

Original Article

Rosiglitazone inhibits lipopolysaccharide-induced A549 cell apoptosis and inflammation

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Abstract: The present study was aimed at clarifying the effects of an anti-inflammatory agent, rosiglitazone, on the progression of acute respiratory distress syndrome (ARDS) *in vitro* model induced by lipopolysaccharide (LPS, 10 µg/ml) in A549 cells. Peroxisome proliferator activated receptor-γ (PPARγ) agonist rosiglitazone (10 mM) was added into the LPS-stimulated A549 cells for 48 h. Cell viability and proliferation of A549 was examined by CCK-8 and Brdu assay, respectively. Cell apoptosis and ROS generation in response to LPS and rosiglitazone were measured by flow cytometry assay. The contents of TNF-α, IL-6 and MCP-1 in supernatants of A549 cells were measured by ELISA. The expression of PPARγ, NF-κBp65, Bax, Bcl-2, NOX4 and iNOS was measured by Real-time PCR and Western blotting. Our results showed that LPS induced cell apoptosis and inflammation response, suggesting a success of ARDS model was established. Rosiglitazone treatment reduced LPS-induced injury in A549 cells, evidenced by inhibiting cell apoptosis, ROS generation and secretion of TNF-α, IL-6 and MCP-1. Furthermore, rosiglitazone also inhibited LPS-induced increased expression of NOX4, iNOS, NF-κBp65 and Bax. Taken together, our data suggest that LPS inhibits A549 cell viability and proliferation and induces apoptosis through inhibiting PPARγ, which was reversed by rosiglitazone, and thus rosiglitazone may serve as an agent for ARDS treatment.

Keywords: Acute respiratory distress syndrome, lipopolysaccharide, rosiglitazone, PPARγ, NF-κBp65

Introduction

Acute respiratory distress syndrome (ARDS) previously called acute lung injury (ALI) was characterized by rapid onset of respiratory failure [1], in the presence of inflammation, alveolar epithelial and epithelial barrier destruction, and activation of neutrophil and platelet, following a variety of direct and indirect lung insults [2, 3]. The pathogenesis of ARDS is complex, including cell and tissue injuries caused by the imbalance of inflammatory and anti-inflammatory reaction [4], microthrombus formation caused by coagulation and/or fiber disorder [5] and systemic involvement caused by severe hypoxic disease [6].

Peroxisome proliferator activated receptors (PPARs) comprise a family of ligand-activated transcription factors belonging to the nuclear hormone receptor family that are related to immunomodulatory and anti-inflammatory

activity in a variety of disease states including type 2 diabetes, atherosclerosis, inflammatory bowel disease, arthritis, myocarditis, cancer, and endotoxic shock [7, 8]. PPAR isoform PPARγ is prominently involved in many feedback loops that normally limit inflammation, such as decreases in cytokines, chemokines, ROS and adhesion molecules [9], through interacting with transcription factor NF-κB preventing its association with DNA sequences [10] and hence suppressing inflammatory gene transcription [11]. Inhibiting PPARγ promotes fibroproliferative ARDS [12] and rosiglitazone, a PPARγ agonist, reduces acute lung injury in endotoxemic rats [13]. However, to date, few studies have tested the mechanism of rosiglitazone as a potential treatment intervention following the onset of pulmonary inflammation and lung injury.

Lipopolysaccharide (LPS), derived from gram-negative bacteria, induces pulmonary inflam-

mation models, which have been used extensively for testing new anti-inflammatory drugs [14]. The inflammatory stimulation of LPS is mainly through Toll like receptor-4 following activating NF- κ B [15], results in the synthesis and release of a variety of inflammatory mediators. In particular, activated alveolar macrophages and neutrophils cause the excessive production of pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), which attract neutrophils and cause the release of further cytokines and chemokines [16]. Moreover, NF- κ B is activated by ROS, which elicits increased expression of the iNOS gene in mouse and man [17], whereas NO may inhibit NOX via a not yet well defined mechanism.

The A549 human alveolar epithelial cell line, which has many of the features of alveolar type II epithelial cells, is often used as a surrogate for human alveolar epithelium. In order to evaluate the effect of rosiglitazone on LPS-induced A549 cells apoptosis and inflammation *in vitro*, we analyzed cell patterns and estimated protein expression. In addition, ELISA and western blot observations for apoptosis and inflammatory response were made on the A549 cells. Thus the present study was conducted to investigate the effects of rosiglitazone on LPS-induced apoptotic and inflammatory signal transduction in human alveolar epithelial A549 cells.

Materials and methods

Cell culture and drug treatment

Human lung carcinoma type II epithelium-like A549 cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and grown in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂. For experiments, cells were seeded at a density of 5 $\times$ 10⁵ cells/well in six-well culture plates or 1 $\times$ 10⁵ cells/well in 24-well culture plates and cultured for 24 h to form an 80-90% confluent monolayer. Cells were divided into three groups (n=3 in each group): control groups (untreated A549 cells), LPS groups (10 μ g/ml) and LPS plus rosiglitazone groups (10 μ g/ml LPS+10 mM rosiglitazone). For the LPS groups, LPS was added to A549 cells for 24 h. For the LPS plus rosigli-

tazone groups, LPS and rosiglitazone were simultaneously added to A549 cells for 24 h. After these additions, the cells were incubated at 37°C in a humidified 5% CO₂ incubator for different periods of time and then the cells or the supernatants were harvested for following measurements.

