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ZLN005 improves the protective effect of mitochondrial function on alveolar epithelial cell aging by upregulating PGC-1 α

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Background: Idiopathic pulmonary fibrosis (IPF) is a chronic and fatal pulmonary interstitial disease that usually occurs in the elderly. The senescence of alveolar epithelial cells (AECs) is an important mechanism of IPF. The AECs of patients with IPF have lower expression of peroxisome proliferator-activated receptor- γ coactivator-1 alpha (PGC-1 α), which has been shown to play an important role in maintaining mitochondrial morphology and energy metabolism. This study sought to explore the mechanism by which ZLN005 improves mitochondrial function by upregulating PGC-1 α to protect AECs from aging.

Methods: Western blot was used to detect the expression of PGC-1 α , mitochondrial synthesis protein nuclear respiratory factor-1 (NRF-1), and p21^{WAF1} in the lung tissue of the IPF patients and the mice with bleomycin (BLM)-induced pulmonary fibrosis. A549 cells and mice AEC2 cells were treated with hydrogen peroxide (H₂O₂) to construct cell senescence models. Cell senescence was detected by senescence-associated beta-galactosidase staining. The mitochondrial respiratory function was measured, including the adenosine triphosphate (ATP) generation, reactive oxygen species (ROS) level, changes in cell membrane potential, and energy metabolism. Using lentivirus as a vector and using gene editing technology to over express (upPGC-1 α) and knockdown PGC-1 α (shPGC-1 α) in the A549 cells. The PGC-1 α agonist ZLN005 was used to pretreat the A549 and shPGC-1 α A549 cells, and cell aging and mitochondrial respiratory function were observed.

Results: The Western blot and immunofluorescence assays showed that the expression of PGC-1 α and NRF-1 was decreased in the lung tissues of the IPF patients and BLM-induced mice pulmonary fibrosis model, while the expression of p21^{WAF1} was increased. The results of the immunofluorescence and mitochondrial function experiments also indicated that the expression of PGC-1 α and mitochondrial synthesis protein NRF-1 were decreased in the senescent cells. Further, the mitochondrial morphology was abnormal and the mitochondrial function was impaired. PGC-1 α was involved in the AEC senescence by

regulating mitochondrial morphology and function. Treatment with the agonist of PGC-1 α (i.e., ZLN005) blocked the H₂O₂-induced cell senescence by enhancing the expression of PGC-1 α .

Conclusions: These results provide preliminary insights into the potential clinical application of ZLN005 as a novel therapeutic agent for the treatment of IPF.

Keywords: Idiopathic pulmonary fibrosis (IPF); cellular senescence; peroxisome proliferator-activated receptor- γ coactivator-1 alpha (PGC-1 α); mitochondrial function; alveolar epithelial cells (AECs)

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive pulmonary interstitial disease characterized by abnormal matrix deposition by lung fibroblasts, alveolar structural disorders, and pulmonary interstitial fibrosis (1). IPF occurs worldwide and is more common in men than women. The incidence and prevalence of IPF increase significantly with age (2). The senescence of alveolar epithelial cells (AECs) is thought to be an important mechanism in the pathogenesis of IPF. A prevailing concept is that alveolar type 2 (AT2) cells can induce a fibrotic response in the lungs (3). Mechanisms of AT2 depletion, particularly in the context

of early events leading to IPF, are not yet fully understood. Many studies have found that IPF lungs have an increased level of cell senescence compared to non-fibrotic lungs (4,5). Mitochondrial dysfunction and senescence have been linked to age-related interstitial lung diseases (ILDs), such as IPF (6).

Mitochondrial dysfunction is a recognized marker of aging and is characterized by the increased production of reactive oxygen species (ROS) and bioenergetics (7,8). Relevant study has found that mitochondrial dysfunction may be related to the pathogenesis of IPF (9). The disturbance and interference of AT2 mitochondrial biogenesis, function, and homeostasis are known profibrotic signals (10-13). The imbalance of mitochondrial dynamics due to impaired mitophagy and the accumulation of mitochondrial DNA damage or irregularities in protein homeostasis lead to endoplasmic reticulum stress and the programmed cell death of AT2 cells. Chronic injury to distal lung tissue results in the loss or alteration of AT2 cell function, which promotes the dysregulated repair and pathogenic activation of fibroblasts (14,15). The secretion of hydrogen peroxide (H₂O₂) by myofibroblasts acts as a signal of diffuse death in lung epithelial cells (16). Evidence suggests that AEC mitochondrial dysfunction plays an important role in mediating lung fibrosis signaling via mechanisms involving imbalances in the levels of ROS, the endoplasmic reticulum stress response, mitophagy, apoptosis, and/or senescence, and inflammatory signaling (17). Mitochondria dysfunction and metabolic reprogramming have been identified in different IPF lung cells (e.g., AECs, fibroblasts, and macrophages) and have been shown to promote low resilience and increase susceptibility to the activation of profibrotic responses (18,19).

Highlight box

Key findings

- Peroxisome proliferator-activated receptor- γ coactivator-1 alpha (PGC-1 α) is involved in alveolar epithelial cell (AEC) senescence by regulating mitochondrial morphology and function.
- The agonist of PGC-1 α (i.e., ZLN005) alleviates hydrogen peroxide (H₂O₂)-induced cell senescence by enhancing the expression of PGC-1 α and improving mitochondrial function.

What is known and what is new?

- The senescence of AECs is an important mechanism in the pathogenesis of idiopathic pulmonary fibrosis (IPF). Mitochondrial dysfunction is a recognized marker of aging. PGC-1 α has been confirmed to play an important role in maintaining mitochondrial morphology and energy metabolism.
- ZLN005 alleviates H₂O₂-induced cell senescence by enhancing the expression of PGC-1 α and improving mitochondrial function.

What is the implication, and what should change now?

- ZLN005 improves mitochondrial function by upregulating PGC-1 α to protect AECs from aging, and thus may serve as a novel therapeutic agent for IPF.

A study has shown that the expression of peroxisome proliferator-activated receptor- γ coactivator-1 alpha (PGC-1 α) is lower in the AECs in the lung tissues of patients with IPF, and PGC-1 α has been confirmed to play an important role in maintaining mitochondrial morphology and energy metabolism (20). PGC-1 α is a powerful transcriptional regulator and was originally identified as a transcriptional coactivator of mitochondrial function (21,22) and brown fat thermogenesis (23). It can effectively regulate oxidation and antioxidant processes (24,25) and is involved in the regulation of mitochondrial function (26) and the development of a variety of diseases (27).

Hypoxia has been shown to reduce the expression of PGC-1 α and cause mitochondrial dysfunction (28,29), significantly reduce the efficiency of oxidative phosphorylation, resulting in membrane potential decomposition, increase ROS production, deplete adenosine triphosphate (ATP), and reduce the mechanism of uncoupled oxidative phosphorylation, leading to endothelial dysfunction (30). IPF is thought to involve lung injury induced by ROS, in particular superoxide anions. The superoxide dismutase catalyzes the dismutation of superoxide anion to H₂O₂ (31). The high expression of PGC-1 α in IPF has a protective effect on fibroblasts (32).

ZLN005 has been identified as an agonist of PGC-1 α . It has been reported that ZLN005 effectively protects RPE (retinal pigment epithelium)-19 cells from cell death caused by H₂O₂, oxidized low-density lipoprotein, and sodium iodate. Moreover, in PGC-1 α silenced cells, ZLN005 lost its protective effect against cell death mediated by H₂O₂ (33). The activation of PGC-1 α expression in prostate cancer cells (PC3) by ZLN005 has been shown to partially reverse tumor protein 53 (p53)-mediated mitochondrial dysfunction (26). However, it is not yet known whether ZLN005 can mitigate the development of IPF by activating the expression of PGC-1 α in AECs and improving the mitochondrial function of AECs.

This study conducted *in vitro* and *in vivo* experiments to preliminarily explore the mechanism by which PGC-1 α is involved in AEC senescence through regulating mitochondrial morphology and function. The protective effect of the PGC-1 α agonist ZLN005 on senile AECs was also examined. Our findings may provide preliminary insights into the potential clinical application of ZLN005 in the treatment of IPF. We present this article in accordance with the ARRIVE reporting checklist (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-23-815/rc>).

Methods

Animal model

The bleomycin (BLM)-induced mice pulmonary fibrosis model is a widely used animal model for pulmonary fibrosis and has been used as a preliminary experiment animal model by our research group (34). The animals used in this experiment were 8-week-old male wild-type C57BL/6 mice purchased from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). All the mice were raised under specific-pathogen-free conditions. The mice were anesthetized with toluenthiiazide hydrochloride and ketamine (1:2 by volume), BLM was injected into a 2.4-mg/mL phosphate buffered saline (PBS) solution, and the mice were given an intratracheal infusion at 5 mg/kg after weighing. The mice were randomly sacrificed on days 7, 14, and 28 after modeling. This animal experiment was performed in the Medical School of Nanjing University [No. SYXK(SU)2019-0056], in compliance with Nanjing University guidelines for the care and use of animals. The BLM hydrochloride used for the injections was purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan).

