Inhibitory effects of rosiglitazone on paraquat-induced acute lung injury in rats

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Aim: To investigate the effects of the PPAR-γ agonist rosiglitazone on acute lung injury induced by the herbicide paraquat (PQ) and the underlying mechanisms of action.

Methods: Male Sprague-Dawley rats were injected with PQ (20 mg/kg, ip). Rosiglitazone (3 or 10 mg/kg, ip) was administered 1 h before PQ exposure. Peripheral blood was collected at 4, 8, 24, and 72 h after PQ exposure for measuring the levels of MDA, TNF-α and IL-1β, and the SOD activity. Lung tissues were collected at 72 h after PQ exposure to determine the wet-to-dry (W/D) ratios and lung injury scores, as well as the protein levels of NF-κBp65, PPAR-γ, Nrf2, IκBα, and pIκBα.

Results: At 72 h after PQ exposure, the untreated rats showed a 100% cumulative mortality, whereas no death was observed in rosiglitazone-pretreated rats. Moreover, rosiglitazone pretreatment dose-dependently attenuated PQ-induced lung edema and lung histopathological changes. The pretreatment significantly reduced the levels of TNF-α, IL-1β, and MDA, increased SOD activity in the peripheral blood of PQ-treated rats. The pretreatment also efficiently activated PPAR-γ, induced Nrf2 expression and inhibited NF-κB activation in the lung tissues of PQ-treated rats. Furthermore, the pretreatment dose-dependently inhibited IκB-α degradation and phosphorylation, thus inhibiting NF-κB activation.

Conclusion: Pretreatment with rosiglitazone protects rats against PQ-induced acute lung injury by activating PPAR-γ, inducing Nrf2 expression and inhibiting NF-κB activation.

Keywords: rosiglitazone; herbicide; paraquat; acute lung injury; PPAR-γ; NF-κB; Nrf2; toxicity
treatment for PQ-induced acute lung injury.

Materials and methods

Animals

Male Sprague-Dawley rats weighing 200–250 g were purchased from the Experimental Animal Center of China Medical University. Rats were housed in cages in a temperature-(20–25 °C) and humidity-controlled (40%–70%) environment with a daily light-dark cycle. The rats had access to food and water ad libitum. All animal experiments were conducted in accordance with the Institutional Animal Ethics Committee and Animal Care Guidelines of China Medical University governing the use of experimental animals.

Reagents

PQ and rosiglitazone were purchased from Sigma-Aldrich (St Louis, MO, USA). Rabbit anti-PPAR-γ, anti-p65, anti-IκBα, anti-pIκBα, and anti-β-actin monoclonal antibodies were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). Rabbit anti-Nrf2 monoclonal antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). A mouse anti-TBP monoclonal antibody was purchased from Abcam (Cambridge, MA, USA). The ELISA kits for TNF-α and IL-1β were purchased from R&D, America. The superoxide dismutase (SOD) and malondialdehyde (MDA) determination kits were purchased from Jiancheng Bioengineering Institute of Nanjing (Nanjing, China). The purity of all of the chemical reagents was of analytical grade or higher.

Experimental protocols

After adapting to the environment, the rats were intraperitoneally administered PQ at doses of 10, 20, 30, 40, and 50 mg/kg. Cumulative PQ poisoning rates and mortalities were observed at different time points (4, 8, 24, 72, and 168 h) after PQ administration. A dose of 20 mg/kg was chosen after preliminary poisoning rate and mortality studies were conducted with different doses of PQ. In the following experiments, the rats were randomly distributed into four groups: a control group (n=10), a PQ-treated group (n=10), a rosiglitazone (3 mg/kg) + PQ-treated group (n=10), and a rosiglitazone (10 mg/kg)+PQ-treated group (n=10). Rosiglitazone was intraperitoneally administered at doses of 3 and 10 mg/kg. After 1 h, PQ was intraperitoneally administered at a dose of 20 mg/kg. The peripheral blood was then collected through the orbital veins at 4, 8, 24, and 72 h, and centrifuged at 4000 r/min for 15 min. The supernatants were collected to measure SOD activity, and MDA, TNF-α and IL-1β levels. Throughout the study period, the rats were killed via exsanguination 72 h after PQ administration. The whole lungs were removed. Each lung was dried, weighed, and then placed in an oven at 80 °C for 48 h to obtain the “dry” weight. The ratio of the wet weight of the lung to the dry one of the lung was calculated to assess tissue edema[16].

