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Angptl2 deficiency attenuates paraquat (PQ)-induced lung injury in mice by altering inflammation, oxidative stress and fibrosis through NF- κ B pathway

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ABSTRACT

Paraquat (PQ) is one of the most extensively used herbicides, possessing high toxicity for humans and animals. The lung is the main target organ by the poisoning of PQ resulting in acute lung injury. Nonetheless, molecular mechanisms underlying PQ-induced lung injury remain unclear. Here, we ask if angiopoietin-like protein 2 (Angptl2), a pro-inflammatory protein, contributes to inflammation that accelerates acute lung injury. The results indicated that abundant Angptl2 expression was observed in lung tissues of PQ-treated mice. Histological analysis revealed that PQ-induced histological changes were alleviated by Angptl2 knockout (Angptl2^{-/-}). Angptl2^{-/-} in PQ-treated mice attenuated acute lung injury progression by reducing the number of total cells, total leukocytes, neutrophils and macrophages in bronchoalveolar lavage fluid (BALF) and reducing inflammatory response through the inactivation of nuclear factor kappa B (NF- κ B) pathway. Angptl2^{-/-} reduced oxidative stress in PQ-treated mice, as evidenced by the enhanced superoxide dismutase (SOD) activity and reduced malondialdehyde (MDA) levels in serum or lung tissue samples, which was accompanied by increased expressions of nuclear respiratory factor 2 (Nrf-2), heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO-1). PQ-induced fibrosis was also improved in Angptl2^{-/-} mice by decreasing pulmonary transforming growth factor (TGF)- β 1 expressions. In vitro, we found that Angptl2 knockdown-suppressed inflammation, oxidative stress and fibrosis was restored by increasing NF- κ B activation in PQ-incubated A549 cells; however, the results above were significantly reversed by inactivating NF- κ B using its inhibitor, Bay 11–7085 or LY2409881. Therefore, Angptl2 could provide therapeutic effects on PQ-induced acute lung injury through inhibiting inflammation, oxidative stress and fibrosis by regulating NF- κ B pathway.

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1. Introduction

Acute lung injury is a severe life-threatening disease, characterized by lung edema, hemorrhage, inflammatory cell infiltration, as well as diffused alveolar capillary injury on pathology, and is mainly presented as dyspnea, continuous hypoxemia, and

tachycardia in clinics [1–3]. Acute lung injury, a leading cause of morbidity and mortality in critically sick patients, could result in persistent respiratory failure or even death [4]. Various factors could induce acute lung injury, including paraquat (PQ) and lipopolysaccharide (LPS) [5,6]. As reported, multiple inflammatory cells and cytokines were involved in the progression of acute lung injury [7].

Angiopoietin-like protein 2 (Angptl2) sustains tissue homeostasis by enhancing adaptive inflammation and subsequent tissue reconstruction [8]. However, excessive Angptl2 activation induced by prolonged stress accelerates breakdown of tissue homeostasis because of inflammation and irreversible tissue remodeling,

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promoting the progression of various diseases, such as atherosclerotic diseases, type 2 diabetes, and some cancers [9–11]. Suppressing excess Angptl2 signaling could represent novel and effective therapeutic strategies against various types of diseases and cancer [12]. However, the effects of Angptl2 on acute lung injury are yet unclear.

In the present study, we aimed to explore the role of Angptl2 in the development of acute lung injury induced by PQ by using the wild type mice (Angptl2^{+/+}) and Angptl2 knockout (Angptl2^{-/-}) in vivo, and the A549 cells with Angptl2 knockdown in vitro. We found that Angptl2 deficiency attenuates PQ-induced acute lung injury in mice by reducing inflammation, oxidative stress and fibrosis through inactivating NF- κ B pathway.

2. Materials and methods

2.1. Animals and treatments

A total of 24 male, wild type (Angptl2^{+/+}), C57BL/6 mice (18–22 g, 8 weeks old, the Center of Animal Experiment, Heilongjiang, China) and 16 male, Angptl2 knockout (Angptl2^{-/-}) C57BL/6 mice (18–22 g, 8 weeks old, Cyagen Biosciences Inc., Guangzhou, China) were maintained in standard cages at controlled conditions of temperature (22 $\pm$ 2 $^{\circ}$ C) with relative humidity of 50 $\pm$ 10%, 12 h light/dark cycle and free access to water and food. All the protocols were conducted according to the ethical standards and approved by the First Affiliated Hospital of Harbin Medical University (Heilongjiang, China). The mice were randomly divided into 5 groups (n=8): (1) wild type control (Angptl2^{+/+}/Con), (2) wild type PQ (Angptl2^{+/+}/PQ), (3) knockout control (Angptl2^{-/-}/Con), (4) knockout PQ (Angptl2^{-/-}/PQ) and (5) wild type LPS. PQ (Sigma-Aldrich, St. Louis, MO, USA), dissolved in sterile saline, was given to mice at a dose of 30 mg/kg by intraperitoneal (i.p.) injection, and the Con mice each received an i. p. injection of an equal volume of saline. The average survival rate of the mice in each group was monitored during the 14-day interval. 14 days post exposure to PQ, all mice were sacrificed for assays. Right lungs were cleared and weighed immediately to obtain the wet weight, and then put in an oven at 60 $^{\circ}$ C for 72 h to obtain the dry weight. The ratio of the wet lung to dry lung was evaluated. Peripheral blood was also collected to obtain serum for biochemical index analysis.