Tissues

All the human lung tissues from the IPF patients (n=4) were obtained from the Department of Lung Transplantation, Wuxi People's Hospital, Wuxi, China. Normal peripheral tissues (n=4), were used as the controls and were obtained from patients who underwent lobectomy at the Thoracic Surgery Department of Nanjing Drum Tower Hospital at the Affiliated Hospital of Nanjing University Medical School. All diagnoses of IPF were made in accordance with the American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Latin American Thoracic Society criteria for IPF (35). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Nanjing Drum Tower Hospital of Medical School of Nanjing University (No. 2021-390-01). Informed consent was obtained from the patients.

Cell culture

Lung cancer human alveolar basal epithelial cells (A549 cells) were provided by the American Type Culture

Collection (ATCC). The mouse AEC2 cells were obtained using a method described previously (22). The A549 cells were cultured in Dulbecco's Modified Eagle Medium high glucose medium (0.1% penicillin and streptomycin) containing 10% fetal bovine serum (FBS). The AEC2 cells were grown in Ham's F12 medium with 10% FBS. All the cells were cultured in a cell incubator, under the following conditions: 37 °C and 5% carbon dioxide (CO₂). The reagents used in the cell culture were purchased from Gibco (New York, USA).

Western blot analysis

Proteins collected from the lung tissue or AEC2 cell lysates were isolated using sodium dodecyl-sulfate polyacrylamide gel electrophoresis and polyvinylidene fluoride membranes. The proteins were incubated with the corresponding antibody. The primary antibodies including PGC-1 α , nuclear respiratory factor-1 (NRF-1), p21^{WAF1} and β -actin were used. The antibodies used in this experiment were provided by Abcam.

Senescence-associated beta-galactosidase (SA- β -gal) activity assays

SA- β -gal activity assays were completed in accordance with the instructions of the SA- β -gal staining kit (Beyotime Biotechnology, Shanghai, China). In brief, after removing the cell growth medium, the cells were rinsed with 1 \times of PBS, and 1 mL of 1X fixative solution was added to each well. The cells were then immobilized at room temperature for 10–15 min. The plate was rinsed with 1 \times PBS, and 1 mL of the prepared β -galactosidase staining solution was added. The cells were then incubated overnight in a CO₂-free dry incubator at 37 °C. Images were captured using a microscope equipped with a digital camera (Eclipse800, Nikon, Tokyo, Japan). For each slide, at least 3 fields were obtained to calculate the SA- β -gal intensity.

Immunofluorescence

The immunofluorescence kit (Cell Signaling Technology, Boston, USA) was used for the immunofluorescence analysis of the antibodies against p21 in accordance with the manufacturer's instructions. The immunofluorescence results were captured by confocal laser microscopy (Leica, Wetzlar in Hesse, Germany). To measure the mitochondrial morphology,

the A549 cells underwent Mitotracker Red (Cell Signaling Technology, Boston, USA) fluorescent dye staining for 30 mins and were then observed by fluorescence microscopy.

Construction of stable PGC-1 α cell lines

The cells were transfected 24 h prior to the experiments with Silencer™ Select small-interfering RNA targeted to PGC-1 α (s21394, sequence 5'→3': *CCUGUUUGAUGACAGCGAATT*) and the non-targeting control (Silencer™ Select Negative Control No. 1) using Lipofectamine RNAiMAX in accordance with the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). The upPGC-1 α A549 cells were treated the same as the shPGC-1 α cells in accordance with the manufacturer's instructions (Thermo Scientific). The lentivirus (shPGC-1 α and upPGC-1 α) was transfected into the A549 cells, and a stable cell line was obtained by screening with puromycin. The expression of PGC-1 α in the experimental group, the negative control group, and the normal A549 group were detected by western blot to verify the interference efficiency.

Related experiments of mitochondrial function detection

The mitochondria extraction was completed using a mitochondrial extraction kit (Sigma-Aldrich, Germany). The ROS products were analyzed according to the instructions of the ROS analysis kit (Biyuntian, Jiang Su, China). The stained A549 cells were examined by a fluorescent probe CM-H2DCFDA, and the ROS analysis was performed by flow cytometry. The ROS relative intensity of the living cells was measured using the logarithm of the fluorescence readings of the corresponding fluorescent probe with the abscissa and the number of cells as the ordinate. Mitochondrial membrane potential was detected using the JC-10 kit (Biyuntian, Jiang Su, China). Apoptosis was induced by adding JC-10 solution with a final concentration of 2 μ M to the prepared cells and Annexin V. Finally, the experimental data were analyzed by flow cytometry. The relevant detection of ATP was completed by the ATP kit (Biyuntian, Jiang Su, China). The Seahorse XF Cell Mitochondrial Stress Test Kit (Seahorse Bioscience, Boston, USA) was used to determine the complete state of the mitochondrial stress metabolism function. In Seahorse Test, the cells were grown on dedicated microplates to measure the oxygen consumption rate (OCR) of the different drugs in real time.

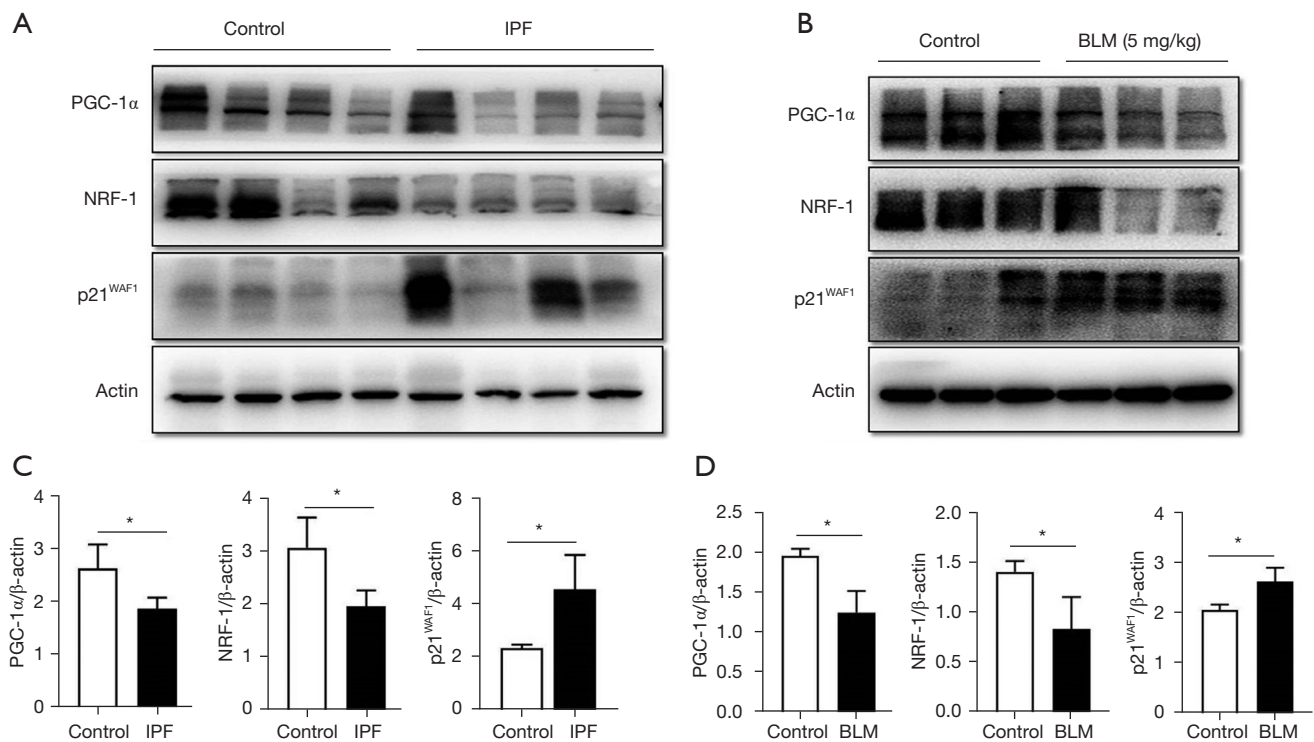


Figure 1 Expression of PGC-1 α , NRF-1, and p21^{WAF1} in idiopathic pulmonary fibrosis. (A,B) Western blot experiments were conducted to analyze the expression of PGC-1 α and mitochondrial synthesis protein NRF-1 in the lung tissues of the IPF patients and BLM-induced pulmonary fibrosis mouse models, and the expression levels of the aging-related marker (p21^{WAF1}) in the lung tissues. (C,D) A quantitative analysis was conducted to examine the Western blot experiment results. β -actin served as an internal parameter. *, $P < 0.05$. PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 alpha; NRF-1, nuclear respiratory factor-1; IPF, idiopathic pulmonary fibrosis; BLM, bleomycin.