Histopathologic evaluations

The rats were killed 72 h after PQ administration. The lungs were then removed and stored in 4% paraformaldehyde for 48 h at 4°C. Hematoxylin and eosin staining was carried out according to the regular staining method, and the slides were evaluated using a semiquantitative scoring method. Lung injury was graded in a blinded fashion from 0 (normal) to 4 (severe) for interstitial inflammation, neutrophil infiltration, congestion, and edema. The total lung injury score was calculated by adding up the individual scores from each category[16].

MDA levels and SOD activity assays

MDA levels and SOD activity in the peripheral blood were spectrophotometrically assayed using assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Both detective procedures were performed according to the manufacturer’s recommended instructions.

TNF-α and IL-1β ELISA assays

The levels of TNF-α and IL-1β in the peripheral blood were measured via ELISA assays using commercially available kits according to the manufacturer’s recommended instructions. The levels of TNF-α and IL-1β in the samples were calculated based on a standard curve. The detection ranges of the TNF-α and IL-1β ELISA assays were 12.5–800 pg/mL and 31.25–2000 pg/mL, respectively. Samples that had a concentration that exceeded the limit of the standard curve were measured after dilution.

Immunohistochemistry

The lung tissues were fixed in 4% paraformaldehyde for 48 h at 4°C and processed for paraffin embedding. Paraffin-embedded blocks were cut into 4 μm thick sections and mounted onto slides. The sections were pretreated at 60 °C for 1 h, then dewaxed in xylene, hydrated, and washed in 0.01 mol/L of citrate buffer. After inhibiting endogenous peroxidase using 3% H2O2 in methanol, the sections were incubated with anti-NF-κBp65, anti-PPAR-γ and anti-Nrf2 polyclonal antibodies overnight at 4°C. The sections were then thoroughly washed with a phosphate-buffered saline (PBS) solution, after which point the corresponding secondary antibodies were applied and incubated at room temperature for 30 min. Reaction products were visualized following incubation with diaminobenzidine (DAB) and then counterstained with hematoxylin. Negative controls were generated by omitting the primary antibodies.

Western blotting

Proteins were extracted from the lungs using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology,
China). The extracts were boiled for 5 min with loading buffer, and the proteins were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels and transferred onto polyvinylidene difluoride sheets. The membranes were washed with PBST and 5% skim milk for 1 h at room temperature. Following three washes with PBST, the membranes were incubated with primary antibodies overnight at room temperature. After three additional washes, secondary antibodies were added and the membranes were incubated for 1 h at room temperature. After three final washes, the blots were developed using Beyo ECL Plus reagent (Beyotime Biotechnology, Haimen, China), exposed to film for 1 min, and placed in developing and fixing solutions for 1 min each.

Statistical analyses
The data are expressed as the mean±SD. Statistical analyses were carried out using SPSS 16.0. One-way ANOVA followed by the Student-Newman-Keuls test was used to compare the results that were obtained in the different treatment groups. Differences were considered to be statistically significant when $P<0.05$.

Results

Cumulative poisoning rates and mortality in rats following PQ exposure
As shown in Figures 1 and 2, the cumulative poisoning rate was low when rats were treated with PQ at a dose of 10 mg/kg. Although the cumulative poisoning rate was satisfactory, the cumulative mortality was too high when rats were treated with 30, 40, or 50 mg/kg of PQ. The cumulative poisoning rate was satisfactory and mortality was low when rats were treated with PQ at a dose of 20 mg/kg. Therefore, we selected 20 mg/kg as the final dosage and 72 h as the observation time. All of the rats in our study displayed the typical symptoms of ALI. No deaths were observed at this dosage and time.

Effects of rosiglitazone on the clinical signs of PQ poisoning in rats
No deaths were observed for the duration of the experimental period, but clinical signs of toxicity, including polypnea, cyanosis, crouch, decreased activity, diarrhea and anorexia were observed in some of the rats that had been exposed to PQ. The rats from the groups that had been pretreated with rosiglitazone displayed few symptoms, which suggested that rosiglitazone treatment could attenuate PQ-induced symptoms.

