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Protein kinase C θ deletion ameliorates bleomycin-induced pulmonary fibrosis via the improvement of SIRT1/AMPK signaling on autophagy and senescence

Wei Li [1], Xue Zhao [1], Mengdie Wang [2], Heling Wang [2], Rongyu Li [3], Guoguang Wang [1]*

[1] Department of Pathophysiology, Wannan Medical College, Wuhu, China
[2] School of Medical Imaging, Wannan Medical College, Wuhu, China
[3] Department of Immunology, Wannan Medical College, Wuhu, China

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ABSTRACT

Objective(s): Protein kinase C theta (PKC θ) is involved in the regulation of various inflammatory diseases, such as allergic asthma and ConA-induced hepatitis. However, its role in pulmonary fibrosis has not been reported. This study aimed to explore the role of PKC θ in bleomycin-induced pulmonary fibrosis and the potential mechanisms involved.

Materials and Methods: WT C57BL/6 and PKC-θ deletion mice were administered a single intratracheal injection of bleomycin at a dosage of 3 mg/kg to prepare an animal model of pulmonary fibrosis. Histopathological changes of lung fibrosis were observed. Blood samples and BALF were collected for biochemical analysis. Protein expression in lung tissues was assessed to investigate a potential mechanism of PKC θ in pulmonary fibrosis.

Results: The results suggested that PKC θ knockout improved pulmonary structure and fibrosis through down-regulating the expression of collagen I and fibronectin, and the TGF-β1 pathway. PKC θ knockout significantly reduced the levels of the inflammatory factors TNF-α, IL-6, and TGF-β in BALF and lung tissues and the NF-κB/IκB-α pathway in the lung. PKC θ knockout also elevated the activities of antioxidant enzymes SOD and CAT, and improved the SIRT1/AMPK-HO-1/Nrf2 pathway. Further, PKC θ knockout ameliorated the expression of the autophagy-related proteins Beclin 1 and p62, and the senescence markers p53 and p21.

Conclusion: These data showed that the improvement in PKC θ knockout in pulmonary fibrosis was associated with enhanced anti-inflammatory and antioxidant effects, activation of autophagy, and reduced senescence effects.

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Introduction

Pulmonary disorders are increasingly among the most common causes of morbidity and mortality. The median survival for patients with pulmonary fibrosis is about 3 to 5 years, and morbidity is increasing globally (1). Explicit mechanisms for idiopathic pulmonary fibrosis (IPF) are unclear. Still, various studies demonstrated that IPF is a progressive interstitial lung disease caused by repetitive diffuse alveolar epithelial cell injury and subsequent proliferation, migration, and activation of fibroblasts, leading to excessive secretion of extracellular matrix (ECM) and eventually promoting the development of pulmonary fibrosis (1). Accumulating evidence shows that oxidative stress and the inflammatory response play pivotal roles in the pathogenesis of IPF (2). It has been demonstrated that inflammatory cytokines, oxidative species, and tissue growth factor beta 1 (TGF-β1) released by inflammatory cells lead to the destruction of epithelial cells and lung tissue and stimulate migration and activation of fibroblasts(3, 4), causing extracellular collagen deposition (5) and excessive production of the ECM (6). It has been demonstrated that inflammation contributes to the development of various respiratory diseases, including acute respiratory distress syndrome (ARDS), pulmonary edema, and asthma (7). Nuclear factor-kappa B (NF-κB), a dimeric transcription factor, plays a key role in various complex biological processes, such as cell proliferation and differentiation (9), and in pathological processes, including chronic inflammation, Alzheimer's disease, immune disorders, and tumors (8). NF-κB bound to the inhibitory IκB is inactive, and NF-κB is activated through phosphorylation of IκB and subsequent dissociation (11). Activated NF-κB translocates to the nucleus, binds to recognition sequences, and mediates transcription of various target genes. Accumulating studies showed that NF-κB is involved in the regulation of cell senescence, oxidative stress, lipid metabolism, and apoptosis (9, 10). Further, NF-κB plays a vital role in coordinating immunological responses and inflammation

***Corresponding author:** Guoguang Wang. Department of Pathophysiology, Wannan Medical College, Wuhu, China. Tel/ Fax: +86-5533932476, Email: guoguangw1226@sina.com

(11). An inhibitor of NF-κB has been reported to suppress excessive inflammatory response and lipid peroxidation (12). Additionally, NF-κB is involved in the pathogenesis of various lung disorders, including lung fibrosis (13).

Protein kinase C theta (PKC θ) is a serine/threonine-specific kinase that belongs to the calcium-independent and lipid-sensitive subfamily of the PKC family (14). PKC θ is first separated from hematopoietic cells and plays an unusual role in the development and activation of T cells. Further studies have shown that PKC θ is expressed in other cell types, including muscle cells, and is involved in numerous physiological and pathophysiological processes. PKC θ plays a key role in regulating the immune system, T cell activation, cell survival, and differentiation (14). PKC θ can activate various crucial transcription factors, such as NF-κB, nuclear factor of T cells (NFAT), and activating protein 1 (AP-1) via activating a series of signaling cascades (15, 16). Activated PKC θ mediates translocation of the NF-κB dimers to the nucleus by degrading the inhibitors from the NF-κB-inhibitor κB (IκB) kinases (IKK) complex (17), where the dimers bind to specific DNA sequences, stimulating the transcription of target genes, including tumor necrosis factor alpha (TNF-α), interleukin (IL) 1B, IL-18, IκB, calcium-calmodulin kinase (CaMK) II, and brain-derived neurotrophic factor (BDNF)(18, 19). Therefore, PKC θ is involved in the regulation of various inflammatory diseases, including allergic asthma, inflammatory bowel disease (20), chronic muscle injury (21), and ConA-induced hepatitis (22). However, the effect of PKC θ on pulmonary fibrosis remains unclear. This study aimed to elucidate the role of PKC θ knockout in modulating pulmonary fibrosis and explore its potential mechanism.