Statistics

Prism Graph Pad 8 was used to analyze the experimental data. Differences between the groups were calculated using the 2-tailed Student *t*-test. The results are presented as the mean \pm standard error of the mean. A *P* value < 0.05 was considered significant.

Results

Expression of PGC-1 α , NRF-1, and p21^{WAF1} in IPF

PGC-1 α has been confirmed to play an important role in maintaining mitochondrial morphology and energy metabolism (20). To investigate the role of PGC-1 α in AEC senescence by regulating mitochondrial morphology and function, we first detected the expression levels of PGC-1 α , NRF-1, and p21^{WAF1} in both the IPF patients and BLM-induced mouse pulmonary fibrosis model. NRF-1 and

p21^{WAF1} were used as senescent markers to detect the appearance of senescence in the IPF lung tissues and AECs. The lung tissues of the IPF transplanted patients and BLM-induced mouse lung fibrosis model specimens underwent a Western Blot analysis and showed decreased expression of PGC-1 α and NRF-1 in both the lung tissues was decreased, while the expression of p21^{WAF1} was increased (Figure 1A-1D). IPF is an aging-related disease. The results of this experiment suggest that cellular senescence and changes in mitochondrial function are involved in the pathogenesis of IPF.

The expression of PGC-1 α and the NRF-1 protein, the determination of mitochondrial morphology and function, and the expression levels of the cell senescence-related markers in the H₂O₂-induced AEC senescence model

In previous experiments, the expression of PGC-1 α was

found to be decreased in the lung tissues of the IPF patients and the BLM-induced mouse pulmonary fibrosis model. It was not known whether these results would be replicated in an AEC senescence model. Thus, we treated the AECs with different concentrations of H₂O₂ to construct a cell senescence model.

H₂O₂ secreted by myofibroblasts acts as a diffuse death signal for lung AECs. The Western blot experiments showed that as the concentration of H₂O₂ increased, the expression of PGC-1 α and NRF-1 in the AECs gradually decreased, and the expression of p21^{WAF1} gradually increased (Figure 2A,2B). The SA- β -gal staining method was used to detect the positive rate of the AEC staining. The results showed that compared to the control group, as the H₂O₂ concentration continuously increased, the positive rate of the SA- β -gal staining of the cells slowly increased (Figure 2C-2F) consistent with the senescence of the cells.

Mitochondrial morphology was detected using the immunofluorescence method, and the results showed that as the H₂O₂ concentration increased, the volume of the aging A549 cells gradually increased. Under confocal microscopy, the tubular network between the mitochondria disappeared, and were observed to have scattered and granular structures (Figure 2G). The expression of the p21^{WAF1} protein was increased (Figure 2H,2I). The results of the mitochondrial function experiments showed that as the H₂O₂ concentration continuously increased, the ATP level and matrix metalloproteinases (MMPs) of the AECs gradually decreased, while the ROS level gradually increased (Figure 3A-3E). The mitochondrial pressure test results showed that the basal OCR and maximum OCR of the A549 senescent cells in the H₂O₂ treatment group were significantly decreased compared to those of the control group (Figure 3F,3G). These results suggest that H₂O₂ induced the senescence of the AECs, and the expression of PGC-1 α and the mitochondrial synthesis protein NRF-1 in the senescent cells decreased. Abnormal mitochondrial morphology and mitochondrial dysfunction manifested as decreased ATP production, MMP, and mitochondrial respiratory function, and increased ROS levels.

The mitochondrial function and senescence expression of the A549 cells were verified by knockout (shPGC-1 α) and overexpression (upPGC-1 α), respectively

Mitochondrial function changed after the expression of PGC-1 α was knocked down. The previous experiments in

this study showed that PGC-1 α expression decreased in the H₂O₂-induced senescence model. To determine whether the regulation of PGC-1 α is a key factor in regulating aging, mitochondrial function and cellular senescence were monitored by changing the expression of PGC-1 α in the A549 cell senescence model that was induced by H₂O₂.

As Figure 4A,4B show, the Western blot analysis revealed that after PGC-1 α gene expression was knocked down in the A549 cells, the expression of p21^{WAF1} was upregulated. The positive rate of the SA- β -gal staining cells was significantly increased (Figure 4C,4D), ATP production was decreased, the ROS level was increased, and the MMP level was significantly decreased (Figure 4E-4I). The basal OCR and maximal OCR of the shPGC-1 α cells were significantly decreased (Figure 4J,4K). When treated with H₂O₂, the expression of p21^{WAF1} in the shPGC-1 α cell group was higher than that in the untreated group, the expression of NRF-1 was decreased (Figure 4A,4B), the positive rate of the SA- β -gal staining cells was significantly increased, ATP production was decreased, the ROS level was increased, and MMP was decreased (Figure 4C-4K), and all the differences were statistically significant.

After upregulating the PGC-1 α gene expression in the A549 cells, the Western blot analysis showed that p21^{WAF1} expression in the A549 cells was decreased, NRF-1 expression was increased (Figure 5A,5B), the positive rate of the SA- β -gal staining cells was significantly decreased, ATP production was increased, and the ROS level was decreased. Additionally, MMP was significantly increased (Figure 5C-5I), and the basal OCR and the maximum OCR of the upPGC-1 α cells were significantly increased (Figure 5J,5K). When treated with H₂O₂, the expression of p21^{WAF1} in the upPGC-1 α cell group was significantly decreased compared to that of the untreated group, the expression of NRF-1 was increased (Figure 5A,5B), the positive rate of the SA- β -gal staining cells was significantly decreased, ATP production was increased, ROS level was decreased, and MMP was significantly increased (Figure 5C-5K), and the differences were statistically significant.

Thus, it can be concluded that after the PGC-1 α gene was knocked down in the A549 cells, cell aging was aggravated, the expression of mitochondrial synthesis protein NRF-1 was decreased, and mitochondrial morphology and dysfunction were abnormal. After PGC-1 α gene expression was upregulated, cell senescence was reduced, mitochondrial synthesis protein NRF-1 expression was increased, and mitochondrial morphology and function were restored.