Effects of rosiglitazone on W/D lung ratios in rats with PQ-induced acute lung injury
W/D lung ratios were evaluated 72 h after PQ administration. As shown in Figures 3A and 3B, lung edema and the W/D lung ratios in the PQ-treated group were significantly higher than those in the control group ($P<0.05$). Rosiglitazone treatment at 3 or 10 mg/kg decreased lung edema and the W/D lung ratios in rats with PQ-induced lung injury ($P<0.05$).

Effects of rosiglitazone on histopathological changes in the lung tissues of rats with PQ-induced acute lung injury
The effects of rosiglitazone on histopathological changes in the lung tissue were determined by histochemical staining with H&E 72 h after PQ administration. As shown in Figures 3C and 3D, the lungs of rats that were exposed to PQ displayed significant inflammatory alterations that were characterized by lung edema, alveolar hemorrhage, inflammatory cell infiltration, and destruction of epithelial and endothelial cell structure. Treatment with rosiglitazone relieved many of the symptoms of PQ-induced acute lung injury that were outlined above.

Effects of rosiglitazone on MDA levels and SOD activity in the peripheral blood of rats with PQ-induced acute lung injury
MDA levels in the peripheral blood were significantly higher...
in the PQ group than those in the control group \( (P<0.05) \), as shown in Figure 4A. The increased levels were significantly inhibited by treatment with rosiglitazone (10 mg/kg) at 24 and 72 h after PQ administration \( (P<0.05) \). As shown in Figure 4B, PQ administration resulted in significant decreases in SOD activity in the peripheral blood \( (P<0.05) \). However, treatment with rosiglitazone counteracted these effects at 8, 24, and 72 h after PQ administration.

**Effects of rosiglitazone on cytokine production in the peripheral blood of rats with PQ-induced acute lung injury**

We used ELISAs to analyze the effects of rosiglitazone on the production of TNF-α and IL-1β in the peripheral blood at 4, 8, 24, and 72 h after PQ administration. As shown in Figure 4C and 4D, treatment with PQ alone caused significant increases in IL-1β and TNF-α compared to the control group \( (P<0.05) \). Rosiglitazone markedly reduced the production of TNF-α at 8, 24, and 72 h after PQ administration and markedly reduced the production of IL-1β at 24 and 72 h after PQ administration compared with the PQ-treated group \( (P<0.05) \).

**Effects of rosiglitazone on PPAR-γ expression in the lung tissues of rats with PQ-induced acute lung injury**

PPAR-γ localization in the lung tissues is illustrated in Figure 5C. In the control group, PPAR-γ positive signals were weakly observed in the lung tissues. At 72 h after PQ administration, PPAR-γ positive signals were further decreased in the lung tissues. Notably, rosiglitazone pretreatment significantly increased PPAR-γ positive signals in the lung tissues. The results of the Western blotting analyses are illustrated in Figure 5A. The PQ-treated group revealed decreased PPAR-γ expression as compared to the control group. Rosiglitazone pretreatment significantly increased PPAR-γ expression in the lung tissues \( (P<0.05) \).

**Effects of rosiglitazone on Nrf2 expression in the lung tissues of rats with PQ-induced acute lung injury**

Nrf2 localization in the lung tissues is illustrated in Figure 5C. In the control group, Nrf2 positive signals were weakly detected in the lung tissues. At 72 h after PQ administration, Nrf2 positive signals were observed to be subtly increased in the lung tissues. Notably, Nrf2 expression in the lung tissues was significantly increased in the groups that had been pretreated with rosiglitazone. The results of the Western blotting analyses are illustrated in Figure 5A. The PQ-treated group...
displayed increased Nrf2 expression compared to the control group. Rosiglitazone pretreatment further increased Nrf2 expression in the lung tissues ($P<0.05$).