Materials and Methods

Materials and reagents

Bleomycin was obtained from Hefei Homei Biotechnology Co., Ltd. (Hefei, China). Primary antibodies used in the experiment, including AMPK (Cat. No. ab32047), p-AMPK (Cat. No. ab133448), PGC-1α (Cat. No. ab106814), PKC-θ (Cat. No. ab185974), p2 (Cat. No. ab109199), Collagen I (Cat. No. Ab21286), Fibronectin (Cat. No. Ab45688), NF-κB (Cat. No. ab16502), p-NF-κB (Cat. No. Ab86299), IκB-α (Cat. No. Ab32518), p-IκB-α (Cat. No. ab133462), and GAPDH (Cat. No. Ab9485) were purchased from Abcam; Nrf 2 (Cat. No. 80593-1-RR) and p53 (Cat. No. 10442-1-AP) were purchased from proteintech Group Inc. (Wuhan, China); HO-1, ERK)1/2 (Cat. No. 4695S), p-ERK1/2 (Cat. No. 4370P), p62 (Cat. No. #5114) and mTOR (Cat. No. #2983) were purchased from Cell Signaling Technology Inc., α-SMA (Cat. No. bs70000) and TGF-β1 (Cat. No. bs1361) were purchased from Bioworld. Specific ELISA kits to detect TNF-α (Cat. No. E-MSEL-M0002) and IL-6 (Cat. No. E-MSEL-M0001) were purchased from Wuhan Eliruit Biotechnology Co., Ltd, (Wuhan, China). Malondialdehyde (MDA) (Cat. No. A003-1-2), catalase (CAT)(Cat. No. A007-1-1), and superoxide dismutase (SOD)(Cat. No. A001-3-2) assay kits were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). HRP-conjugated goat anti-rabbit IgG secondary antibodies (Cat. No. BST11112B54) and rabbit Anti-goat IgG secondary antibodies (Cat. No. BA1060) were purchased from Wuhan Boster Biological Technology, Ltd.

Mice

Male C57BL/6 mice aged eight to ten weeks were provided by Changsha Tianqin Biotechnology Co., Ltd (Changsha, China). Age-matched male PKC-θ-deleted mice on a C57BL/6 background were purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). All mice were raised in the animal laboratory at 23±1 ˚C, 50±5% humidity, and a 12-hour light-dark cycle. The Ethics Committee of Experimental Animals of Wannan Medical College approved the experiments on mice.

Animal model of pulmonary fibrosis

Bleomycin sulfate, dissolved in saline, was used to establish an animal model of pulmonary fibrosis. Pulmonary fibrosis was induced as reported previously (23). Briefly, after being anesthetized with an injection of pentobarbital (IP, 50 mg/kg), the C57BL/6 (n=10) and gene-deletion mice (n=10) received a single intratracheal injection of bleomycin at 3 mg/kg. On day 21 of bleomycin treatment, the mice were sacrificed. The lung tissues and blood samples were collected (Figure 1).

Bronchoalveolar lavage fluid (BALF)

BALF was collected as reported previously (24). The mice were sacrificed on Day 21 of bleomycin treatment. After the trachea was separated and exposed, the mice were cannulated with a small-caliber cannula into the left bronchus and lavaged with 0.5 ml of PBS three times. BALF was collected and centrifuged at 500 g for 10 min at 4 ˚C. The supernatants were used to detect the total protein levels and cytokines such as tumor necrosis factor (TNF)-α, Interleukin (IL)-6, and transforming growth factor (TGF)-β1 by commercial specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions, and the total protein was measured. The cells in the sedimented pellet from BAL fluid were collected, resuspended in 0.5 ml of PBS, and counted.

Histological examination

The left lung tissues were collected, immediately dissected, and fixed in 4% neutral paraformaldehyde for 48 hr. Then,

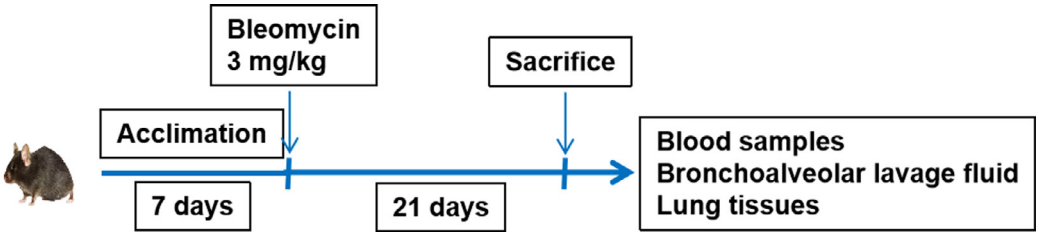

Figure 1. Experimental animal model diagram

the paraffin-embedded specimens were sectioned at 5 μm after dehydration in an ascending alcohol series. To observe histopathological changes, tissue sections were stained with hematoxylin and eosin (H&E) at room temperature. Masson's Trichrome staining was used to assess collagen deposition by measuring the percentage of stained area in lung tissues.

Immunohistochemistry

The expression of TGF-β1 and α-SMA was evaluated with immunohistochemical staining. Briefly, paraffin-embedded sections of lung tissue were incubated with rabbit anti-TGF-β1 (diluted 1:100) or anti-α-SMA (diluted 1:100) overnight at 4 ˚C. Subsequently, the sections were incubated with anti-rabbit IgG secondary antibody for 1 hr at 25 ˚C. Antigen in the sections was stained with DAB solution. Antigen staining was observed under a light microscope.

Measurement of hydroxyproline

Pulmonary tissues were homogenized in ice-cold PBS. Hydroxyprolines were measured in lung tissues according to the manufacturer's instructions.