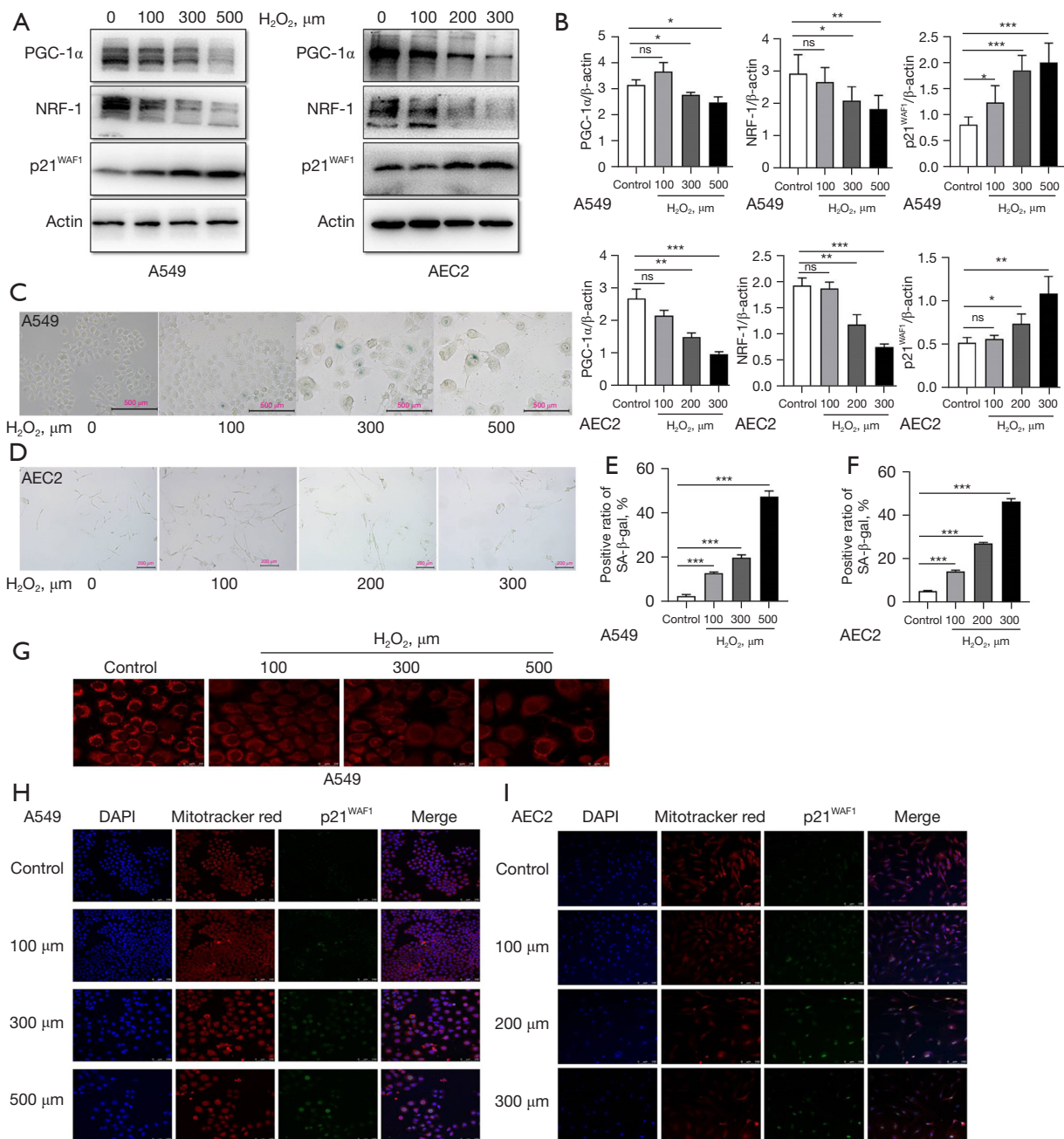


Figure 2 Determination of PGC-1 α , NRF-1 protein, mitochondrial morphology, and function, and the expression levels of cell senescence-related markers (i.e., p21^{WAF1} and SA- β -gal) in the H_2O_2 -induced AEC senescence model. The A549 cells and mice AEC2 cells were cultured in serum-free medium and treated with different concentrations of H_2O_2 for the following experiments: (A) Western blot was used to detect the expression levels of PGC-1 α , NRF-1, and p21^{WAF1} in the AECs; (B) a quantitative analysis of the Western blot experiment results was conducted; (C,D) SA- β -gal staining of the A549 and AEC2 cells treated with different concentrations of hydrogen peroxide was performed ($\times 100$); (E,F) the positive rates of the SA- β -gal staining in the A549 and AEC2 cells was determined; (G) immunofluorescence experiments were conducted to determine the mitochondrial morphological changes in the A549 cell (Mitotracker Red, $\times 800$); and (H,I) immunofluorescence experiments were conducted to determine of the mitochondrial morphological changes and the expression levels of p21^{WAF1} in the AECs ($\times 200$). β -actin served as an internal parameter. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, no significance. PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 alpha; NRF-1, nuclear respiratory factor-1; DAPI, 4',6-diamidino-2-phenylindole; SA- β -gal, senescence-associated beta-galactosidase; AEC, alveolar epithelial cell.

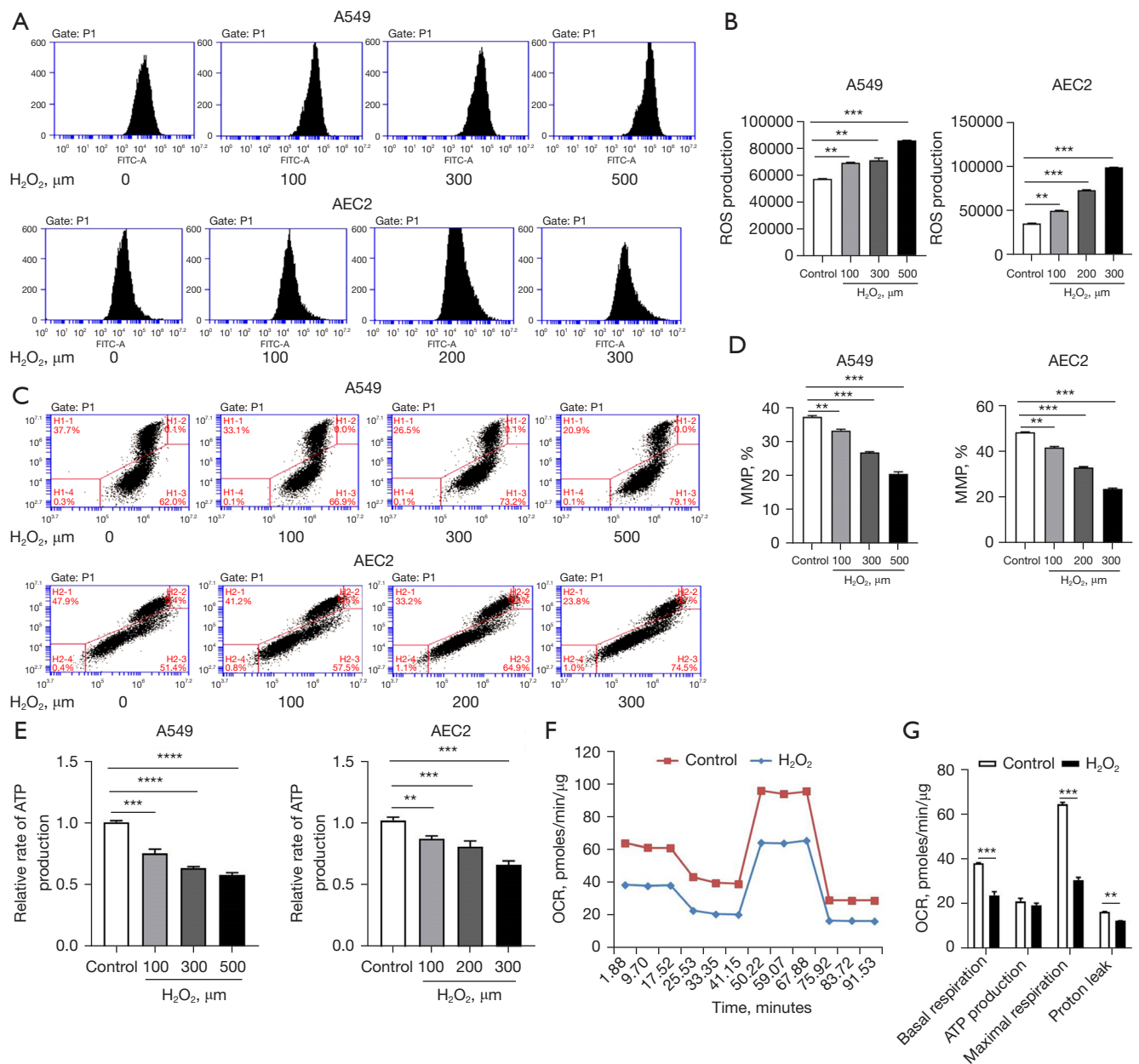


Figure 3 Detection of mitochondrial function (i.e., ATP production, changes in ROS levels, MMP, and mitochondrial respiratory function) in the AEC senescence model induced by H_2O_2 . The A549 cells and mice AEC2 cells were cultured in serum-free medium and treated with different concentrations of H_2O_2 for the following experiments: (A) flow cytometry was used to detect ROS in the A549 cells and mice AEC2 cells; (B) a quantitative analysis of the ROS products was conducted; (C) flow cytometry was used to detect the mitochondrial membrane potential of the A549 cells and mice AEC2 cells; (D) a quantitative analysis was conducted to compare the cell membrane potential percentage between the different concentrations group; (E) a quantitative analysis was conducted to determine the ATP production of the 2 types of cells; (F) a Seahorse Extracellular Flux analysis was conducted to determine the bioenergy profiles of the cells; and (G) a quantitative analysis was conducted to determine the basal respiration, maximal respiration, ATP production, and proton leak. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. FITC-A, fluorescein isothiocyanate isomer I; ROS, reactive oxygen species; MMP, matrix metalloproteinase; ATP, adenosine triphosphate; OCR, oxygen consumption rate; AEC, alveolar epithelial cell.

