Angiopoietin-like 4 knockdown attenuates cigarette smoke extract-induced oxidative stress and apoptosis in lung bronchial epithelial cells by inhibiting NADPH oxidase

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Received 28 March 2022; Accepted 9 May 2022
Available online: 1 September 2022

\textbf{KEYWORDS}
ANGPTL4; lung bronchial epithelial cells; NAPDH oxidase; cigarette smoke extract; oxidative stress

\textbf{Abstract}
It has been found that angiopoietin-like 4 (ANGPTL4) expression is increased in the serum of patients with chronic obstructive pulmonary disease (COPD). Herein, cigarette smoke extract (CSE) was used to stimulate oxidative stress in bronchial epithelial cells BEAS-2B, and the role and potential mechanism of ANGPTL4 in smoking-induced lung dysfunction were explored. The roles of different concentrations of CSE (0, 1, 2.5, 5, or 10%) in cell viability and ANGPTL4 levels were evaluated. Following ANGPTL4 being knocked down, the effects of ANGPTL4 knockdown on oxidative stress and apoptosis were determined. Moreover, the level of NADPH oxidase 2 (NOX2) was upregulated to assess the mediated role of NOX in the regulation of ANGPTL4, along with JNK/p38 MAPK signaling. CSE treatment elevated the level of ANGPTL4, and ANGPTL4 knockdown reduced CSE-induced oxidative stress, apoptosis, and NOX level in BEAS-2B cells. The greatest degree of alteration was found in NOX2, and additional NOX2 overexpression broke the inhibitory influences of ANGPTL4 knockdown on oxidative stress and apoptosis. Otherwise, ANGPTL4 knockdown hindered the activation of JNK/p38 MAPK signaling, whereas NOX2 overexpression activated this signaling pathway. Together, ANGPTL4 knockdown attenuated CSE-induced oxidative stress, apoptosis, and activation of JNK/MAPK signaling by inhibiting NOX.

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic bronchitis, chronic airway obstruction, and emphysema, which can lead to a progressive and irreversible decline in lung function.1,2 Emphysema is the major pathological diagnosis of COPD affecting the distal space of terminal bronchioles and is associated with mechanisms such as irregular inflammatory responses to toxic particles or gases, and oxidative imbalance.3 COPD is a widespread respiratory disease worldwide, which can drive numerous hospitalizations and deaths annually, and increase the medical burden on individuals and society.4 Epidemiological and genetic risk factors for COPD include chronic smoking, chronic exposure to air pollution, respiratory infections, biofuel fumes, and occupational dust inhalation.5 Therein, smoking is one of the most important risk factors for the development of COPD,4 and its damage to bronchial epithelial cells is produced through various complex mechanisms, of which oxidative stress is a noticeable part.6 Because cigarette smoke is a key inducer of reactive oxygen species (ROS) in the respiratory tract, inhalation causes oxidative stress, and an increasing number of apoptotic and necrotic lung cells.7 Meanwhile, it leads to depletion of antioxidants, downregulation of antioxidant pathways, and further induction of the development of chronic airway disease.9,10

Full-length angiopoietin-like protein 4 (ANGPTL4), also known as a fasting-induced adipokine, is cleaved into different domains by different protein convertases in different tissues and organs and exerts different functions.11 ANGPTL4 is an important protein that regulates glucose metabolism, lipid metabolism, and insulin sensitivity, and is mainly expressed in organs that are rich in epithelial components and relevant to lipid metabolism.12,13 In small intestinal epithelial cells, ANGPTL4 is thought to be involved in short-chain fatty acid-induced gut microbiota regulation, inhibiting fatty acid uptake by macrophages and reducing oxidative stress.14 In retinal melanocytes and skin epithelial cells, ANGPTL4 is involved in the regulation of high glucose-induced retinopathy15 and wound healing.16 ANGPTL4, on the other hand, exhibits pro-inflammatory effects in adipocytes, brain tissue, and models of coronary atherosclerosis.17,18 In the respiratory system, abnormally high expression of ANGPTL4 is thought to be associated with lung cancer progression.19 Furthermore, RNA sequencing of mouse type 2 alveolar epithelial cells reveals that ANGPTL4 is a novel downstream target of the mTOR pathway, which may be associated with the etiology of pulmonary fibrosis.20 Novelty, it has been found that ANGPTL4 expression is increased in the serum of patients with COPD,21 and anti-ANGPTL4 antibody treatment can reduce pulmonary edema and damage in secondary pneumococcal pneumonia.22 Based on the fact that smoking is a risk factor for COPD, we hypothesized that downregulation of ANGPTL4 levels might alleviate smoking-induced lung damage.