Assessment of anti-oxidant effects and inflammatory cytokines

Lung tissues were lysed in ice-cold PBS and centrifuged at 13000 g for 15 min at 4 ˚C. The supernatants were used to measure malondialdehyde (MDA) content, the activities of the antioxidant enzymes SOD and CAT, and the levels of TNF-α and IL-6 using commercial assay kits according to the manufacturers' instructions.

Western blotting analysis

The lung tissues were lysed in lysis buffer with protease inhibitor to extract total proteins, and the lysates were centrifuged at 13000 g for 15 min at 4 ˚C. The protein in the lysates was quantified by the bicinchoninic acid (BCA) method. The proteins were isolated by SDS-PAGE and subsequently transferred onto PVDF membranes. The membranes were blocked in 10 mM Tris-buffered saline (pH 7.5) with 0.1% Tween-20 and 2% bovine serum albumin, and then incubated with the following primary specific antibodies against p21 (1:1000), p53 (1:1000), α-SMA (1:2000), AMPK (1:1000), p-AMPK (1:1000), SIRT1 (1:1000), PGC-1α (1:1000), TGF-β1 (1:1000), Collagen I (1:1000), NF-κB (1:1000), p-NF-κB (1:1000), IκB-α (1:1000), p- IκB -α (1:1000), HO-1 (1:1000), Nrf 2 (1:1000), and GAPDH (1:1000) overnight at 4 ˚C. After washed three times, the membranes were incubated with a secondary HRP-conjugated goat anti-rabbit IgG antibody (1:10000) for 1h at room temperature. After being treated with the ECL chemiluminescence substrate kit, the proteins in the membranes were visualized using the chemiluminescence imaging system.

Statistical analysis

All data were presented as the means±standard deviation (SD). Statistical differences were analyzed using one- or two-way analysis of variance (ANOVA), followed by the Bonferroni or Dunnett's *post hoc* test. Statistical analysis was performed by SPSS 22.0 (SPSS Inc., Chicago, USA). Values of $P<0.05$ were considered significant.

Results

PKC θ knockout weakened lung injury induced by bleomycin in mice

To examine the role of PKC θ in pulmonary fibrosis, the PKC θ knockout mice were treated with bleomycin to induce pulmonary fibrosis. Therefore, PKC θ protein expression was analyzed in the lung tissue of the mice. The result showed that no PKC θ protein expression was detected in the PKC θ knockout mice (Figure 2E).

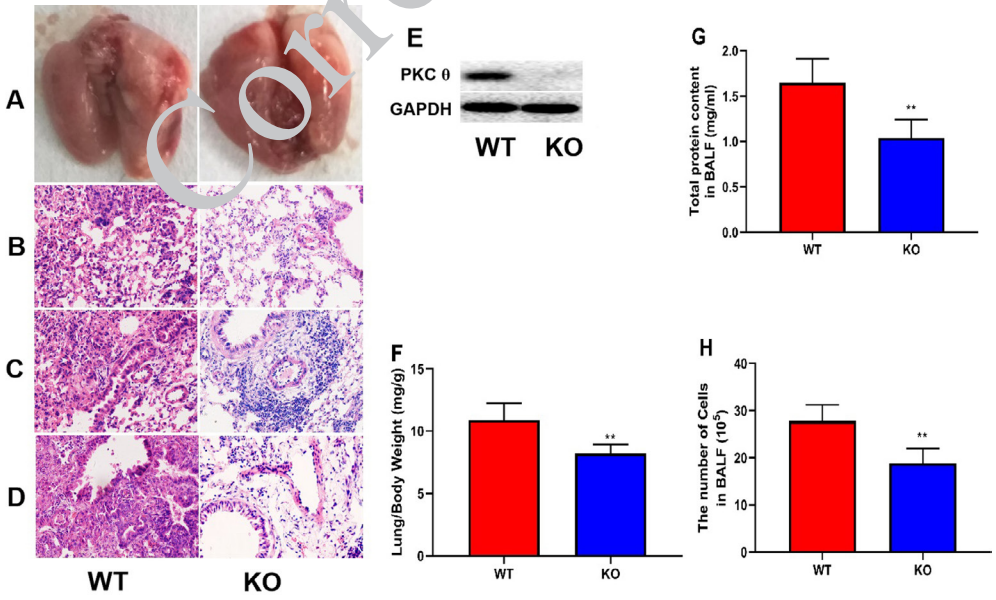

Figure 2. Effect of protein kinase C θ (PKC θ) knockout on bleomycin-induced pulmonary fibrosis
(A) Feature of pulmonary fibrosis. (B) PKC θ knockout eased inflammatory cell infiltration in the lung and extracellular matrix exudation (400×). (C) Changes of myointimal thickening (400×). (D) PKC θ knockout attenuated bronchial injury (400×). (E) PKC θ expression in lung tissue. (F) Change of lung to body weight ratio. (G) Total protein content in BALF. (H) Change of the number of cells in BALF. The results were expressed as mean±SD. Statistical analyses were performed by One-way ANOVA with Bonferroni test. ** *P-value* <0.01 compared with WT mice
BALF: Bronchoalveolar lavage fluid; WT: Wild type mice; KO: PKC-θ knockout mice

Further, we investigated the role of PKC θ in pulmonary injury through gene deletion in mice. The results of the study indicated that hemorrhage and nodules were markedly observed in the lungs of WT mice treated with bleomycin (Figure 2A), and PKC θ deletion reduced the hemorrhage and nodules in the lungs. The lung in PKC θ-deleted mice was lighter gray than that of WT mice (Figure 2A). PKC θ deletion significantly decreased the lung/body weight ratio compared with the WT group (P<0.01)(Figure 2F).