Assay of cell viability

After drug treatment for 0, 24, 48 or 72 h, the Cell Counting Kit-8 (CCK-8) solution mixed with FBS-free RPMI-1640 (v/v, 1:10) was added into the A549 cells (100 μ l/well) and cultured 37°C in a 5% CO₂ incubator for 1 h, according to the manufacturer's instructions. Absorbance of the supernatant for each well was measured at 450 nm using the Multiskan EX plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

BrdU incorporation

After drug treatment for 48 h, the A549 cells were harvested at a density of 1 $\times$ 10⁵ cells/well and incubated with 10 μ M BrdU for 60 min in the dark at 37°C. Then the cells were incubated with 1 ml of BrdU Staining Buffer for 2 h and washes with PBS prior to incubated with 5 μ l of anti-BrdU for 1 h in the dark at room temperature. Data was acquired by flow cytometry (BD Biosciences, San Diego, CA, USA).

Quantification of apoptotic cells by flow cytometry

Cell apoptosis analysis was performed using flow cytometry and an Annexin V apoptosis detection kit (eBioscience, Inc., San Diego, CA, USA). Briefly, after drug treatment for 48 h, the harvested A549 cells were plated in 6-well plates at a density of 1 $\times$ 10⁵ cells/well and incubated with 195 μ l Annexin V and 5 μ l propidium iodide for 15 min in the dark at 4°C. The early and late apoptotic cells were represented in the lower right and upper right quadrant of the fluorescence-activated cell sorting histogram.

Quantification of intracellular ROS by flow cytometry

Detection of ROS was performed using flow cytometric analysis as described previously. In brief, after drug treatment for 48 h, A549 cells were washed with PBS, resuspended in complete medium and incubated with 0.5 μ M dihy-

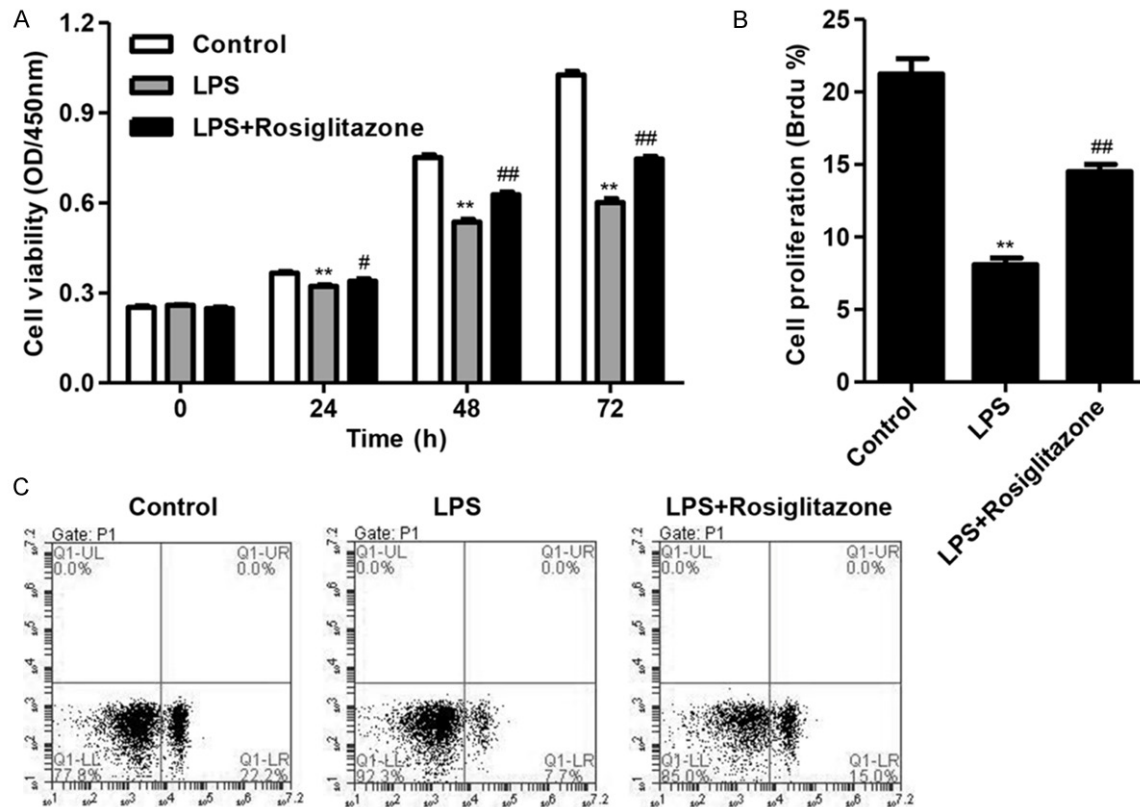


Figure 1. Effect of rosiglitazone on LPS-induced cell viability and proliferation of A549 cells. Exposure of A549 cells to 10 μ g/ml LPS with or without 10 mM rosiglitazone simultaneously for 24, 48 and 72 h, the cell viability of A549 cells was measured by CCK-8 assay. After treatment for 48 h, the cell proliferation of A549 cells was measured by BrdU assay. * P <0.05, ** P <0.01 compared with control; ## P <0.01 compared with LPS.

drorhodamine 123 (Sigma Aldrich; Merck Millipore) for 30 min at 37°C. ROS fluorescence intensity was determined by flow cytometric analysis, with excitation at 490 nm and emission at 520 nm.

