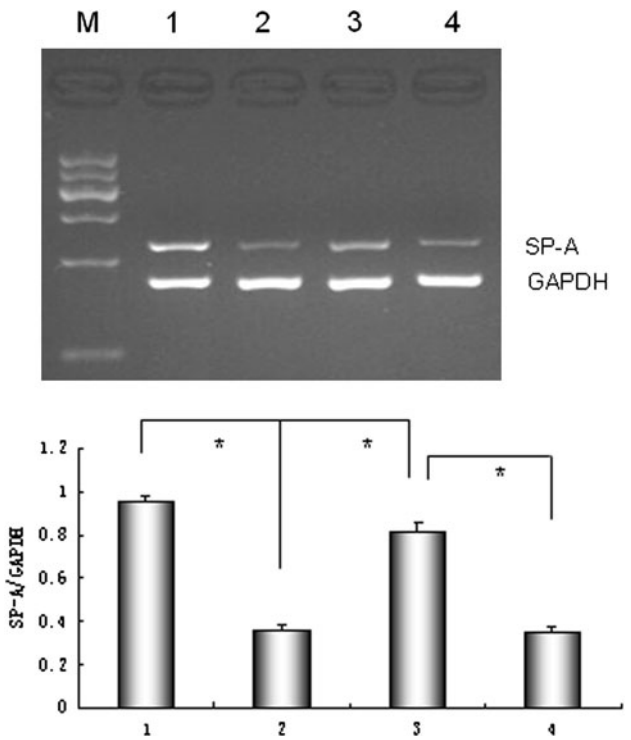

Fig. 2 Troglitazone increased the SP-A transcription in alveolar type II epithelial cells. RT-PCR showed that LPS exposure significantly reduced mRNA transcription of SP-A in alveolar type II epithelial cells ($P < 0.05$). Treatment with troglitazone significantly increased SP-A mRNA transcription ($P < 0.05$). GW9662 blocked the effect of troglitazone. *1* control, *2* LPS, *3* troglitazone–LPS, *4* troglitazone–GW9662–LPS