In this study, cigarette smoke extract (CSE) was used to stimulate oxidative stress in bronchial epithelial cells BEAS-2B, and the role and potential mechanism of ANGPTL4 in this type of lung dysfunction were explored. This paper expands the pathogenesis of COPD, and the disease can be alleviated by the development of specific ANGPTL4 inhibitors.

Methods

Cell culture and treatment

The BEAS-2B cells were purchased from the American Type Culture Collection. Cells were cultured in DMEM (Gibco) containing 10% FBS (Solarbio, Beijing) and 1% penicillin-streptomycin, and maintained in an incubator with 5% CO₂ at 37°C. CSE was acquired as previously described.24 Cigarette smoke was inhaled into a glass syringe containing 10 mL of serum-free DMEM. After filtration, it was titrated with sodium hydroxide to pH 7.35-7.45. The prepared liquid was regarded as a 100% concentration of CSE. It was diluted further with DMEM to obtain the appropriate concentration (1, 2.5, 5, or 10%). Cells were treated with CSE for 24 h. Untreated cells were the control.

Cell transfection

BEAS-2B cells were transfected with small interfering (si) RNA to knock down ANGPTL4, and the nontargeted siRNA was used as the negative control (NC). Cells were transfected with the pcDNA3.1 vector to overexpress NOX2, and the empty vector (GenePharma, Shanghai, China) was used as the NC. Steps were performed according to the instructions of the FuGENE HD reagent (Roche, Shanghai).

CCK-8

BEAS-2B cells (5 × 10³/well) were seeded into 96-well plates and cultured in an incubator containing 5% CO₂ at 37°C overnight. To examine the effect of CSE on cell survival, CSE (0, 1, 2.5, 5, or 10%) was used to treat the cells at 37°C for 24 h. Viable cells were measured after the addition of 10 µl of CCK-8 solution at 37°C for another 2 h. Optical density (OD) was measured at a wavelength of 450 nm using a microplate reader (Hiwell-Diatek, Wuxi, China).

RT-qPCR

Total RNA was isolated from cells using TRIzol® reagent (Thermo Fisher Scientific), and cDNA was primed using a Reverse Transcriptase kit (Thermo Fisher Scientific). The reverse transcription product was diluted and RT-qPCR was performed using a QuantiTect® SYBR Green PCR kit (Qiagen). The ΔΔCT method was used to analyze the data. β-actin was used for normalization.

Western blotting

Protein extraction was performed and a BCA method was used for protein quantification. Ten per cent SDS-PAGE was performed to separate protein samples (25 µg/lane), and the separated protein was then transferred to a PVDF membrane, followed by incubation in blocking fluid. Later, the blots were incubated with primary antibodies, and the strips were incubated at room temperature with an HRP-conjugated secondary antibody. ECL was performed for...
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Statistics analysis

GraphPad Prism 8.0 (GraphPad Software, Inc.) was used for statistical analysis. Data are presented as the mean ± SD. Statistical significance was determined via one-way ANOVA with Tukey’s post-hoc test. All experiments were carried out three times. P < 0.05 was considered to be statistically significant.

Results

ANGPTL4 knockdown attenuates CSE-induced oxidative stress

The effects of different concentrations of CSE (0, 1, 2.5, 5, or 10%) on cell viability were evaluated using a CCK-8 assay. The viability of BEAS-2B cells decreased with the increase in CSE concentration in a concentration-dependent manner (Figure 1A). Meanwhile, RT-qPCR and western blotting were used to determine the expression level of ANGPTL4 in each group of cells. The results displayed that the ANGPTL4 level also increased in a concentration-dependent manner with the concentration of CSE (Figure 1B-C). To investigate the role of ANGPTL4 in BEAS-2B cells, the expression of ANGPTL4 was knocked down through transfection, which could be proved from the results of RT-qPCR and western blotting (Figure 2A-B). The siRNA-ANGPTL4-1 group was selected for the following assays due to the lower ANGPTL4 level. BEAS-2B cells and transfected cells were exposed to 10% CSE, and the levels of ROS, MDA, SOD, GSH, and CAT were determined using the corresponding assay kits. CSE treatment markedly improved the level of ROS, whereas ANGPTL4 knockdown could prevent the ROS level from rising (Figure 2C). The level of MDA represented a similar

Oxidative stress indices

The determination of ROS was performed using a DCFH-DA assay kit (E004-1-1). Diluted DCFH-DA was added to the wells and cells were incubated for 30 min. Next, cells were collected and washed with PBS twice. Following centrifugation at 1000 rpm/min for 5 min, the supernatant was removed, and the cells were suspended in PBS for detection. The results were photographed under a fluorescence microscope (>100; Olympus Corporation). Malondialdehyde (MDA; A003-4-1), SOD (A001-1), GSH (A005-1), and catalase (CAT, A007-1-1, JianCheng, Nanjing) assay kits were used to assess the level of the corresponding function indices. Briefly, BEAS-2B cells were collected and lysed, and samples were obtained according to the different requirements of the kits. The OD values were measured using a microplate reader.