Enzyme-linked immunosorbent assay (ELISA)

The cell culture supernatants were collected from A549 cells 48 h after treatment. The concentrations of TNF- α , IL-6 and MCP-1 were measured with ELISA technology.

Quantitative real-time RT-PCR analysis

The cell samples were harvested at 48 h after treatment to analyze the expression of NOX4 and iNOS mRNAs as described previously [18]. Target mRNA was quantified by real-time PCR using the SYBR® Premix Ex Taq™ kit on a Bio-Rad iCycler iQ5 Real-time Detection System. Oligonucleotides for these PCR analyses were designed and synthesized by Clontech Laboratories (Palo Alto, CA, USA). The oligonucleotide sequences of the respective upstream

and downstream primers for these mRNA analyses were 5'-AGGAGAACCAGGAGATTG-3' and 5'-GAAGTTGAGGGCATTAC-3' for NOX4, 5'-CC-CAGCCTCAAGTCTTATTTCC-3' and 5'-ACTCAGC-AGCAAGTTCATC-3' for iNOS, and 5'-CACCCA-CTCTCCACCTTTG-3' and 5'-CCACCACCCTGTT-GCTGTAG-3' for GAPDH. Relative mRNA quantities were determined by using the $2^{-\Delta\Delta Ct}$ method with data normalized to the GAPDH housekeeping gene.

Western blot analysis

Protein analyses were carried out according to a previously described method [19]. After drug treatment, proteins were prepared in an ice-cold radioimmunoprecipitation assay (RIPA) buffer. The protein content was determined using the BCA kit. Proteins (15 μ l) were subjected to SDS-PAGE, and transferred to nitrocellulose membranes. After blocking, the membranes were immunodetected using primary antibodies: anti-iNOS (1:500; abcam), anti-NOX4 (1:2000; abcam), anti-PPAR γ (1:1000;

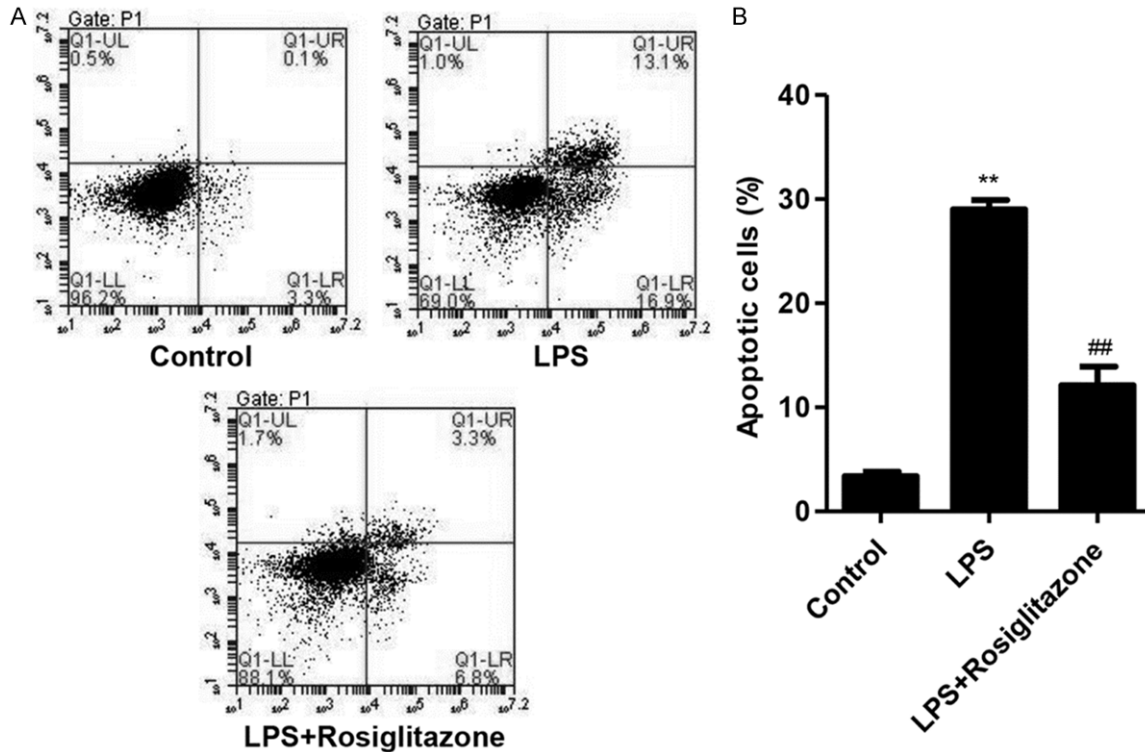


Figure 2. Effect of rosiglitazone on LPS-induced cell apoptosis of A549 cells. A, B. Exposure of A549 cells to 10 μ g/ml LPS with or without 10 mM rosiglitazone simultaneously for 48 h, the apoptosis of A549 cells was measured by flow cytometry assay. ** P <0.01 compared with control; ## P <0.01 compared with LPS.

abcam), anti-Bcl-2 (1:300; santa), anti-Bax (1:300; santa), anti-NF- κ Bp65 (1:1000; Cell Signaling Technology) and anti-GAPDH (1:2000; Cell Signaling Technology) at 4°C overnight. The membranes were washed three times with PBST (PBS+0.1% Tween 20) and then incubated with horseradish peroxidase (HRP)-conjugated antibodies at room temperature for 1 h. The blots were visualized using enhanced chemiluminescence (Millipore, Billerica, MA, USA) and signal intensity was determined using a digital imaging system (UVtec, Cambridge, UK).