Histological examination showed that the thickness of the alveolar septum and wall was significantly reduced in PKC θ-deleted mice compared with WT mice (Figure 2B). PKC θ deletion was observed to ease inflammatory cell infiltration in the lung and extracellular matrix exudation in the alveoli compared with the WT group (Figure 2B). Additionally, PKC θ deletion alleviated myointimal thickening (Figure 2C) and bronchial injury (Figure 2D) in the lungs of bleomycin-treated mice compared with WT mice.

Additionally, PKC θ deletion was determined to decrease the total protein content significantly and the number of cells in BALF compared with the WT group (P<0.01) (Figure 2G, H).

PKC θ knockout attenuated pulmonary fibrosis induced by bleomycin in mice

Masson's trichrome staining is used to detect changes in collagen levels in organs, indicating fibrosis. In this study, lung tissue sections stained with Masson's trichrome showed that collagen levels were lower in the PKC θ-deleted group than in the WT group (Figure 3A). Furthermore, the expression of collagen I and fibronectin in the lung was assessed, and PKC θ deletion significantly reduced their expression compared with the WT group (P<0.01)(Figure 3B-D). The results from this study suggested that PKC θ deletion significantly reduced hydroxyproline content in the lung tissue compared with the WT group (P<0.01)(Figure 3E).

PKC θ knockout down-regulated the TGF-β1/α-SMA pathway in the lung of the mice treated with bleomycin

To explore the effects of PKC θ knockout on the TGF-B1/α-SMA pathway, TGF-β1 and α-SMA levels were measured in the lung. Immunohistochemical examination showed that PKC θ knockout significantly reduced TGF-β1 and α-SMA-positive staining compared with the WT group (Figure 4A, B). The expression of TGF-β1 and α-SMA in the lung was significantly decreased in the KO group compared with the WT group (P<0.01)(Figure 4C-E).

PKC θ knockout improved oxidative stress induced by bleomycin

In this study, we examined changes in markers of oxidative stress to explore the effects of PKC θ knockout on oxidative stress in the lung of mice treated with bleomycin. The results showed that PKC θ knockout significantly decreased the content of MDA (P<0.01)(Figure 5C), and elevated the activities of SOD and CAT in the lung tissues compared with the WT group (P<0.01)(Figure 5A, B), suggesting that PKC θ knockout mitigated oxidative stress induced by bleomycin.

PKC θ knockout reduced the levels of inflammatory cytokines in the mice induced by bleomycin

This study explored the effects of PKC θ knockout on inflammatory cytokine levels in bleomycin-treated mice. As shown in Figure 6, PKC θ knockout significantly reduced the levels of TGF-β, TNF-α and IL-6 in BALF when compared to the WT group (P<0.01)(Figure 6A-C). Further, PKC θ knockout significantly decreased the levels of TNF-α and IL-6 in the lung compared with the WT group (P<0.01) (Figure 6D, E).

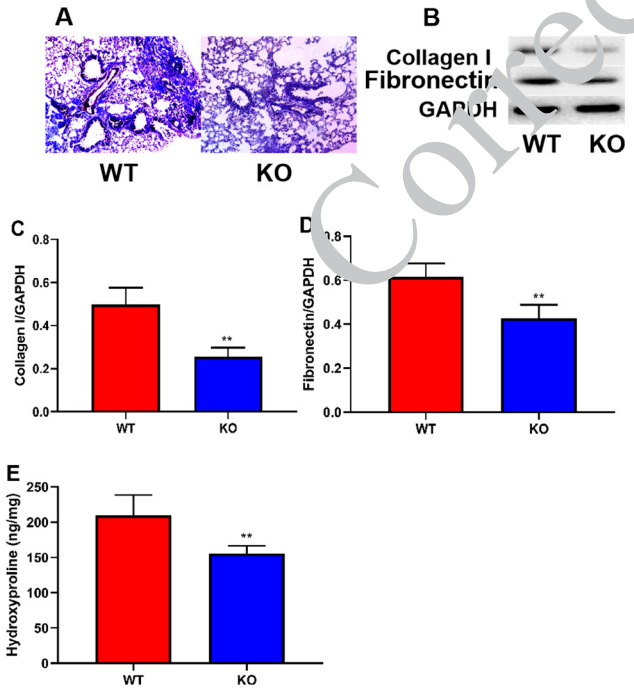

Figure 3. Effect of PKC θ knockout on the expression of fibrosis-related protein in the lung induced by bleomycin
(A) Analysis of Masson's trichrome staining (400×). (B) Expression of collagen I and fibronectin protein in the lung tissue. (C) Relative level of collagen I. (D) Relative level of fibronectin. (E) The hydroxyproline content in lung tissues. The results were expressed as mean±SD. Statistical analyses were performed by One-way ANOVA with Bonferroni test. ¨*P-value* <0.01 compared with WT mice
WT: Wild type mice; KO: PKC-θ knockout mice; PKC θ: Protein kinase C θ

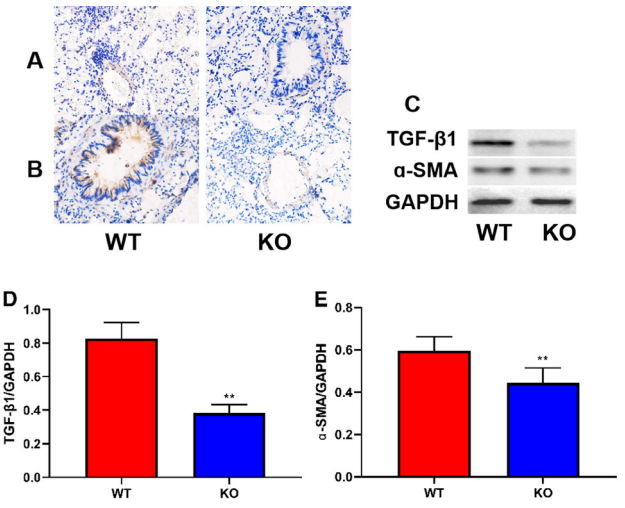

Figure 4. Effect of PKC θ knockout on the expression of TGF-β1 and α-SMA protein in the lung
(A) Immunohistochemical staining of TGF-β1 (400×). (B) Immunohistochemical staining of α-SMA (400×). (C) Expression of TGF-β1 and α-SMA protein in the lung. (D) Relative level of TGF-β1. (E) Relative level of α-SMA. The results were expressed as mean±SD. Statistical analyses were performed by Two-way ANOVA with Bonferroni test. ¨*P-value* <0.01 compared with WT mice
WT: Wild type mice; KO: PKC-θ knockout mice; PKC θ: Protein kinase C θ