TUNEL

BEAS-2B cells (5 × 105/well) were seeded in a 24-well plate and cultured until they reached 80% confluence. They were then treated with 10% CSE for 24 h. The cell smears were immersed in the fixative solution and fixed for 30 min, followed by washing with PBS. Prepared working fluid in the TUNEL assay kit (cat. no. E-CK-A334; Elabscience) was added according to the instructions. The coverslip was removed, sealed, and observed under a fluorescence microscope (>200).

Figure 1 CSE treatment elevates ANGPTL4 expression level. (A) The effects of different concentrations of CSE (0, 1, 2.5, 5, or 10%) on cell viability were evaluated using a CCK-8 assay. (B) The expression level of ANGPTL4 in each group of cells was determined using RT-qPCR and (C) western blotting. *P < 0.05, **P < 0.01, ***P < 0.001 versus 0% CSE.
trend to the ROS level, whereas SOD, GSH, and CAT levels represented the opposite trend. They were increased following the CSE treatment and elevated when ANGPTL4 was knocked down (Figure 2D).

**ANGPTL4 knockdown attenuates CSE-induced cell apoptosis**

Afterward, cell apoptosis was assessed using the TUNEL assay and western blotting. The fluorescence emitted by apoptotic cells in the CSE treatment group was significantly enhanced compared to that in the control group. The fluorescence in the ANGPTL4 knockdown group was significantly weakened compared to the NC group (Figure 3A-B). In addition, the expression level of Bcl2 declined in the CSE treatment and partly increased when ANGPTL4 was knocked down. The alterations in the Bax and cleaved caspase 3 levels in the CSE treatment group and the CSE+siRNA-ANGPTL4 group were both contrary to the Bcl2 level (Figure 3C).

**ANGPTL4 knockdown suppresses the expression of NADPH oxidase**

Thereafter, the expression levels of NOX1, NOX2, and NOX4 were assessed with RT-qPCR and western blotting. With the increase in CSE concentration, their levels were all elevated and concentration-dependent (Figure 4A-B). Moreover, the impacts of ANGPTL4 knockdown on their levels were also assessed. ANGPTL4 knockdown significantly declined their levels compared with the NC group (Figure 4C-D).

**ANGPTL4 knockdown attenuates CSE-induced oxidative stress and apoptosis by inhibiting NOX2**

Therein, the greatest degree of change was found in NOX2. To highlight the influence of NOX on cells, NOX2 was overexpressed through transfection. Its overexpression was confirmed with RT-qPCR and western blotting (Figure 5A-B). Subsequently, the levels of the aforementioned oxidative
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Figure 3 ANGPTL4 knockdown attenuates CSE-induced cell apoptosis. (A) Cell apoptosis was assessed using a TUNEL assay. (B) Semi-quantitative result of TUNEL assay. (C) The expression levels of apoptosis-related proteins were determined with western blotting. ***P < 0.001 versus Control; ##P < 0.01, ###P < 0.001 versus 10% CSE + siRNA-NC.

stress indices were evaluated. The results indicated that NOX2 overexpression reversed the effects of ANGPTL4 knockdown on the ROS, MDA, SOD, GSH, and CAT levels (Figure 5C-D). Otherwise, the influence of NOX2 overexpression on cell apoptosis was also determined. The result of the TUNEL assay revealed that NOX2 overexpression promoted the apoptosis of BEAS-2B cells, reversing the inhibitory role of ANGPTL4 knockdown in the apoptosis (Figure 6A-B). Consistently, the western blotting results demonstrated that the Bcl2 level was dropped whereas Bax and cleaved caspase 3 levels were elevated following NOX2 was overexpressed, which provided evidence that NOX2 overexpression promoted cell apoptosis (Figure 6C).

**ANGPTL4 knockdown blocks the JNK/p38 MAPK signaling by inhibiting NOX2**

Furthermore, the roles of ANGPTL4 knockdown and NOX2 overexpression in the JNK/p38 MAPK signaling were assessed using western blotting. Single CSE treatment elevated the expression level of phosphorylated (p)-JNK and p-p38 MAPK, and additional ANGPTL4 knockdown significantly reduced their level. In addition, combined NOX2 overexpression partially reversed the suppressive effects of ANGPTL4 knockdown, and the levels of p-JNK and p-p38 MAPK manifested an upward trend (Figure 7).