Statistical analysis

Statistical analyses were performed with SPSS 19.0 statistical software. Results were expressed as mean $\pm$ SD. Statistical analysis between groups was carried out by unpaired, two-pair t test. A p value of less than 0.05 was defined statistically significant.

Results

Rosiglitazone inhibits LPS-induced decreased cell viability and proliferation of A549 cells

Exposure of A549 cells to 10 μ g/ml LPS for 24, 48 and 72 h caused significant 12.03%,

28.83% and 41.50% decreases, respectively, in the proliferation of A549 cells compared with control groups (**Figure 1**). After exposure to 10 μ g/ml LPS and 10 mM rosiglitazone simultaneously for 24, 48 and 72 h, proliferation of A549 cells was significantly increased by 5.39%, 17.27 and 24.39%, respectively, compared with LPS treatment alone. Thus, 10 μ g/ml LPS with or without 10 mM rosiglitazone simultaneous treatment for 48 h were used in our following experiments. After drug treatment for 48 h, the cell proliferation was also measured by Brdu assay. As shown in **Figure 1B** and **1C**, the cell proliferation was significantly decreased after 10 μ g/ml LPS treatment, which was inhibited by 10 mM rosiglitazone treatment.

Rosiglitazone inhibits LPS-induced cell apoptosis of A549 cells

Exposure of A549 cells to 10 μ g/ml LPS for 48 h caused significant 7.62-fold increase in the apoptosis of A549 cells compared with control groups (**Figure 2A** and **2B**). After exposure to 10 μ g/ml LPS and 10 mM rosiglitazone simultaneously for 48 h, apoptosis of A549 cells was significantly decreased by 58.22% compared with LPS treatment alone.

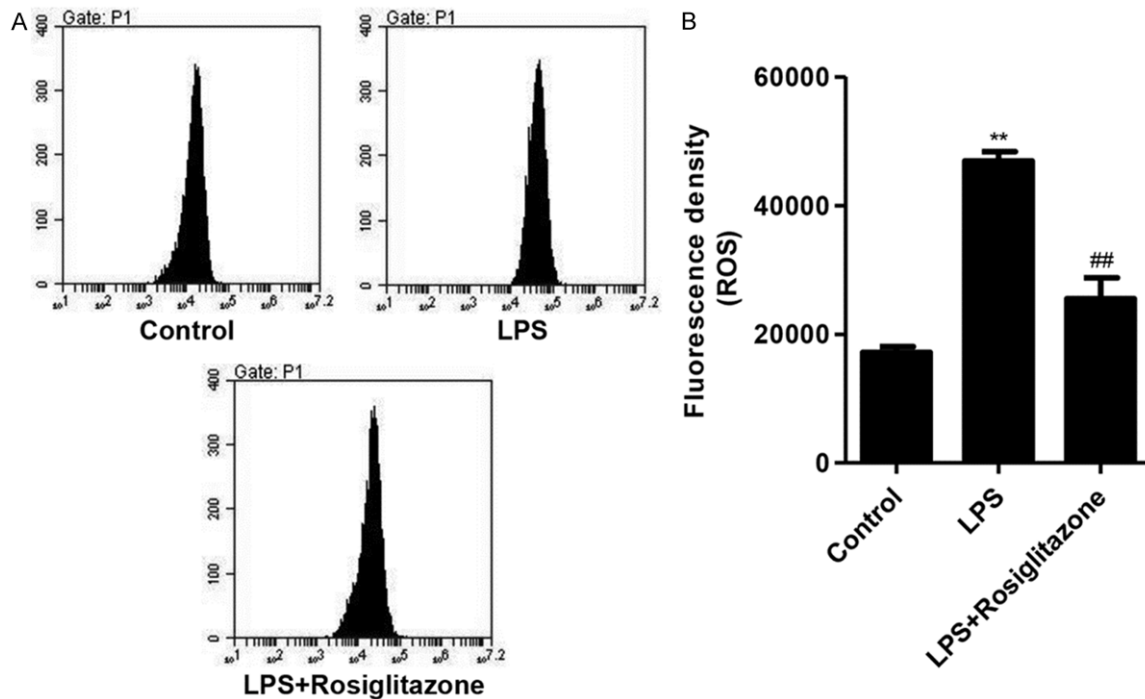


Figure 3. Effect of rosiglitazone on LPS-induced intracellular ROS level of A549 cells. A, B. Exposure of A549 cells to 10 μ g/ml LPS with or without 10 mM rosiglitazone simultaneously for 48 h, the intracellular ROS of A549 cells was measured by flow cytometry assay. ** P <0.01 compared with control; ## P <0.01 compared with LPS.