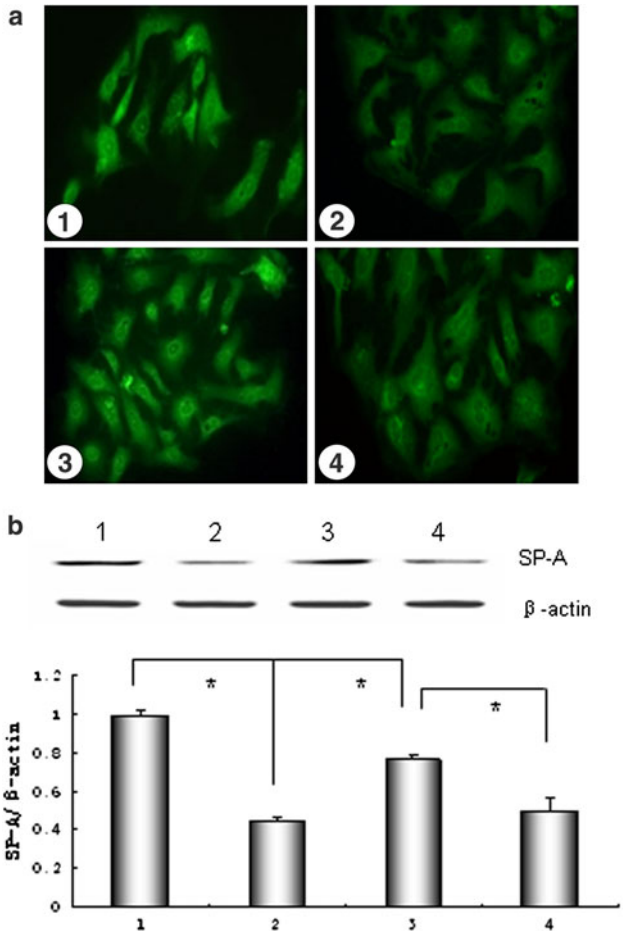

Fig. 3 Troglitazone increased the SP-A expression in alveolar type II epithelial cells. **a** Immunofluorescence showed that SP-A was mainly expressed in cytoplasm of alveolar type II epithelial cells, and that exposure to LPS decreased SP-A expression. Treatment with troglitazone significantly increased the expression of SP-A compared to the LPS group (×100). **b** Western blot showed that LPS exposure reduced the protein expression of SP-A in alveolar type II epithelial cells ($P < 0.05$). Troglitazone treatment increased SP-A expression relative to the LPS group ($P < 0.05$). GW9662 blocked the effect of troglitazone on SP-A expression. *1* control, *2* LPS, *3* troglitazone–LPS, *4* troglitazone–GW9662–LPS

($P < 0.05$), while troglitazone significantly reduced apoptosis and necrosis in these cells ($P < 0.05$). GW9662 could block the effect of troglitazone.

Discussion

ALI commonly results from the excessive inflammatory response caused by endotoxin in sepsis, and its incidence is regulated by inflammatory mediators and cytokines together [36]. NF-κB is considered the key nuclear factor of transcriptional regulation for many inflammatory mediators [37, 38], including TNF-α, IL-1, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion

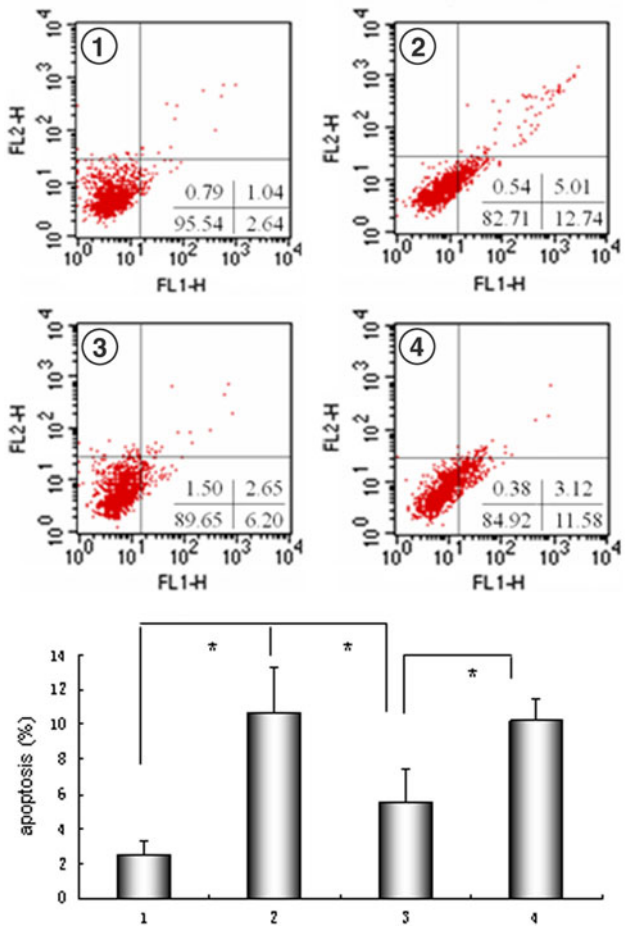

Fig. 4 Troglitazone reduced the cell apoptosis in alveolar type II epithelial cells. Flow cytometry showed that LPS significantly increased the rates of apoptosis and necrosis in rat alveolar type II epithelial cells, while compared to the LPS group ($P < 0.05$), those rates were significantly reduced when the cells were treated with troglitazone ($P < 0.05$). GW9662 blocked the effect of troglitazone. The *X*- and *Y*-axis were corresponded to the fluorescence intensity of Annexin V and PI, respectively. The lower left area represented the normal cells. The lower right area showed the early apoptotic cells, while the upper and lower right areas together characterized all the apoptotic and necrosed cells

