FGF10 mediates protective anti-oxidative effects in particulate matter-induced lung injury through Nrf2 and NF-κB signaling

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Background: Particulate matter (PM), a well-known environmental pollutant, is an independent risk factor associated with the morbidity of various respiratory diseases. Oxidative stress is an important pathophysiological mechanism related to PM exposure, which mediates redox-sensitive inflammatory signaling, leading to lung injury. Fibroblast growth factor 10 (FGF10), a paracrine fibroblast growth factor that mediates mesenchymal to epithelial signaling, participates in epithelial repair during lung injury. However, whether FGF10-mediated repair in PM-induced lung injury is related to the regulation of oxidative stress remains to be elucidated.

Methods: *In vivo*, the C57BL/6 mice were randomly divided, with intratracheal instillation of 5 mg/kg FGF10 1 h before 4 mg/kg PM for 2 consecutive days. *In vitro*, the BEAS-2B cells were pretreated with 10 ng/mL FGF10 before exposed to 200 μg/mL PM. Besides, the specific Nrf2 inhibitor ML385 was adopted *in vitro*. The harvested lung tissues were pathologic grading scored. The state of oxidative stress was assessed with dihydroethidium (DHE) staining, malondialdehyde (MDA) activity, hydrogen peroxide (H_2O_2) assays and reactive oxygen species (ROS). The contents of IL-6 and IL-8 in bronchoalveolar lavage (BAL) as well as culture supernatant were quantified by ELISA. The protein levels of nuclear factor erythroid 2 related factor 2 (Nrf2) and nuclear factor-κB (NF-κB) signaling from lung tissue as well as cell lysate were determined by Western blot.

Results: In this study, recombinant FGF10 administration relieved the degree of lung injury, which is characterized by bronchitis, in a mouse model of PM exposure. In addition, reduced ROS levels, which are indicative of restrained oxidative stress, were also observed. Moreover, two redox-sensitive signaling pathways, Nrf2 and NF-κB, were found to be differentially regulated by FGF10. Using a cellular model of PM exposure, we found that the anti-inflammatory effect of FGF10 on NF-κB signaling was mediated through the regulation of oxidative stress. The anti-oxidative effect relied on the stimulation of Nrf2 signaling. Blockade of Nrf2 signaling with ML385 significantly compromised the anti-inflammatory effect of FGF10.

Conclusions: These results underscore that the protective anti-oxidative effects of FGF10 in lung injury are mediated by the stimulation of Nrf2 signaling and inhibition of the NF-κB pathway.

Keywords: Particulate matter (PM); fibroblast growth factor 10 (FGF10); oxidative stress; nuclear factor-κB (NF-κB); nuclear factor erythroid 2 related factor 2 (Nrf2)

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Introduction

Industrialization and urbanization directly impact atmospheric pollution, which is recognized as a global environmental threat to human health. Regarded as the fundamental constituent of atmospheric pollutants, particulate matter (PM) poses a considerable health risk (1). As a chemical mixture of solid particles and liquid droplets, PM mainly consists of exhaust particles from vehicles and those released as a result of the incomplete combustion of coal. Due to their aerodynamics characteristics in the context of a functional lung, PM deposits in the bronchial and alveolar epithelium (2). The deposition of PM in the pulmonary epithelium inevitably leads to premature epithelial senescence and secretion of pro-inflammatory cytokines, which closely relates to the oxidative stress and lays the foundation for the pathological characteristics associated with PM-induced lung injury (3).

As documented in both epidemiological investigations and fundamental research, short- or long-term PM exposure is closely related to the morbidity and mortality of individuals suffering from various respiratory diseases, such as bronchitis and alveolitis (4). However, the molecular mechanism underlying the toxic effects of PM has not yet been fully elucidated. Besides symptomatic management such as inhalation of budesonide, there are only partially effective clinical interventions for protecting the lung from the harmful effects of PM. Considering the continuous exposure to PM in our daily lives and the heavy socio-economic burden of respiratory diseases, further investigation of the molecular mechanism mediating the deleterious effects of PM is urgently required to identify novel preventive and therapeutic strategies.

Oxidative stress, which is characteristic of an imbalance between the intrinsic oxidation and anti-oxidation systems, is a typical cellular response to PM and is reported as a pivotal pathophysiological event in PM-induced lung injury (5). As the first mechanical and immunological surface proximal to PM exposure, the pulmonary epithelium is the primary source of the overproduction of reactive oxygen species (ROS). ROS excess not only drives lipid peroxidation and protein denaturation, which mediates the cytotoxic effects of PM leading to premature epithelial senescence, but also functions as an essential regulator of redox-sensitive signaling, contributing to secretion of pro-inflammatory cytokines (6).