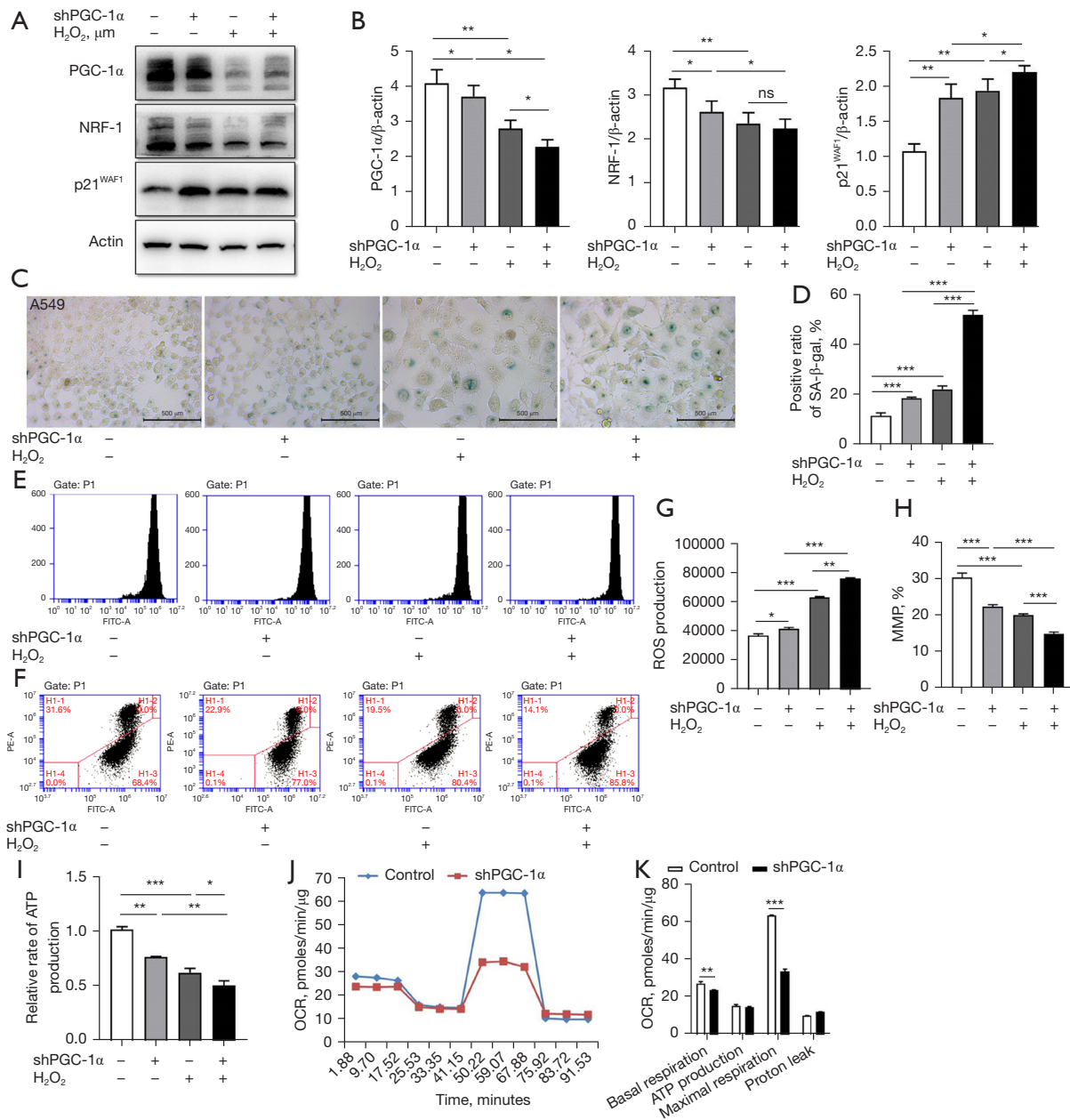


Figure 4 The mitochondrial function and the senescence expression of the A549 cells were verified after PGC-1 α expression was knocked down. The following experiments were performed after the PGC-1 α gene was knocked down in the A549 cells transfected with the shPGC-1 α lentivirus: (A) A Western blot analysis was conducted to detect the expression of PGC-1 α , NRF-1, and p21^{WAF1} in the A549 cells after PGC-1 α was knocked down; (B) a quantitative analysis was conducted of the Western blot results; (C) SA- β -gal staining of the A549 cells treated with different concentrations of hydrogen peroxide was performed ($\times 100$); (D) a quantitative analysis of the SA- β -gal staining in the A549 cells was performed; (E) flow cytometry was used to detect the ROS levels in the A549 cells; (F) flow cytometry was used to detect the mitochondrial membrane potential of the A549 cells; (G) a quantitative analysis of the ROS products was conducted; (H) a quantitative analysis of the cell membrane potential percentage was conducted; (I) a quantitative analysis of the ATP production of the A549 cells was conducted; (J) a Seahorse Extracellular Flux analysis was conducted to measure the bioenergy profiles of the cells; and (K) a quantitative analysis was conducted to examine basal respiration, maximal respiration, ATP production, and proton leak. β -actin served as an internal parameter. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, no significance. PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 alpha; NRF-1, nuclear respiratory factor-1; shPGC-1 α , knockdown PGC-1 α (shPGC-1 α); SA- β -gal, senescence-associated beta-galactosidase; PE-A, phycoerythrin; FITC-A, fluorescein isothiocyanate isomer I; ROS, reactive oxygen species; MMP, matrix metalloproteinase.

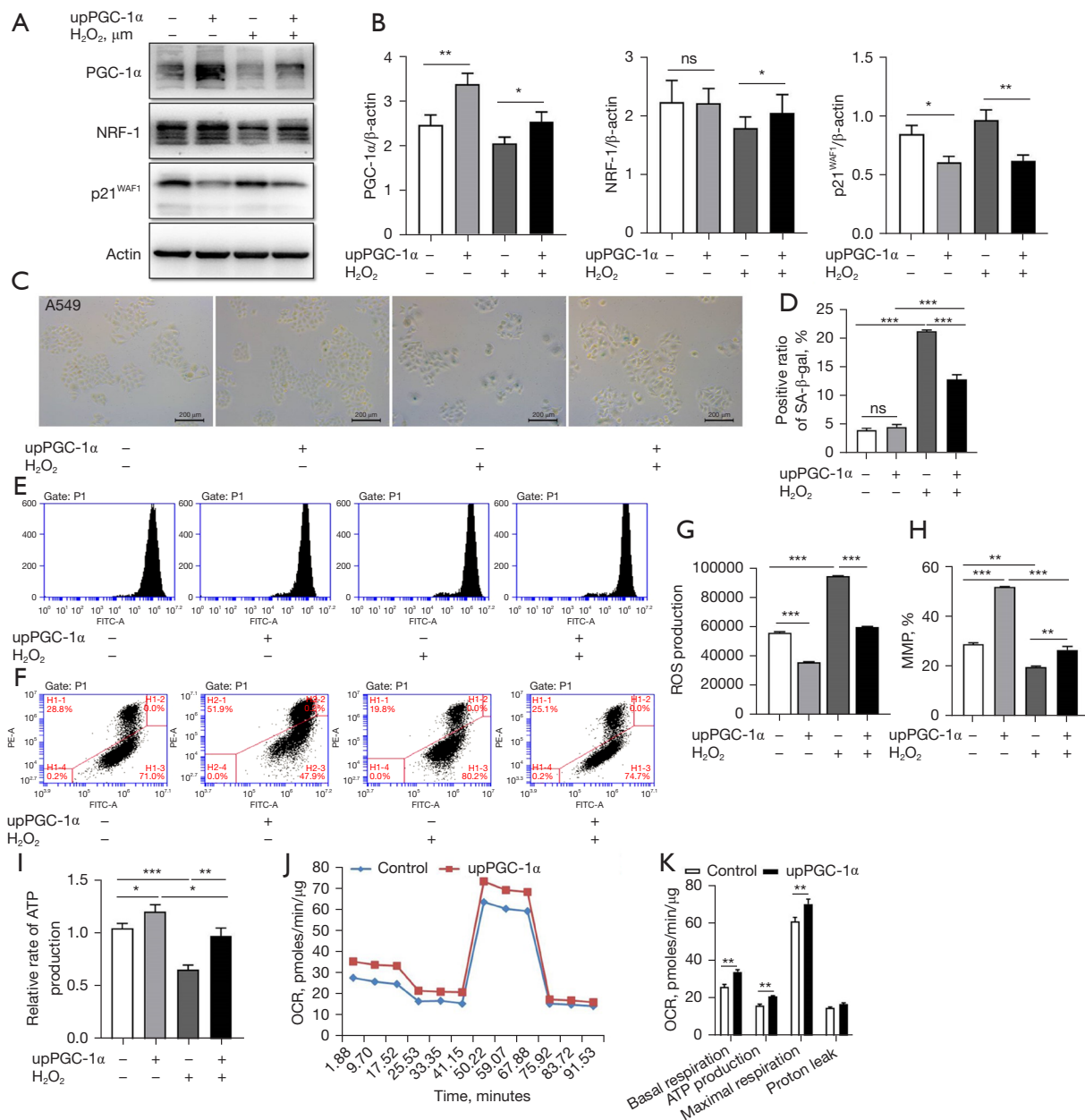


Figure 5 The mitochondrial function and senescence expression of the A549 cells were verified after the overexpression of the upPGC-1 α . After the PGC-1 α gene was overexpressed in the A549 cells transfected with the upPGC-1 α lentivirus, the following experiments were conducted: (A) Western blot was used to detect the expression of PGC-1 α , NRF-1, and p21^{WAF1} in the A549 cells after PGC-1 α was overexpressed; (B) a quantitative analysis of the Western blot experiment results was conducted; (C) SA- β -gal staining of the A549 cells treated with different concentrations of hydrogen peroxide was performed ($\times 100$); (D) a quantitative analysis of the SA- β -gal staining in the A549 cells was conducted; (E) flow cytometry was used to detect ROS in the A549 cells; (F) flow cytometry was used to detect the mitochondrial membrane potential of the A549 cells. (G) a quantitative analysis of the ROS products was conducted; (H) a quantitative analysis of the cell membrane potential percentage was conducted; (I) a quantitative analysis of the ATP production of the A549 cells was conducted; (J) a Seahorse Extracellular Flux analysis was conducted to measure the bioenergy profiles of the cells; and (K) a quantitative analysis was conducted to examine basal respiration, maximal respiration, ATP production, and proton leak. β -actin served as an internal parameter. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, no significance. PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 alpha; NRF-1, nuclear respiratory factor-1; upPGC-1 α , over express PGC-1 α ; FITC-A, fluorescein isothiocyanate isomer I; ROS, reactive oxygen species; MMP, matrix metalloproteinase; ATP, adenosine triphosphate; OCR, oxygen consumption rate.