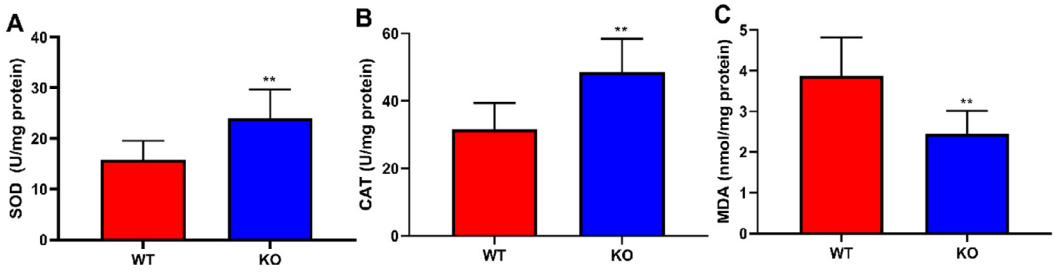

Figure 5. Effect of PKC θ knockout on markers of oxidative stress in the lung tissue induced by bleomycin
(A) Change of SOD in the lung. (B) Change of CAT activity. (C) MDA content in the lung. The results were expressed as mean±SD. Statistical analyses were performed by One-way ANOVA with Bonferroni test. ** *P-value* <0.01 compared with WT mice
WT: Wild type mice; KO: PKC-θ knockout mice; PKC θ: Protein kinase C θ

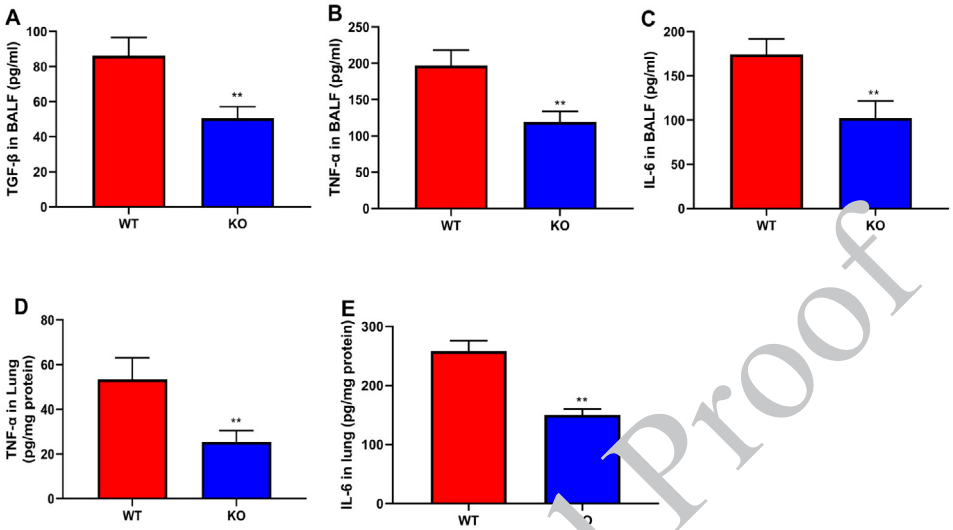

Figure 6. Effect of PKC θ knockout on inflammation induced by bleomycin
(A) Levels of TGF-β in BALF. (B) Levels of TNF-α in BALF. (C) Levels of IL-6 in BALF. (D) Levels of TNF-α in the lung. (E) Levels of IL-6 in the lung. The results were expressed as mean±SD. Statistical analyses were performed by One-way ANOVA with Bonferroni test. ** *P-value* <0.01 compared with WT mice
WT: Wild type mice; KO: PKC-θ knockout mice; PKC θ: Protein kinase C θ

PKC θ knockout suppressed the NF-κB/IKB-α pathway in the lungs of the mice treated with bleomycin

PKC θ has been reported to activate NF-κB by degrading inhibitors of the IKK complex, and the NF-κB pathway was detected in this study. Our results showed that PKC θ knockout significantly reduced the levels of phosphorylated NF-κB, IκB-α, and ERK in the lungs of mice treated with bleomycin compared with the WT group (P<0.01)(Figure 7A-D).

PKC θ knockout up-regulated the HO-1/Nrf2-SIRT1/AMPK pathway in the lungs of the mice treated with bleomycin

The expressions of HO-1, Nrf2, and SIRT1 proteins and the level of phosphorylated AMPK (p-AMPK) in the lung were determined in this study. The results showed that PKC θ knockout significantly reduced the expression of HO-1, Nrf2, and SIRT1 proteins in the lungs of bleomycin-treated mice compared with the WT group, along with the level of p-AMPK (P<0.01)(Figure 8A-E).

PKC θ knockout ameliorated the expression of autophagy-related protein Beclin 1 and p62 in the lung tissue

To further explore the mechanisms of PKC θ knockout in pulmonary fibrosis, the expression of Beclin 1 and p62 protein in the lung was determined. PKC θ knockout significantly elevated Beclin 1 protein expression (P<0.01) (Figure 8A, B) and reduced p62 protein expression in the lungs of mice treated with bleomycin compared with the WT group (P<0.01)(Figure 9A, C).