molecule-1 (ICAM-1) and inducible nitric oxide synthase (iNOS). TNF-α is one of the most important inflammatory mediators regulated by NF-κB, as it can act directly on inflammatory effectors at a cellular level, promote the release of other inflammatory mediators and participate in the inflammation response cascade. Therefore, the level of TNF-α may be a direct reflection of the severity of inflammation [39]. Studies have shown that LPS-induced ALI is associated with increased TNF-α in the lung, suggesting that inhibition of TNF-α in lung tissues may help decrease inflammatory cell infiltration and protect the lung [40, 41].

Injury of the alveolar epithelial cells is an important pathological change of ALI [42–44]. Alveolar epithelial cells include two types of alveolar cells: type I and type II. Type II cells can proliferate and transform to type I cells during the alveolar damage [7], repairing the injury to the alveolar wall [45, 46]. And another important function of alveolar type II epithelial cells is to synthesize and secrete the surfactant to improve lung compliance and effectively maintain the stability of alveolar structure and the normal physiological function of the lung tissues [8]. Surfactant is composed of lipids (mostly phospholipids) and proteins, in which the main functional hydrophilic protein, SP-A, accounts for about 50% of the total protein. Surfactant plays an important role in maintaining lung homeostasis, and SP-A is considered as an indicator of the function of alveolar type II epithelial cells and an important indicator of the severity and prognosis of ALI [47]. It was reported that in patients with ALI, decease in SP-A in bronchoalveolar lavage fluid can be found in the early stage, and its rate of decline is positively correlated with disease severity [48].

Our study demonstrated that LPS exposure could significantly decrease the expressions of SP-A in alveolar type II epithelial cells while concurrently increase the release of TNF-α and other inflammatory factors as well as the reduction of SP-A expression. Flow cytometry showed that LPS exposure could increase both cell necrosis and apoptosis. Troglitazone, the PPARγ activator, could significantly inhibit the release of TNF-α, upregulate the expression of SP-A and reduce alveolar type II epithelial cell necrosis and apoptosis. Conversely, GW9662, the PPARγ antagonist, could block all effects of troglitazone on lung cells. Miakotina reported that the reduction of SP-A expression might be primarily caused by the inhibition of TNF-α during ALI, besides the injury of the alveolar type II epithelial cells [49]. Michael reported that the activation of PPARγ might not directly affect the expression of SP-A [50]. Therefore, we speculate that troglitazone may upregulate SP-A during inflammation by inhibiting NF-κB activity and reducing TNF-α release, both of which result in attenuated cell injuries. Excessive apoptosis and necrosis of alveolar epithelial cells can also lead to ALI, and the Fas/Fas ligand (Fas L) is the primary pathway through which LPS induces alveolar type II epithelial cell apoptosis [51–54]. White reported that SP-A could regulate the activation of Fas/FasL pathway [55], an important factor in inhibiting alveolar type II epithelial cell apoptosis. During ALI, excessive release of inflammatory factors can inhibit the synthesis of SP-A, which can increase the activity of the Fas/FasL pathway and promote the apoptosis of alveolar epithelial cells. Increased apoptosis can further reduce SP-A expression, resulting in exacerbation of the lung injuries. Activation of PPARγ by troglitazone could decrease NF-κB nuclear translocation, leading to reduced formation of its downstream inflammatory factors. The

decrease in inflammatory mediators could restore the function of SP-A, and therefore SP-A could reduce the cell apoptosis and have a protective effect on the lungs.

In conclusion, the activation of PPARγ by troglitazone reduced the LPS-induced release of TNF-α. By decreasing the levels of inflammatory mediators, PPARγ increased the expression of SP-A, and reduced the necrosis and apoptosis of alveolar type II epithelial cells. The numerous positive effects on lung tissues suggest that PPARγ may have a potential therapeutic effect on ALI.

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