Effects of the PGC-1 α agonist ZLN005 on the mitochondrial function improvement of the H₂O₂ cell aging model and the protective effect of AEC aging

ZLN005, a PGC-1 α transcriptional activator, upregulates PGC-1 α in skeletal muscle myotubes, but not in AECs. Because the action of ZLN005 appears cell-specific, the effect of ZLN005 on AECs was not investigated. The AECs were treated with 10 μ M of ZLN005 for 24 h, and a significant increase in PGC-1 α protein levels was observed. ZLN005 has been reported to be effective in protecting retinal pigment epithelium cells from cytotoxic oxidative damage (33). We sought to investigate whether ZLN005 improves mitochondrial function in a PGC-1 α -dependent manner and reduces oxidation-induced cellular senescence. We first pretreated the serum-free AECs with ZLN005 for 24 h, and then treated them with H₂O₂ to establish the cell senescence model. The results showed that the PGC-1 α agonist ZLN005 significantly decreased the expression of p21^{WAF1} and increased the expression of NRF-1 (Figure 6A,6B). The positive rate of the SA- β -gal staining was decreased. The immunofluorescence assays showed that the volume and mitochondrial morphology of the A549 senescent cells were more improved in the drug treatment group than the H₂O₂ group, and the expression of the PGC-1 α protein was increased in the A549 cells, while the expression of p21^{WAF1} protein was decreased (Figure 6C-6F). The mitochondrial function experiment results showed that the ZLN005 treatment improved the mitochondrial function of the AECs, which showed increased ATP production, a decreased ROS level, and increased MMP. The results of the mitochondrial pressure test showed that the basal OCR and maximum OCR of the aging A549 cells in the drug treatment group were significantly higher than those in the H₂O₂ group (Figure 6G-6M).

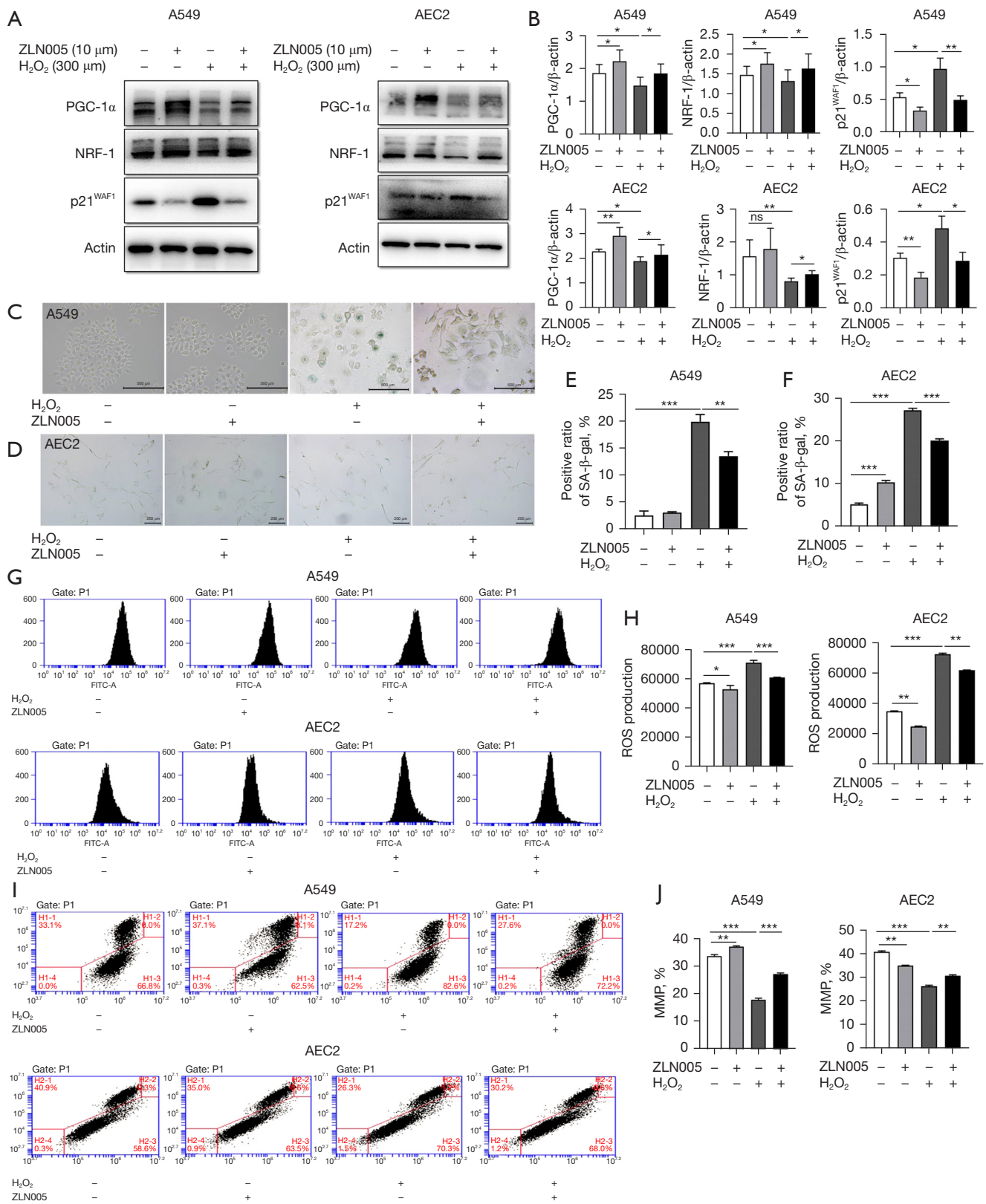
To determine if this protection against cell senescence was dependent on ZLN005's upregulation of PGC-1 α , the A549 cells with reduced PGC-1 α (shPGC-1 α) activity and the associated control (shControl) cells were exposed to H₂O₂. When the A549 cells with reduced PGC-1 α gene expression were exposed to H₂O₂ to induce the cell senescence model, the differences in NRF-1 and p21^{WAF1} between the two groups were insignificant after PGC-1 α agonist ZLN005 treatment (Figure 7A,7B). Additionally, there was no significant difference in the positive rate of the SA- β -gal staining (Figure 7C,7D). The mitochondrial function test results showed that the drug treatment group did not improve the mitochondrial function of the shPGC-1 α

A549 cells, which showed ATP production, and there was no statistically significant difference in the ROS and MMP levels. The results of the mitochondrial pressure test showed that the maximum OCR of the aging A549 cells of the drug treatment group was significantly decreased compared to that of the H₂O₂ group (Figure 7E-7K). Pretreatment with ZLN005 did not protect the shPGC-1 α cells from the H₂O₂-mediated induction of cell senescence but did cause a decrease in the shControl cells, which suggests that PGC-1 α is required for ZLN005 anti-senescence function.

Discussion

Overall, a decrease in PGC-1 α expression in the lung tissues of patients with IPF was found. The expression of PGC-1 α and mitochondrial synthetic protein NRF-1 in the senescent cells was decreased, and the morphology of the cell mitochondria was abnormal consistent with mitochondrial dysfunction. PGC-1 α participates in the senescence of AECs by regulating the morphology and function of the mitochondria. The PGC-1 α agonist ZLN005 improved mitochondrial function and reduced the cell senescence induced by H₂O₂. After the expression of the PGC-1 α gene was reduced, the protective effect of the drug was weakened, which demonstrates that effect of ZLN005 depends on PGC-1 α , and the molecular mechanism of such induction is dependent on increasing the expression of PGC-1 α gene targets that promote mitochondrial biogenesis and function. Further research involving the *in vivo* testing of ZLN005 in the lung is necessary but the current study suggests ZLN005 has a potential therapeutic effect against IPF pathogenesis.