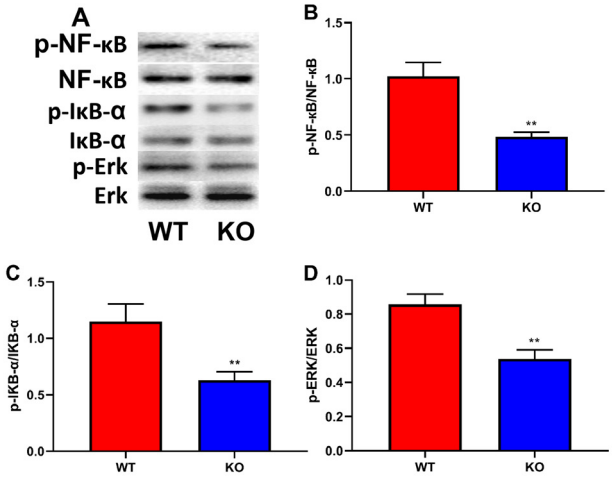

Figure 7. Effect of PKC θ knockout on expression of inflammation signaling protein
(A, B) Expression of p-NF-κB, p-IKB-α and p-ERK in the lung. (B) Relative level of p-NF-κB g. (C) Relative level of p-IKB-α. (D) Relative level of p-ERK. The results were expressed as mean±SD. Statistical analyses were performed by Two-way ANOVA with Bonferroni test. ** *P-value*<0.01 compared with WT mice
WT: Wild type mice; KO: PKC-θ knockout mice; PKC θ: Protein kinase C θ

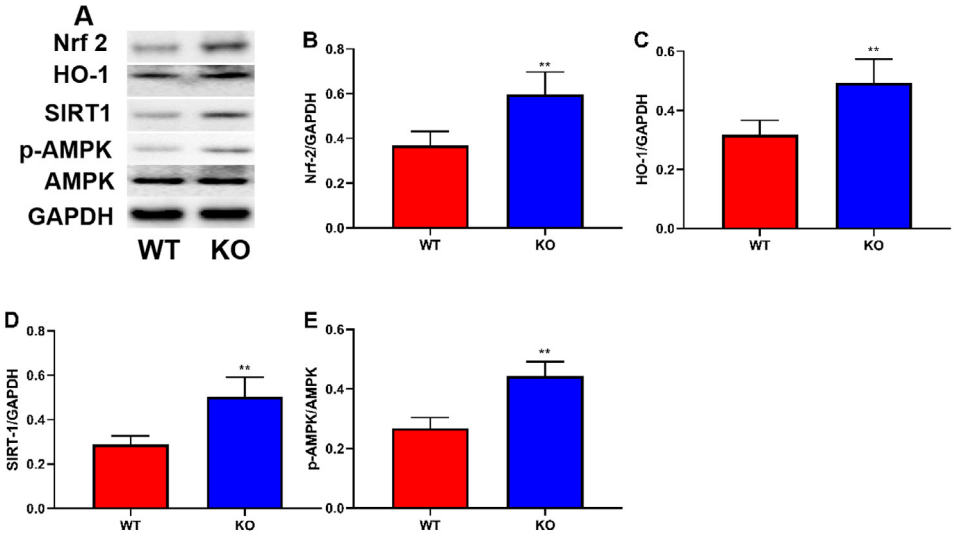

Figure 8. Effect of PKC θ knockout on HO-1/Nrf2-SIRT1/AMPK signaling pathway
(A) Expression of Nrf2, HO-1 and SIRT1 protein in lung tissue. (B) Relative level of Nrf2. (C) Relative level of HO-1. (D) Relative level of SIRT1. (E) Relative level of p-AMPK. The results were expressed as mean±SD. Statistical analyses were performed by Two-way ANOVA with Bonferroni test. ** *P-value* <0.01 compared with WT mice
WT: Wild type mice; KO: PKC-θ knockout mice; PKC θ: Protein kinase C θ; Nrf2: Nuclear factor E2-related factor 2; HO-1: Heme oxygenase 1; SIRT1: Sirtuin 1; Nrf2: Nuclear factor E2-related factor 2; AMPK: Activated protein kinase

PKC θ knockout abated the expression of senescence markers p53 and p21 in the lung

To investigate the effect of PKC θ knockout on senescence, we examined changes in the expression of senescence markers, including p53 and p21. The results showed that PKC θ knockout significantly reduced the protein expression of p53 and p21 in the lung tissue of mice treated with bleomycin compared with the WT group (P<0.01)(Figure 10A-C).

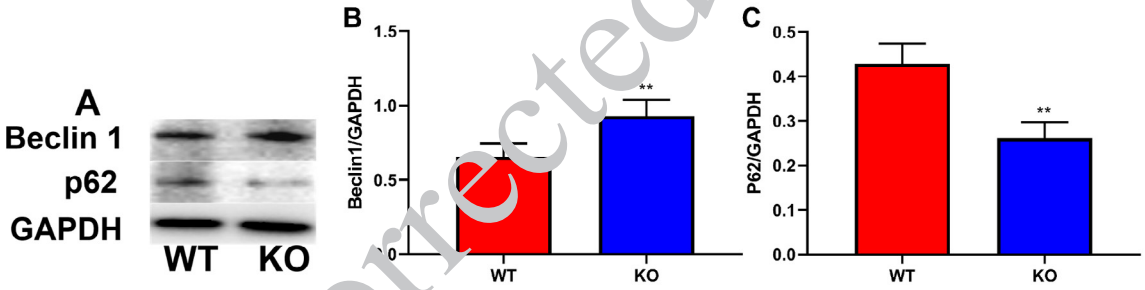

Figure 9. Effect of PKC θ knockout on autophagy related protein expression in the lung
(A) Expression of beclin 1 and p62 protein in the lung. (B) Relative expression of beclin 1 protein. (C) Relative expression of p62 protein. The results were expressed as mean±SD. Statistical analyses were performed by Two-way ANOVA with Bonferroni test. ** *P-value* <0.01 compared with WT mice
WT: Wild type mice; KO: PKC-θ knockout mice; PKC θ: Protein kinase C θ