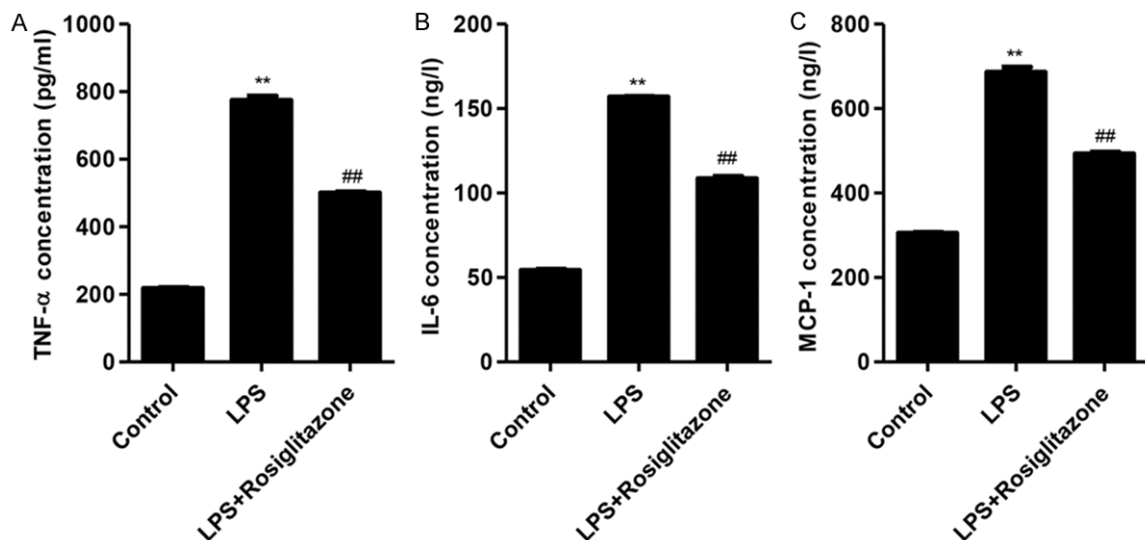


Figure 4. Effect of rosiglitazone on LPS-induced TNF- α , IL-6 and MCP-1 concentration of A549 cells. Exposure of A549 cells to 10 μ g/ml LPS with or without 10 mM rosiglitazone simultaneously for 48 h, the TNF- α (A), IL-6 (B) and MCP-1 (C) concentration of A549 cells was measured by ELISA. ** P <0.01 compared with control; ## P <0.01 compared with LPS.

Rosiglitazone inhibits LPS-induced intracellular ROS generation of A549 cells

Exposure of A549 cells to 10 μ g/ml LPS for 48 h caused significant 1.73-fold increase in the intracellular ROS of A549 cells compared

with control groups (Figure 3A and 3B). After exposure to 10 μ g/ml LPS and 10 mM rosiglitazone simultaneously for 48 h, intracellular ROS of A549 cells was significantly decreased by 45.63% compared with LPS treatment alone.

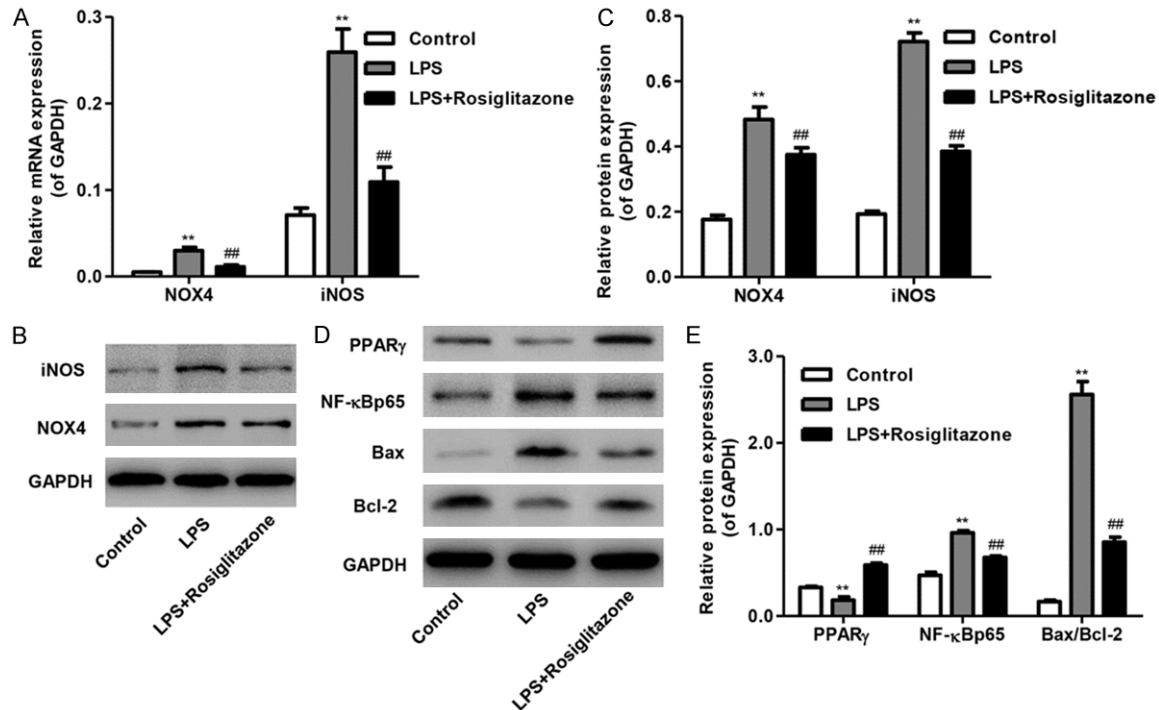


Figure 5. Effect of rosiglitazone on LPS-induced the expression of NOX4, iNOS, NF- κ Bp65, Bax and Bcl-2 in A549 cells. Exposure of A549 cells to 10 μ g/ml LPS with or without 10 mM rosiglitazone simultaneously for 48 h, the expression of NOX4 and iNOS of A549 cells was measured by Real-time PCR (A) and Western blot (B, C), and the expression of PPAR γ , NF- κ Bp65, Bax and Bcl-2 in A549 cells was also measured by Western blot (D, E). ** P <0.01 compared with control; ## P <0.01 compared with LPS.