### Effects of rosiglitazone on NF-κB signal transduction in the lung tissues of rats with PQ-induced acute lung injury

As shown in Figure 5A and 5B, the PQ-treated group displayed significant IκBα degradation and phosphorylation compared to the control group. In contrast, IκBα degradation and phosphorylation in the rosiglitazone-pretreated groups were significantly reduced compared to the PQ-treated group ($P<0.05$). The nuclear extracts of PQ-treated rats displayed increases in the levels of the p65 subunit of NF-κB. In contrast, rosiglitazone treatment significantly decreased the levels of the p65 subunit of NF-κB in the nuclear extracts ($P<0.05$).

### Discussion

The main target organ of PQ poisoning is the lungs. Edema is a representative symptom of PQ-induced acute lung injury. To quantify the magnitude of pulmonary edema, we detected the W/D ratios of sampled lung tissues. Our experiments suggested that treatment with rosiglitazone significantly inhibited lung edema, because the W/D ratio in the rosiglitazone-treated group was significantly lower than that in the PQ-treated group. We also observed PQ-induced pathological alterations, including alveolar edema, hemorrhage, inflammatory cell infiltration, and diffuse alveolar collapse that was accompanied by wall thickening. However, pretreatment with rosiglitazone was very effective at preventing PQ-induced lung tissue damage in rats.

Free radicals are known to play a crucial role in PQ-induced lung intoxication\[17\]. PQ-induced toxicity has been reported to be related to redox cycling of an iron-PQ complex, which in turn catalyzes the formation of reactive oxygen species (ROS) and ultimately progresses to lipid peroxidation\[18\]. In the present study, we found that MDA levels were significantly increased, while SOD activity was significantly decreased, in the peripheral blood of rats after PQ exposure. However, rosiglitazone treatment significantly increased SOD activity and decreased MDA levels in the peripheral blood.

Nrf2 is a transcription factor that induces antioxidant defenses and gene expression by binding to cis-acting antioxidant response elements (AREs)\[19\] and is essential for tissue protection against various oxidants and xenobiotics\[20\]. The protective role of the Nrf2-ARE pathway has been demonstrated in oxidant-mediated inflammatory lung injury (for example, hyperoxia toxicity)\[21–30\]. In the present study, we demonstrated that PQ treatment alone induced the expression of Nrf2 in the lung tissues. In addition, we demonstrated that rosiglitazone could further increase Nrf2 expression. Therefore, the potent anti-oxidant effects of rosiglitazone may involve Nrf2 activation. Although the cellular targets and

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**Figure 4.** Effects of rosiglitazone on the cytokine production, SOD activity and MDA level, in peripheral blood of PQ-induced acute lung injury rats. Rosiglitazone was intraperitoneally administered 1 h before intraperitoneal administration of PQ. Peripheral blood was collected after PQ administration for 4, 8, 24, and 72 h to analyze MDA level and SOD activity (A, B), levels of IL-1β and TNF-α (C, D). RSGL: rosiglitazone at the dose of 3 mg/kg, RSGH: rosiglitazone at the dose of 10 mg/kg. The values presented are the mean±SD (n=6). $^bP<0.05$ vs PQ group.
molecular mechanisms leading to rosiglitazono-induced Nrf2 activation remain to be elucidated, our study has shed some light on them.

Several important inflammatory and chemotactic cytokines, such as TNF-α and IL-1β, are involved in the inflammatory process during acute lung injury. These cytokines, as well as other pro-inflammatory compounds, initiate, amplify, and perpetuate the inflammatory response during acute lung injury. TNF-α is the primary endogenous mediator of the inflammatory process. TNF-α may play a role in the initiation or progression of multiple organ failure during endotoxic shock, and it has also been shown to be a particularly important mediator of acute lung injury[31]. IL-1β plays a key role in the development of acute lung injury, and can inhibit fluid transport across the distal lung epithelium[32] causing surfactant abnormalities[33] and increasing protein permeability across the alveolar-capillary barrier[34]. In this experiment, we found that the levels of TNF-α and IL-1β were significantly increased in the peripheral blood of rats after PQ exposure. However, when the rats were pretreated with rosiglitazone, the levels of TNF-α and IL-1β in the peripheral blood of PQ-poisoned rats were significantly decreased.