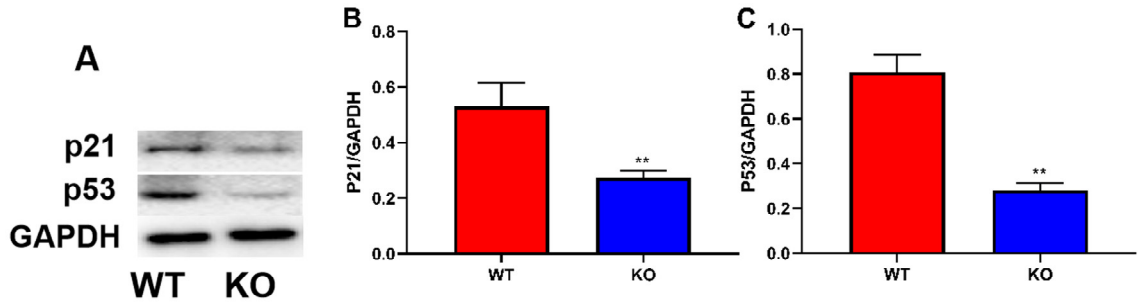

Figure 10. Effect of PKC θ knockout on senescence-related protein expression in the lung
(A) Expression of p21 and p53 protein in the lung. (B) Relative expression of p21 protein. (C) Relative expression of p53 protein. The results were expressed as mean±SD. Statistical analyses were performed by Two-way ANOVA with Bonferroni test. ** *P-value* <0.01 compared with WT mice
WT: Wild type mice; KO: PKC-θ knockout mice; PKC θ: Protein kinase C θ

Discussion

Some lung diseases inevitably progress to pulmonary fibrosis and lead to respiratory failure, causing high mortality (25). Unclear pathogenesis mechanisms of pulmonary fibrosis contribute to the challenge of clinical treatment (25). Therefore, the effect of PKC θ gene knockout on pulmonary fibrosis is investigated in the present study. TGF-β and α-SMA play vital roles in stimulating the fibrotic response, which is considered an important driver and marker of tissue fibrosis (26). Our findings show that PKC θ knockout attenuates bleomycin-induced pulmonary fibrosis in mice and reduces the expression of TGF-β and α-SMA protein in the lung. Furthermore, this study also indicates that PKC θ knockout decreases the expression of collagen I and fibronectin, and the level of hydroxyproline in the lung. Pulmonary fibrosis is attributed to excessive deposition of collagen I and fibronectin induced by TGF-β1 stimulation (27). Hydroxyproline, a collagen deposition marker, is considered a marker of pulmonary fibrosis (28).

Bleomycin is a chemotherapy drug for many tumors (29). Further studies suggested that bleomycin can induce hypersensitivity pneumonitis, bronchiolitis obliterans, and pulmonary fibrosis (30). It has been demonstrated that treatment with bleomycin up-regulates profibrosis signaling pathways and inflammatory response (30). Chronic inflammation plays a vital role in pulmonary fibrosis (31). Bleomycin impairs DNA, triggering oxidative stress via the production of free radicals, resulting in inflammation and subsequent fibrosis (32). Additionally, bleomycin leads to apoptosis and necrosis of alveolar type I cells and stimulates the proliferation and differentiation of alveolar type II cells (33). Meanwhile, fibroblasts are induced to migrate to interepithelial cell defects and to be activated, thereby causing pulmonary fibrosis (34). Thus, bleomycin is associated with pulmonary fibrosis(35). PKC θ, one of the calcium-independent PKC isoenzymes, is involved in various physiological processes by regulating gene expression, cell differentiation and proliferation, ion channels, and angiogenesis (36). PKC θ has been shown to activate NF-κB, which plays an important role in the inflammatory response by translocating to the nucleus, stimulating the production of inflammatory cytokines such as interleukin-2, TNF-α, and IL-6. Furthermore, the release of inflammatory cytokines stimulates oxidative stress via generation of ROS, while oxidative stress induces an inflammatory response (37, 38). In this study, the data show that PKC θ gene knockout in mice decreases the levels of the inflammatory cytokines TNF-α and IL-6 in BALF and the lungs, and increases SOD and CAT activities and reduces MDA content in the lung. PKC θ gene knockout down-regulated the NF-κB/IκB-α signaling and the level of phosphorylated Erk in the lungs. The results suggested that PKC θ gene knockout attenuates pulmonary fibrosis by reducing the inflammatory response. However, PKCδ, another PKC isoform, has been reported to alleviate bleomycin-induced pulmonary fibrosis by inhibiting the NF-κB pathway (45), and PKCα up-regulates collagen production in pulmonary fibroblasts. Furthermore, Down-regulation of PKC θ has been shown to have no effect on the expression of other PKC isoforms, including PKCα, PKCβ, and PKCδ (39).