Rosiglitazone inhibits LPS-induced increased TNF- α , IL-6 and MCP-1 concentration of A549 cells

Exposure of A549 cells to 10 μ g/ml LPS for 48 h caused significant 2.55, 1.89 and 1.25-fold increases, respectively, in the TNF- α , IL-6 and MCP-1 concentration in the culture supernatants of A549 cells compared with control groups (Figure 4A-C). After exposure to 10 μ g/ml LPS and 10 mM rosiglitazone simultaneously for 48 h, TNF- α , IL-6 and MCP-1 concentration in A549 cells was significantly decreased by 35.38%, 30.76% and 28.18%, respectively, compared with LPS treatment alone.

Rosiglitazone inhibits LPS-induced increased expression of NOX4, iNOS, NF- κ Bp65, and Bax/Bcl-2 ratio in A549 cells

Exposure of A549 cells to 10 μ g/ml LPS for 48 h caused significant 4.82 and 2.66-fold increases in the mRNA expression of NOX4 and iNOS in A549 cells compared with control

groups (Figure 5A). After exposure to 10 μ g/ml LPS and 10 mM rosiglitazone simultaneously for 48 h, the mRNA expression of NOX4 and iNOS in A549 cells was significantly decreased by 62.25% and 57.86% compared with LPS treatment alone. Similar results were also found in the protein expression of NOX4 and iNOS in A549 cells with LPS with or without rosiglitazone treatment (Figure 5B and 5C). Moreover, exposure of A549 cells to 10 μ g/ml LPS for 48 h caused significant 44.63% and 52.23% decreases in the protein expression of PPAR γ and Bcl-2 in A549 cells compared with control groups, but increased NF- κ Bp65 and Bax protein expression by 1.03 and 6.36-fold (Figure 5D and 5E). After exposure to 10 μ g/ml LPS and 10 mM rosiglitazone simultaneously for 48 h, the protein expression of PPAR γ and Bcl-2 in A549 cells was significantly increased by 2.21 and 0.46-fold compared with LPS treatment alone, and the NF- κ Bp65 and Bax protein expression in A549 cells was significantly decreased by 29.63% and 51.43%, respectively.

Discussion

The acute respiratory distress syndrome (ARDS) is a rapid onset of respiratory failure characterized by severe impairment in gas exchange and lung mechanics with a high case mortality rate of estimated at 26% to 58% [20, 21], and has not significantly improved for the past several decades. LPS, which is a major component of the cell wall of Gram-negative bacteria, mimics ARDS [22], leading to the recruitment of neutrophils and finally the impairment of gas exchange. LPS can also cause inflammatory responses by human alveolar epithelial A549 cells that is induced cell death by several bioactive mediators [23], including proteases, reactive oxygen species, eicosanoids, phospholipids, and cytokines, that perpetuate inflammatory responses. The results in this study showed that the PPAR γ agonist rosiglitazone induced a significant increase in LPS-induced A549 cell proliferation and cell viability, and decreases in cell apoptosis, intracellular ROS and inflammation response. Rosiglitazone also inhibited NF- κ B activation and decreased the ratio of Bax/Bcl-2 expression. Overall these results suggest that rosiglitazone has potential clinical applications for ARDS.

Apoptosis is an energy-dependent type of programmed cell death. A variety of intrinsic and extrinsic factors are involved in regulating cell apoptosis [24, 25]. Reactive oxygen species (ROS) are apoptotic factors that can cause oxidative stress and subsequent cell apoptosis. Previous study showed that exposure of A549 cells to LPS increased the levels of cellular nitric oxide (NO) and ROS, and the activities of caspase-9 and caspase-6 were also augmented [26]. Our studies showed that exposure of A549 cells to LPS caused apoptosis and ROS production. Moreover, the expression of iNOS, NOX4 and Bax/Bcl-2 ratio was also increased by LPS stimulation. iNOS is one of three key enzymes generating NO from the amino acid L-arginine, plays an important role in numerous physiological and pathophysiological conditions [27]. NOX4 was strong expression in hyperplastic alveolar type II cells and induced ROS generation, and it is a key player in epithelial cell death leading to pulmonary fibrosis [28]. However, treatment of rosiglitazone significantly inhibited LPS-induced apoptosis and ROS production in A549 cells. In line with our

results that PPAR γ is a member of the family of ligand-activated transcription factors, and its ligand rosiglitazone has the ability to selectively activate PPAR γ in cancer cells, including hepatocellular carcinoma Hep3B cells [29], renal cancer Caki cells [30], and non-small cell lung carcinoma A549 cells [31].