This study had a number of limitations. First, only a preliminary conclusion can be drawn. As is well known, IPF is an irreversible, continuous, and destructive ILD (36). It is characterized by damage to AEC and the transformation of fibroblasts and myofibroblasts (37,38). Fibroblast differentiation, collagen deposition, and epithelial to mesenchymal transition eventually lead to irreversible progressive respiratory insufficiency (1,39,40). The pathogenesis of IPF is not yet fully understood, increasingly, the accelerated aging mechanism, including cell senescence, is thought to play an important role in the pathogenesis of IPF (15,19). Through *in vivo* and *in vitro* experiments, we studied the mechanism of PGC-1 α involved in the aging of AECs by regulating mitochondrial morphology and



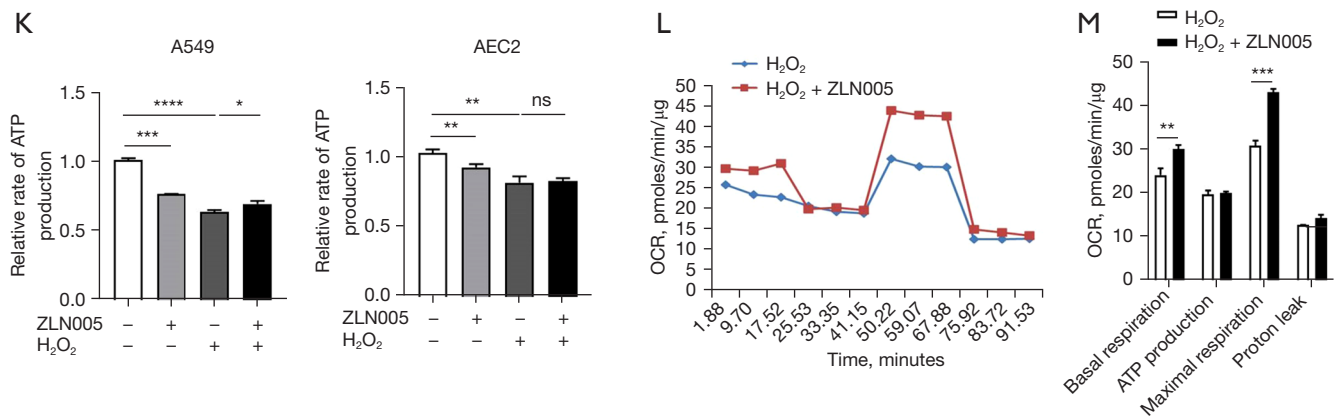
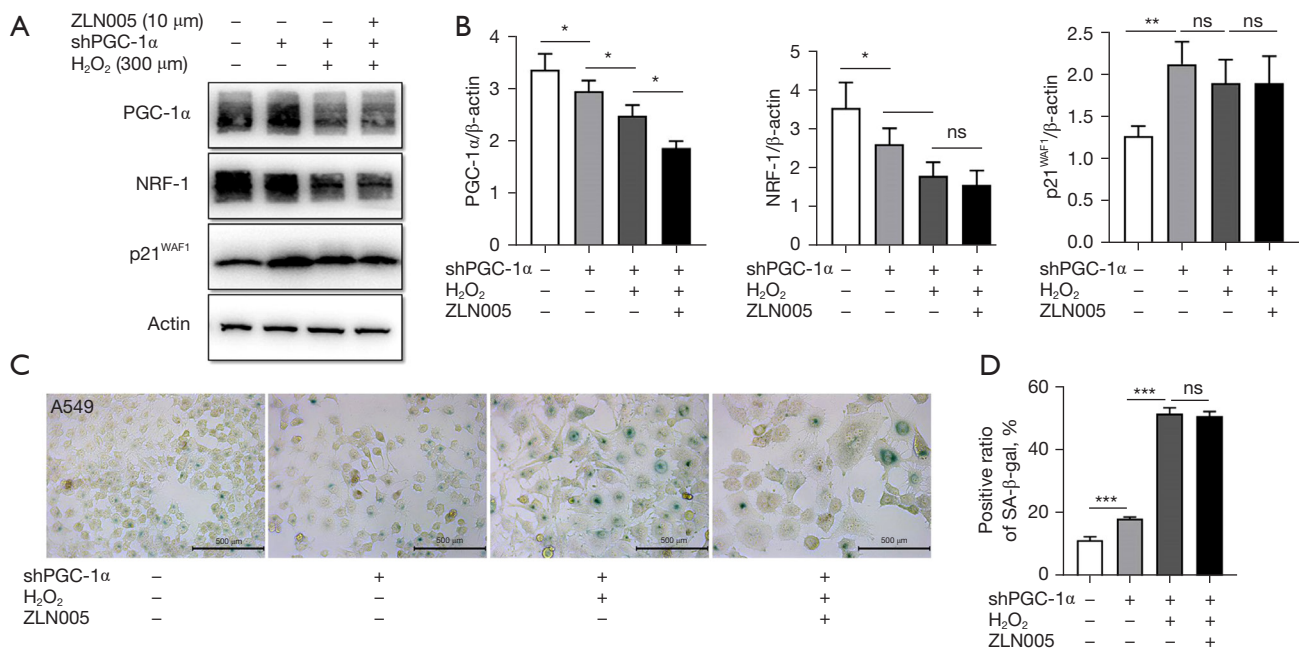


Figure 6 ZLN005 alleviates H₂O₂-induced cell senescence by enhancing the expression of PGC-1 α and improving mitochondrial function. The AECs were cultured in serum-free medium; the cells were pretreated with ZLN005 for 24 h, and were then treated with H₂O₂ before the following experiments were performed: (A) Western blot was used to detect the expression of PGC-1 α , NRF-1, and p21^{WAF1} in the AECs; (B) a quantitative analysis of the Western blot experiment results was conducted; (C,D) SA- β -gal staining of the AECs treated with different concentrations of hydrogen peroxide was performed ($\times 100$); (E,F) a quantitative analysis of the SA- β -gal staining in the AECs was conducted; (G) flow cytometry was used to detect the ROS of the AECs; (H) a quantitative analysis of the ROS products was conducted; (I) flow cytometry was used to detect the mitochondrial membrane potential of the AECs; (J) a quantitative analysis of the cell membrane potential percentage was conducted; (K) a quantitative analysis of the ATP production of the AECs was conducted; (L) a Seahorse Extracellular Flux analysis was conducted to measure the bioenergy profiles of the A549 cells; and (M) a quantitative analysis was conducted to examine basal respiration, maximal respiration, ATP production, and proton leak. β -actin served as an internal parameter. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, no significance. PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 alpha; NRF-1, nuclear respiratory factor-1; AEC, alveolar epithelial cell; FITC-A, fluorescein isothiocyanate isomer I; ROS, reactive oxygen species; MMP, matrix metalloproteinase; ATP, adenosine triphosphate; OCR, oxygen consumption rate; SA- β -gal, senescence-associated beta-galactosidase.