**Discussion**

COPD is characterized by persistent airflow limitation that develops progressively with increased chronic inflammatory responses in the airways and lungs to noxious particles or gases, and smoking is a conspicuous risk factor as described above. It is estimated that by 2030, smoking-related deaths will reach 8 million per year. Cigarette smoke contains complex components such as nicotine, tar, etc., as well as plenty of carcinogenic compounds, which cause lung dysfunction through their overall effect.
Figure 4  ANGPTL4 knockdown suppresses the expression of NAPDH oxidase. (A) The expression levels of NOX1, NOX2, and NOX4 were assessed with RT-qPCR and (B) western blotting. (C) The impacts of ANGPTL4 knockdown on NOX1, NOX2, and NOX4 levels were assessed with RT-qPCR and (D) western blotting. *P < 0.05, **P < 0.01, ***P < 0.001 versus 0% CSE or Control; ###P < 0.001 versus 10% CSE + siRNA-NC.

Cigarette smoke not only stimulates airway mucus gland hyperplasia and hypersecretion but also causes apoptosis, shedding, and bronchial smooth muscle cell proliferation. It can also stimulate the activation of macrophages and neutrophils in the lungs, leading to chronic airway inflammation. In this study, a CSE-induced cell damage model was constructed to simulate the pathogenesis of COPD caused by smoking and to explore the regulatory role of ANGPTL4 in oxidative stress and apoptosis of bronchial epithelial cells. We found that ANGPTL4 expression was increased in CSE-treated BEAS-2B cells, and declined ANGPTL4 expression alleviated the increased ROS levels in BEAS-2B cells induced by CSE exposure. ROS is a variety of chemical molecules released from activated inflammatory cells, including superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl radical (-OH),...
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Figure 5  ANGPTL4 knockdown attenuates CSE-induced oxidative stress by inhibiting NOX2. (A) NOX2 was overexpressed through transfection and the efficacy was confirmed with RT-qPCR and (B) western blotting. (C) The levels of ROS, (D) MDA, SOD, GSH, and CAT were determined using the corresponding assay kits. ***P < 0.001 versus Ov-NC or Control; ###P < 0.001 versus 10% CSE; @@P < 0.01, @@@P < 0.001 versus 10% CSE + siRNA-ANGPTL4 + Ov-NC.

and nitric oxide (NO). NOX reduces oxygen molecules in vivo to superoxide anion through NADPH-dependent one-electron reduction, which is the primary source of ROS and the only enzyme that directly generates ROS in the body. ROS catalyzed by NOX plays an important role in the intracellular oxidative stress response and in accelerating pathological processes such as fibrosis, inflammation, and tumor formation. We speculate that the differences in their levels are due to the regulatory subunits in the activation of NOX2. Afterward, we found that NOX2 overexpression triggered intracellular oxidative stress, partially counteracting the protective effect of ANGPTL4 knockdown.

In addition, the inhibitory effect of AMPK on endoplasmic reticulum stress-mediated apoptosis has been demonstrated in various tissue cells, including bronchial epithelial cells HBEpCs. Therefore, we examined the related protein levels of the JNK/p38 MAPK pathway and found that CSE exposure promoted the phosphorylation of JNK and p38 proteins in cells, whereas NOX2 overexpression reversed the inhibition of signaling activation by ANGPTL4 knockdown. This suggests that ANGPTL4 activates ROS through NOX and further promotes JNK/p38MAPK-mediated apoptosis. Nevertheless, as a preliminary exploration, there are many areas to be improved in this experimental study. The occurrence of COPD is the result of the combined action of many internal and external factors. This study only clarifies the role of ANGPTL4 in the occurrence of COPD from a cytological point of view, which merits further confirmation with animal studies.
Figure 6  ANGPTL4 knockdown attenuates CSE-induced apoptosis by inhibiting NOX2. (A) The influence of NOX2 overexpression on cell apoptosis was determined with a TUNEL assay. (B) Semi-quantitative result of TUNEL assay. (C) The expression levels of apoptosis-related proteins were assessed using western blotting. ***P < 0.001 versus Control; ###P < 0.001 versus 10% CSE; @P < 0.05, @@@P < 0.001 versus 10% CSE + siRNA-ANGPTL4 + Ov-NC.

Figure 7  ANGPTL4 knockdown blocks the JNK/p38 MAPK signaling by inhibiting NOX2. The roles of ANGPTL4 knockdown and NOX2 overexpression in the JNK/MAPK signaling were assessed using western blotting. ***P < 0.001 versus Control; ###P < 0.001 versus 10% CSE; @P < 0.01 versus 10% CSE + siRNA-ANGPTL4 + Ov-NC.
Conclusion

In conclusion, ANGPTL4 knockdown attenuates COPD-induced oxidative stress, apoptosis, and activation of JNK/MAPK signaling by inhibiting NOX. It is hoped that these findings can promote the progression of COPD treatment.

References