On the other hand, PPAR γ and its respective ligand negatively control pro-inflammatory gene expression. The agonist reduces inflammation and vascular leakage in animal ARDS experimental models [13]. Furthermore, cytokines, including TNF- α and IL-6, play important roles in mobilizing neutrophils and promoting LPS-induced ARDS [32, 33]. In addition, it has previously been reported that IL-6 mediated MCP-1 production by endothelial cells [34]. In the present study, we found that rosiglitazone inhibited the expression of TNF- α , IL-6 and MCP-1 in LPS-induced A549 cells. NF- κ B is an important transcription factor which regulates the expression of many cytokines, chemokines, adhesion molecules, inducible enzymes and growth factors [35]. During inflammatory cascade activation, the p65 subgroup of NF- κ B translocates to the nucleus. In LPS-relevant ARDS, LPS binds Toll-like receptor 4 (TLR-4) on the surface of epithelial cells and activates NF- κ B signal transduction [18], and then induces the expression of many inflammatory cytokines and cell-adhesion molecules, finally triggering the relative pathological processes [36, 37]. In this experiment, NF- κ B was activated after LPS stimulation and the addition of rosiglitazone inhibited NF- κ Bp65 nuclear translocation.

In this study, rosiglitazone alleviated LPS-induced apoptosis and inflammation via the augment of PPAR γ expression, leading to the abrogation of NF- κ B activation in human alveolar epithelial cell line A549. These results extend our understanding of the molecular mechanisms underlying the applications of rosiglitazone for ARDS.

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Disclosure of conflict of interest

None.

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References

- [1] Modrykamien AM, Gupta P. The acute respiratory distress syndrome. *Proceedings* 2015; 28: 163.
- [2] ARDS Definition Task Force, Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L, Slutsky AS. Acute respiratory distress syndrome. *JAMA* 2012; 307: 2526-2533.
- [3] Peter JV, John P, Graham PL, Moran JL, George IA, Bersten A. Corticosteroids in the prevention and treatment of acute respiratory distress syndrome (ARDS) in adults: meta-analysis. *BMJ* 2008; 336: 1006-1009.
- [4] Meduri GU, Annane D, Chrousos GP, Marik PE, Sinclair SE. Activation and regulation of systemic inflammation in ARDS: rationale for prolonged glucocorticoid therapy. *Chest J* 2009; 136: 1631-1643.
- [5] MacLaren R, Stringer KA. Emerging role of anticoagulants and fibrinolytics in the treatment of acute respiratory distress syndrome. *Pharmacotherapy* 2007; 27: 860-73.
- [6] Fröhlich S, Boylan J, McLoughlin P. Hypoxia-induced inflammation in the lung: a potential therapeutic target in acute lung injury? *Am J Respir Cell Mol Biol* 2013; 48: 271-279.
- [7] Belvisi MG, Hele DJ. Peroxisome proliferator-activated receptors as novel targets in lung disease. *Chest* 2008; 134: 152-157.
- [8] Spears M, Mcsharry C, Thomson NC. Peroxisome proliferator-activated receptor-gamma agonists as potential anti-inflammatory agents in asthma and chronic obstructive pulmonary disease. *Clin Exp Allergy* 2006; 36: 1494-1504.
- [9] Lee KS, Kim SR, Park SJ, Park HS, Min KH, Jin SM, Lee MK, Kim UH, Yong CL. Peroxisome proliferator activated receptor- γ modulates reactive oxygen species generation and activation of nuclear factor- κ B and hypoxia-inducible factor 1 α in allergic airway disease of mice. *J Allergy Clin Immunol* 2006; 118: 120-127.
- [10] Kato Y, Ying H, Zhao L, Furuya F, Araki O, Willingham MC, Cheng S. PPAR γ insufficiency promotes follicular thyroid carcinogenesis via activation of the nuclear factor- κ B signaling pathway. *Oncogene* 2006; 25: 2736-2747.
- [11] Reddy RC, Standiford TJ. Nrf2 and PPAR (γ): PPARTnering against oxidant-induced lung injury. *Am J Respir Crit Care Med* 2010; 182: 134-135.
- [12] Jain M, Budinger GRS, Lo A, Urlich D, Rivera SE, Ghosh AK, Gonzalez A, Chiarella SE, Marks K, Donnelly HK. Leptin promotes fibroproliferative acute respiratory distress syndrome by inhibiting peroxisome proliferator-activated receptor- γ . *Am J Respir Crit Care Med* 2011; 183: 1490-1498.
- [13] Liu D. Protective effects of rosiglitazone, a peroxisome proliferator-activated receptor-gamma agonist, on endotoxin-induced acute lung injury in rats. *Crit Care Med* 2005; 9: 1-2.
- [14] Abraham E, Nick JA, Azam T, Kim SH, Mira JP, Svetkauskaite D, He Q, Zamora M, Murphy J, Park JS. Peripheral blood neutrophil activation patterns are associated with pulmonary inflammatory responses to lipopolysaccharide in humans. *J Immunol* 2006; 176: 7753-7760.
- [15] Wu TT, Chen TL, Loon WS, Tai YT, Cherng YG, Chen RM. Lipopolysaccharide stimulates syntheses of toll-like receptor 2 and surfactant protein-A in human alveolar epithelial A549 cells through upregulating phosphorylation of MEK1 and ERK1/2 and sequential activation of NF- κ B. *Cytokine* 2011; 55: 40-47.
- [16] Speyer CL, Neff TA, Warner RL, Guo RF, Sarma JV, Riedemann NC, Murphy ME, Murphy HS, Ward PA. Regulatory effects of iNOS on acute lung inflammatory responses in mice. *Am J Pathol* 2003; 163: 2319-2328.
- [17] Aldieri E, Riganti C, Polimeni M, Gazzano E, Lussiana C, Campia I, Ghigo D. Classical inhibitors of NOX NAD(P)H oxidases are not specific. *Curr Drug Metab* 2008; 9: 686-696.
- [18] Song L, Zhu Y, Jin M, Zang B. Hydroxysafflower yellow a inhibits lipopolysaccharide-induced inflammatory signal transduction in human alveolar epithelial A549 cells. *Fitoterapia* 2013; 84: 107-114.
- [19] Huang J, Sun C, Wang S, He Q, Li D. microRNA miR-10b inhibition reduces cell proliferation and promotes apoptosis in non-small cell lung cancer (NSCLC) cells. *Mol Biosyst* 2015; 11: 2051-2059.
- [20] Estenssoro E, Dubin A, Laffaire E, Canales H, Sáenz G, Moseinco M, Pozo M, Gómez A, Baredes N, Jannello G. Incidence, clinical course, and outcome in 217 patients with acute respiratory distress syndrome. *Crit Care Med* 2002; 30: 2450-2456.
- [21] Albert RK. Higher versus lower positive end-expiratory pressures in patients with the acute respiratory distress syndrome. *N Engl J Med* 2005; 351: 327-336.