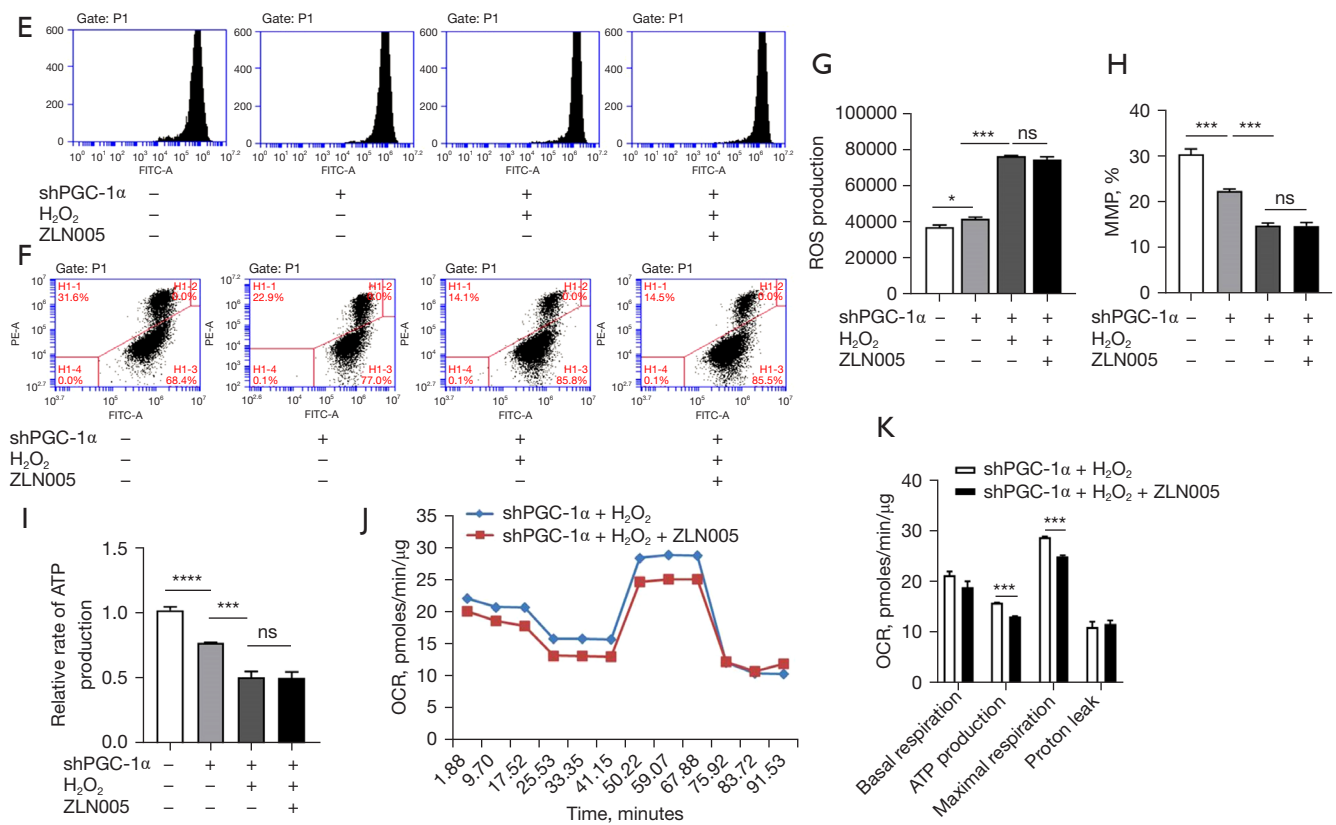


Figure 7 The protective effect of agonist ZLN005 on cell senescence was weakened after the expression of PGC-1 α gene was knocked down. The expression of PGC-1 α in the A549 cells was knocked down, the ZLN005 cells were pretreated for 24 hours, and were then treated with H₂O₂, and the following experiments were conducted: (A) Western blot was used to detect the expression of PGC-1 α , NRF-1, and p21^{WAF1} in the shPGC-1 α cells; (B) a quantitative analysis of the Western blot experiment results was conducted; (C) SA- β -gal staining of the A549 cells treated with different concentrations of hydrogen peroxide was performed ($\times 100$); (D) a quantitative analysis of the SA- β -gal staining in the A549 cells was conducted; (E) flow cytometry was used to detect ROS in the A549 cells; (F) flow cytometry was used to detect the mitochondrial membrane potential of the A549 cells; (G) a quantitative analysis of the ROS products was conducted; (H) a quantitative analysis of the cell membrane potential percentage was conducted; (I) a quantitative analysis of the ATP production of the A549 cells was conducted; (J) a Seahorse Extracellular Flux analysis was conducted to measure the bioenergy profiles of the A549 cells; and (K) a quantitative analysis was conducted to determine basal respiration, maximal respiration, ATP production, and proton leak. β -actin served as an internal parameter. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, no significance. PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 alpha; NRF-1, nuclear respiratory factor-1; SA- β -gal, senescence-associated beta-galactosidase; PE-A, phycoerythrin; FITC-A, fluorescein isothiocyanate isomer I; ROS, reactive oxygen species; MMP, matrix metalloproteinase; ATP, adenosine triphosphate; OCR, oxygen consumption rate.

function and explored the protective effect of PGC-1 α agonist ZLN005 on aging AECs.

In recent years, there has been increasing evidence that IPF is an epithelial-driven disease. The expression of protein senescence markers, including p16, p21, and p53, are significantly increased in IPF lung tissues, especially in AECs (14,41). The senescence of AEC maybe the initiating factor promoting fibrosis in IPF (42,43). This

study found the expression of PGC-1 α and mitochondrial synthesis protein NRF-1 was decreased in the lung tissues of the IPF patients and BLM-induced pulmonary fibrosis model mice. The expression of aging-related protein p21^{WAF1} was increased, which suggests that cellular aging and mitochondrial function changes are involved in the pathogenesis of IPF.

Recent evidence suggests that mitochondrial dysfunction

plays an important role in the occurrence and development of many human diseases, including chronic lung diseases (22). AEC2 cells in the lungs of IPF patients show significant malformation and dysfunctional mitochondrial accumulation (22,44,45). Mitochondria are important sources of energy supply in cells and play an important role in maintaining homeostasis. It has been found that mitochondria also participate in the regulation of cell growth and the cell cycle, cell differentiation, cell information transmission, apoptosis, and other processes, providing energy for cells. The homeostasis and normal function of mitochondria are crucial for the normal physiological state of different tissues and organs (8).

Morphological changes of damaged mitochondria and the increase of mitochondrial ROS were detected in the IPF AECs, which can lead to the oxidative damage of intracellular DNA, lipids and proteins, and accelerate cell senescence (22). Mitochondria regulate cell function not only through energy generation, but also through cell phenotypes associated with aging. A study has shown that the mechanisms of mitochondrial dysfunction that cause aging include increased levels of ROS and changes in the NAD/NADH ratio (11). Other mitochondrial changes that promote cell aging are abnormal mitochondrial dynamics, defects in oxidative phosphorylation, and disorders of calcium homeostasis (46,47). Consistent with the above view, we found that the expression of PGC-1 α and mitochondrial synthesis protein NRF-1 was decreased in the AECs induced by H₂O₂. At the same time, abnormal cellular mitochondrial morphology and mitochondrial dysfunction were observed, which were manifested by decreased ATP production, increased ROS levels, decreased MMP, and decreased mitochondrial respiratory function.

PGC-1 α is a transcriptional regulator that induces oxidative stress and regulates oxidative and antioxidant processes. PGC-1 α induces oxidative stress and destroys insulin secretion by activating adenosine monophosphate (AMP)-activated protein kinase, leading to obesity (48). PGC-1 α signal transduction ameliorates myocardial apoptosis induced by myocardial ischemia/reperfusion. Patients with higher levels of PGC-1 α showed improvements in lung and heart function (49). The increased expression of PGC-1 α may prevent p53-induced cell death by maintaining a balance between oxidative phosphorylation and glycolysis, thereby preventing the development of tumors (50). Hypoxia reduces the expression of PGC-1 α and causes mitochondrial dysfunction, while also significantly reducing the efficiency

of oxidative phosphorylation, resulting in membrane potential decomposition, increased ROS production, the depletion of ATP, and reductions in the mechanism of uncoupled oxidative phosphorylation, leading to endothelial dysfunction (45).

Our study confirmed a decline in PGC-1 α expression in the AECs in the lung tissues of patients with IPF. PGC-1 α was also found to be involved in AEC senescence by regulating mitochondrial morphology and function. After PGC-1 α gene expression was knocked down in the A549 cells, cell senescence was aggravated, mitochondrial synthesis protein NRF-1 expression was decreased, and mitochondrial morphology was abnormal and there was functional dysfunction. However, after the upregulation of PGC-1 α gene expression, cell senescence was reduced, mitochondrial synthesis protein NRF-1 expression was increased, and mitochondrial morphology and function were restored. The agonist of PGC-1 α (i.e., ZLN005) alleviated H₂O₂-induced cell senescence by enhancing the expression of PGC-1 α and improving mitochondrial function; however, the protective effect of this drug was weakened after the expression of the PGC-1 α gene was reduced.

ZLN005, an agonist of PGC-1 α appears to work by an upregulation of PGC-1 α activity and providing a protective effect of mitochondrial function in AECs. These effects could provide a role in the treatment of IPF. However, this study only represents the first small step in the exploration of the mechanism and potential efficacy of ZLN005. For the clinical treatment of IPF patients, more in-depth investigations are needed to support these preliminary findings.

Conclusions

PGC-1 α appears to be involved in AEC senescence by regulating mitochondrial morphology and function. We found an agonist of PGC-1 α (i.e., ZLN005) alleviated H₂O₂-induced cell senescence by enhancing the expression of PGC-1 α and improved mitochondrial function. However, after the expression of the PGC-1 α gene was reduced, the protective effect of this drug was weakened. Thus, ZLN005 could become a potential treatment for IPF.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-23-815/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The animal experiment was performed in the Medical School of Nanjing University [No. SYXK(SU)2019-0056], in compliance with Nanjing University guidelines for the care and use of animals. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Written informed consent was obtained from all the subjects in the study, and the study was approved by the Ethics Committee of Nanjing Drum Tower Hospital of Medical School of Nanjing University (No. 2021-390-01).

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