The NF-κB transcription factor plays a critical role in a number of different cellular processes. NF-κB is normally sequestered in the cytoplasm by a family of inhibitory proteins known as IκBs. A wide variety of stimuli, which have been extensively studied over the past two decades, can cause IκBα phosphorylation, a process that is followed by its ubiquitina-

Figure 5. Effects of rosiglitazone on Nrf2, PPAR-γ, and NF-κB signal transduction in lung tissues of paraquat (PQ)-induced acute lung injury rats. Rosiglitazone was intraperitoneally administered 1 h before intraperitoneal administration of PQ. Lung tissue was collected after PQ administration for 72 h, to determine the expression of Nrf2, PPAR-γ, and NF-κBp65 by immunohistochemistry (C, ×400) and Western blotting (A), IκB-α and pIκB-α by Western blotting (B). RSGL: rosiglitazone at the dose of 3 mg/kg, RSGH: rosiglitazone at the dose of 10 mg/kg. The values presented are the mean±SD (n=5). *P<0.05 vs PQ group.
tion and subsequent degradation. The loss of IκBα results in the release of the free NF-κB subunit p65, which translocates from the cytoplasm to the nucleus where p65 activates the expression of pro-inflammatory mediators (TNF-α, IL-1β, iNOS, COX2, etc)\textsuperscript{[35]}. NF-κB plays an important role in lung injury in PQ-poisoned rats\textsuperscript{[36]}. Our results demonstrated that PQ induced IκBα degradation and phosphorylation, which increased the levels of the p65 subunit of NF-κB in the nuclear extracts of lung tissues. In contrast, rosiglitazone treatment inhibited the degradation and phosphorylation of IκBα and decreased the levels of the p65 subunit of NF-κB in the nuclear extracts of the lung tissues. Therefore, the potent anti-inflammatory effects of rosiglitazone may be suggested to involve IκBα activation, which precipitates the translocation of the p65 subunit from the cytoplasm to the nucleus. However, the cellular targets and molecular mechanisms that lead to rosiglitazone-mediated IκBα activation remain to be elucidated. Li et al\textsuperscript{[37]} found that activation of Nrf2-antioxidant signaling attenuated the NF-κB inflammatory response. In our study, rosiglitazone activated Nrf2, which suggested that rosiglitazone may activate IκBα and attenuate the NF-κB-induced inflammatory response via activation of Nrf2. However, this hypothesis remains to be further elucidated.

PPAR-γ, a member of the nuclear receptor family that plays an important role in lipid and carbohydrate homeostasis, regulates the expression of several genes, including anti-inflammatory and antioxidant genes\textsuperscript{[13–15]}. To further investigate the mechanisms underlying the effects of rosiglitazone on PQ-induced acute lung injury, we detected PPAR-γ expression in the lungs of PQ-poisoned rats using immunohistochemistry and Western blotting. The results of these analyses revealed that the PPAR-γ expression in PQ-poisoned rats was lower than that in control rats. However, rosiglitazone treatment activated PPAR-γ and increased its expression, which suggested that rosiglitazone may exert an inhibitory effect on NF-κB and an activating effect on Nrf2 via PPAR-γ activation. This hypothesis requires further verification. For example, Nrf2 and NF-κB expression may need to be detected after rosiglitazone treatment.

In summary, the results of the present study revealed that rosiglitazone had a protective effect against PQ-induced acute lung injury. Pretreatment with rosiglitazone attenuated pulmonary histological injuries and lung edema, decreased the levels of inflammatory cytokines in the peripheral blood, and reduced the concentrations of MDA and enhanced SOD activity in the serum. These data strongly suggested that rosiglitazone has potent anti-inflammatory and antioxidant activities and may offer a novel strategy for the modulation of PQ-induced acute lung injury. Furthermore, we found that rosiglitazone inhibited NF-κB activation by decreasing the phosphorylation and degradation of IκBα. Rosiglitazone also activated Nrf2 and PPAR-γ, which may partially explain its underlying mechanism of action. We hypothesize that rosiglitazone may activate PPAR-γ and subsequently induce Nrf2 expression and inhibit NF-κB activation. However, further in vitro investigations are required before conclusions can be drawn. More comprehensive studies are still required before this drug could be used clinically to treat PQ-induced acute lung injury.

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Author contribution
Min ZHAO designed the research. Qiang ZHENGYong, Hong-yu ZHAO, Wei-jian HOU, and Shu-ling BAI provided helpful advice regarding the research.

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