- [22] Oliveira MC Jr, Greiffo FR, Rigonato-Oliveira NC, Custódio RW, Silva VR, Damaceno-Rodrigues NR, Almeida FM, Albertini R, Lopes-Martins RÁ, de Oliveira LV, de Carvalho Pde T, Ligeiro de Oliveira AP, Leal EC Jr, Vieira RP. Low level laser therapy reduces acute lung inflammation in a model of pulmonary and extrapulmonary LPS-induced ARDS. *J Photochem Photobiol B* 2014; 134: 57-63.
- [23] Han S, Mallampalli RK. The acute respiratory distress syndrome: from mechanism to translation. *J Immunol* 2015; 194: 855-860.
- [24] Chen TG, Chen TL, Chang HC, Tai YT, Cherng YG, Chang YT, Chen RM. Oxidized low-density lipoprotein induces apoptotic insults to mouse cerebral endothelial cells via a Bax-mitochondria-caspase protease pathway. *Toxicol Appl Pharmacol* 2007; 219: 42-53.
- [25] Ferrari R, Ceconi C, Campo G, Cangiano E, Cavazza C, Secchiero P, Tavazzi L. Mechanisms of remodelling: a question of life (stem cell production) and death (myocyte apoptosis). *Circ J* 2009; 73: 1973-1982.
- [26] Chuang CY, Chen TL, Cherng YG, Tai YT, Chen TG, Chen RM. Lipopolysaccharide induces apoptotic insults to human alveolar epithelial A549 cells through reactive oxygen species-mediated activation of an intrinsic mitochondrion-dependent pathway. *Arch Toxicol* 2011; 85: 209-218.
- [27] Lechner M, Lirk P, Rieder J. Inducible nitric oxide synthase (iNOS) in tumor biology: the two sides of the same coin. *Semin Cancer Biol* 2005; 15: 277-289.
- [28] Carnesecchi S, Deffert C, Donati Y, Basset O, Hinz B, Preynatseauve O, Guichard C, Arbiser JL, Banfi B, Pache JC. A Key role for NOX4 in epithelial cell death during development of lung fibrosis. *Antioxid Redox Signal* 2011; 15: 607-619.
- [29] Cao LQ, Chen XL, Wang Q, Huang XH, Zhen MC, Zhang LJ, Li W, Bi J. Upregulation of PTEN involved in rosiglitazone-induced apoptosis in human hepatocellular carcinoma cells. *Acta Pharmacol Sin* 2007; 28: 879-887.
- [30] Kim YH, Jung EM, Lee TJ, Sang HK, Choi YH, Park JW, Park JW, Choi KS, Kwon TK. Rosiglitazone promotes tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by reactive oxygen species-mediated up-regulation of death receptor 5 and down-regulation of c-FLIP. *Free Radic Biol Med* 2008; 44: 1055-1068.
- [31] Wang JJ, Mak OT. Induction of apoptosis by 15d-PGJ2 via ROS formation: an alternative pathway without PPAR γ activation in non-small cell lung carcinoma A549 cells. *Prostaglandins Other Lipid Mediat* 2011; 94: 104-111.
- [32] Önnheim K, Bylund J, Boulay F, Dahlgren C, Forsman H. Tumour necrosis factor (TNF)- α primes murine neutrophils when triggered via formyl peptide receptor-related sequence 2, the murine orthologue of human formyl peptide receptor-like 1, through a process involving the type I TNF receptor and subcellular gran. *Immunology* 2008; 125: 591-600.
- [33] Jones MR, Mizgerd JP. Roles of interleukin-6 in activation of STAT proteins and recruitment of neutrophils during escherichia coli pneumonia. *J Infect Dis* 2006; 193: 360-369.
- [34] Chatterjee PK, Alabed Y, Sherry B, Metz CN. Cholinergic agonists regulate JAK2/STAT3 signaling to suppress endothelial cell activation. *Am J Physiol Cell Physiol* 2009; 297: 1294-1306.
- [35] Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol* 2009; 1: a001651.
- [36] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; 124: 783-801.
- [37] Martin TR, Frevert CW. Innate Immunity in the Lungs. *Proc Am Thorac Soc* 2004; 2: 403-411.