For LPS group, mice were treated once by intratracheal instillation with 5 mg/kg of LPS (Sigma Aldrich) in saline. After LPS treatment for 6 h, mice were sacrificed and lung tissues were isolated.

2.2. Cells and culture

Human lung cancer cell line A549 was purchased from KeyGen Biotech (Nanjing, China). The cells were cultured in RPMI-1640 containing 10% calf serum in an environment with 5% CO₂ at 37 $^{\circ}$ C. NF- κ B GFP Reporter Plasmid (pGMNF-KB-GFP, 5'-TAG-CAAATAGGCTGTCCC-3') was obtained from ZYBscience (Shanghai, China). Angptl2 siRNA and the negative control (NC) siRNA sequences were designed and synthesized by Shanghai Generay Biotech Co., Ltd (Shanghai, China). Lipofectamine 2000 (Invitrogen, USA) was used for cell transfection following the manufacturer's protocols. Bay 11–7085 and LY2409881 were purchased from Selleckchem (USA).

2.3. Collection of bronchoalveolar lavage fluid (BALF) and analysis

BALF was collected as previously recorded [13]. Then, BALF was mixed well and centrifuged (1500 rpm, 4 $^{\circ}$ C) for 10 min. Supernatants were stored at –80 $^{\circ}$ C for total protein analysis and cytokine

analysis. Total protein levels in BALF were assessed using Coomassie Brilliant Blue G-250 kits (Solarbio, Beijing, China) following the manufacturer's protocols. Pellets were prepared for inflammatory cell counts with a hemocytometer.

2.4. Measurements of cytokines, SOD and MDA

IL-1 β , TNF- α and IL-6 levels in serum or BALF were measured using enzyme-linked (ELISA) kits (Bioss, Beijing, China) following the manufacturer's instructions. MDA levels and SOD activity in serum or lung tissues were determined using commercial kits (Beyotime, Nantong, China) following the manufacturer's instructions.

2.5. Isolation of RNA and real-time PCR and western blot analysis

Trizol (Invitrogen, United States) was used to extract and purify the total RNA from lung tissues or cells. The protein extraction was from lung tissue samples or cells using lysis buffer (KeyGen Biotech). Then, standard protocols were performed for RT-qPCR and western blot analysis [14]. The sequences for RT-qPCR and primary antibodies for western blot were listed in [Supplementary table 1 and table 2](#).

2.6. Histopathology

Left upper lung tissues were fixed with 10% neutral formalin, embedded in paraffin, and sliced (5 μ m thickness). Histology of lung was examined under the microscope with hematoxylin-eosin (H&E) staining. We also performed Masson's trichrome staining to evaluate fibrosis (collagen fibers) [15].

2.7. Immunohistochemistry (IHC)

Paraffin sections (4 μ m thick) of mouse lungs were deparaffinized prior to antigen retrieval. Sections were blocked with normal goat plasma for 20 min at 37 $^{\circ}$ C and then incubated overnight at 4 $^{\circ}$ C with primary antibodies against the following: Angptl2 antigen (dilution 1:200; Abcam) and TGF- β 1 antigen (dilution 1:100; Abcam), which was followed by incubation with secondary antibody (KeyGen Biotech) for 30 min. Diaminobenzidine (DAB, KeyGen Biotech) and hematoxylin were used for color development and counterstaining. The slides were examined under a light microscope.

2.8. Immunofluorescence (IF)

Protein expression in cells was evaluated by IF staining using TGF- β 1 antigen (dilution 1:100; Abcam) diluted with 2% bovine serum albumin (Sigma) in PBS as described earlier [16]. The fluorescence signal was observed using a fluorescence microscope.

2.9. DCF-DA analysis

The levels of ROS were detected with a ROS assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. The fluorescence signal was observed using a fluorescence microscope.

2.10. Statistical analysis

The data are presented as the means $\pm$ SEM. The differences between multiple groups were analyzed with one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. P < 0.05 was considered statistically significant.

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