Sirtuin 1 (SIRT1), a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase, participates in multiple pathophysiological processes, including cell senescence, energy metabolism through the mitochondria, autophagy, Inflammation, and oxidative stress (40, 41). SIRT1 has been shown to reduce acetylation of the NF-κB p65 subunit at lysine 310 and decrease NF-κB activity, thereby down-regulating the expression of inflammatory cytokines (42, 43). Furthermore, SIRT1 suppresses PKC-ζ activity in cardiac hypertrophy, and knockdown of PKC-ζ, a member of the PKC family, reduces NF-κB activity and ERK1/2 activation. However, PKC-ε overexpression activates SIRT1 and improves Doxorubicin-Induced Cardiotoxicity (44). SIRT1 can also activate nuclear factor E2-related factor 2 (Nrf2) through deacetylation, thereby exerting antioxidant effects by eliminating reactive oxygen species (ROS) and regulating the expression of heme oxygenase 1 (HO-1)(45). SIRT1 has been shown to deacetylate and activate the AMP-dependent kinase (AMPK) activating kinase, thereby activating AMPK (46). Activated AMPK induces peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) activation and mitochondrial biogenesis, stimulating the expression of HO-1 and decreasing the generation of ROS (47, 48). HO-1, an inducible antioxidative enzyme, can degrade heme into bilirubin, carbon monoxide, and iron, exerting antioxidative and anti-inflammatory effects by HO-1 and its catalytic byproduct (49). SIRT1 is also confirmed to regulate mitochondrial biogenesis and function, and oxidative stress, via activation of AMPK and PGC-1α (47, 50). This study showed that PKC θ knockout reduces SIRT1 expression, AMPK activity, and HO-1 and Nrf2 levels in the lung. These findings suggested that PKC θ knockout attenuates inflammatory response and oxidative stress in the lung treated with bleomycin by elevating SIRT1/AMPK signaling.

Autophagy is a complex endogenous defense mechanism to mediate cellular and organismal homeostasis via eliminating damaged tissue fragments (51). Autophagy plays a critical role in regulating cellular homeostasis, including lipid and protein metabolism (52). Various pathological processes, such as excessive endoplasmic reticulum-phagy, viral infections, and organelle degradation, can activate autophagy (53). Accumulating evidence demonstrated that activation of autophagy can ameliorate various diseases, such as hepatic steatosis, diabetic cardiomyopathy, and myocardial ischemia-reperfusion injury (54, 55). The induction of autophagy has also been reported to improve lung fibrosis (56). Some studies have demonstrated that SIRT1 is involved in regulating every step of autophagy (52). SIRT1 mediates autophagy by separating beclin 1 from the Bcl2-beclin 1 complex and by down-regulating mechanistic target of rapamycin 1 (mTORC1) via its deacetylase activity (52). In addition, activated AMPK directly stimulates autophagy by activating the Unc51-like kinase (ULK) complex and indirectly triggers autophagy by suppressing mTORC1 (57). P62 (SQSTM1) is a key marker that regulates autophagy and down-regulates autophagy activity (58). Increased expression of SIRT1 is reported to lower the expression of p62 protein and elevate autophagic flux (59). Some studies showed that activated AMPK decreases the expression of p62 (60). In the present study, the results indicated that PKC θ knockout significantly increases beclin-1 levels and reduces p62 expression in the lung. The data suggested that PKC θ knockout improves autophagy in the lung treated with bleomycin through the SIRT1/AMPK pathway.

Age, together with age-related mechanisms, is associated with an increased incidence of IPF. Cellular senescence is a major contributor to aging and a hallmark of aging (61). Various studies have reported that inhibition of senescence attenuates pulmonary fibrosis (56, 62, 63). Multiple factors, such as oxidative stress, mitochondrial damage, and inflammation, have been demonstrated to contribute to cellular senescence (63, 64). Administration of bleomycin increases the expression of senescence markers, such as p16 and p21, thereby inducing pulmonary fibrosis (56). In addition to mediating the activity of antioxidant enzymes through acetylation, SIRT1 exerts an anti-senescent role by regulating p16 and p21 (65). Additionally, NF-κB signaling is involved in regulating senescence (66). In this study, the results revealed that PKC θ knockout down-regulates the expression of p21 and p16 proteins in the lungs of bleomycin-treated mice. These findings suggested that PKC θ knockout ameliorates senescence via the mediation of the SIRT1/AMPK pathway.

Conclusion

In conclusion, our results demonstrated that PKC θ knockout attenuated inflammation, oxidative stress, and pulmonary fibrosis in the bleomycin-treated mice. These beneficial effects were associated with the induction of autophagy and the reduction of senescence through up-regulation of SIRT1/AMPK signaling. Taken together, these findings may identify a promising therapeutic target for pulmonary fibrosis (Figure 11).

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Authors' Contributions

W L performed the experiment and participated in the design and interpretation of the data. X Z, M W, and H W performed the experiments and collected the data. X Z and RY L participated in the experiments, collected the data, and provided some experimental materials. GG W initiated and designed the study, analyzed the data, and wrote the paper. All authors read, reviewed, and approved the final manuscript.

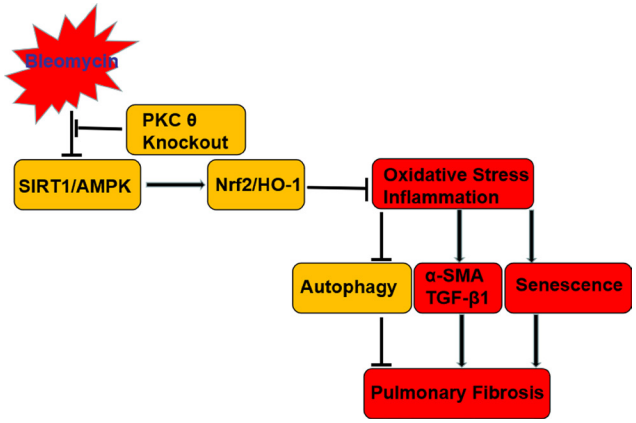

Figure 11. Schematic diagram of the mechanism by which PKC θ knockout attenuates bleomycin-induced pulmonary fibrosis
PKC θ knockout improves impaired SIRT1/AMPK-Nrf2/HO-1 signaling by bleomycin, which reduces oxidative stress and inflammation. Consequently, PKC θ knockout ameliorates autophagy and senescence. Furthermore, PKC θ knockout mitigates pulmonary fibrosis

Conflicts of Interest

All authors declare no conflicts of interest regarding the present manuscript.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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