Nuclear factor-κB (NF-κB) signaling and nuclear factor erythroid 2 related factor 2 (Nrf2) signaling are the two well-known redox-sensitive signaling pathways (7). NF-κB initiates and also sustains the inflammatory response. Wang *et al.* reported that oxidative stress-induced NF-κB signaling was essential for the elevated secretion of the pro-inflammatory cytokines, interleukin (IL)-6 and IL-8, from bronchial epithelial cells exposed to PM (8). In contrast to the pro-inflammatory role of NF-κB signaling, Nrf2 signaling plays an intrinsic cytoprotective role against oxidative stress; it regulates the expression of numerous antioxidant factors, including hemeoxygenase-1 (HO-1) and quinine oxidoreductase 1 (NQO1), through the antioxidant response element (ARE) to eliminate the excess ROS (9).

It has been reported that herbal extracts from plants such as Djulis and curcumin exert a regulatory effect on Nrf2 signaling to protect pulmonary epithelial cells from oxidative injury induced by PM exposure (10,11). Moreover, the activation of Nrf2 signaling is essential for the inhibitory effect of sythiaside on NF-κB signaling, which exerts an anti-inflammatory effect against cigarette smoke (12). Considering the protective anti-oxidative role of Nrf2 signaling during oxidative stress, a potential endogenous modulator of Nrf2 signaling might preserve the redox state, thereby providing a promising therapeutic strategy for the treatment of PM-induced lung injury.

Fibroblast growth factor 10 (FGF10), which acts as a canonic member of the FGF superfamily, is a typical paracrine FGF secreted by mesenchymal cells (13). FGF10 specifically interacts with FGF receptor 2b (FGFR2b), and FGFR2b is exclusively expressed by epithelial cells, enabling FGF10-mediated mesenchymal-to-epithelial transition during development, differentiation, and regeneration (14). The pleiotropic role of FGF10 and the associated molecular mechanism involved in the epithelial repair process of PM-induced lung injury is a significant focus of current research. Our previous studies have identified the protective role of FGF10 in PM-induced lung injury. In particular, we reported that blockade of high-mobility group box 1 (HMGB1)-toll-like receptor 4 (TLR4) inflammatory signaling and dysregulation of endoplasmic reticulum stress constituted the underlying molecular mechanisms (15,16). Given the vital role of oxidative stress in mediating the toxic effects of PM, it is critical to evaluate whether FGF10 impacts oxidative stress during PM-induced lung injury. It is known for the first time to explore whether FGF10 exerts an anti-oxidative effect in PM-induced lung injury and potential molecular mechanism, which might be a therapeutic candidate for environmentally-induced lung injury.

Recently, the anti-oxidative effect of FGF10 in different

organs, except for the lung, has been reported. For example, Dong *et al.* demonstrated that FGF10 activated the Phosphatidylinositide 3-kinases/protein kinase B (PI3K/Akt) signaling-induced antioxidant response to promote peripheral nerve regeneration (17). Moreover, FGF10 was reported to activate the Akt-dependent Nrf2 signaling axis to enable a protective effect against hepatic ischemia-reperfusion injury (18). Based on these findings, FGF10 may exert an anti-oxidative effect against PM exposure. The current study tested the hypothesis that FGF10 is protective against PM-induced lung injury via Nrf2-dependent suppression of NF-κB signaling. To test this hypothesis, C57BL/6 mice were subjected to PM exposure with or without FGF10 administration. Human bronchial epithelial cells were utilized to investigate the mechanism associated with FGF10-mediated regulation of the redox-sensitive NF-κB and Nrf2 signaling. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-4389/rc).

Methods

Reagents and antibodies

The standard reference airborne (1649b) was purchased from NIST (Gaithersburg, MD, USA). For *in vivo* animal experiments, exogenous recombinant FGF10 was obtained from the School of Pharmaceutical Science affiliated with Wenzhou Medical University. For *in vitro* cell experiments, exogenous recombinant FGF10 was purchased from PeproTech (Shanghai, China). Antibodies against FGF10, phospho-p65, p65, phospho-IκBα, IκBα, Nrf2, Kelch-like ECH-associated protein 1 (KEAP1), heme oxygenase 1 (HO-1), and Quinone Oxidoreductase 1 (NQO1) were purchased from Cell Signaling Technology (Beverly, MA, USA). The enzyme linked immunosorbent assay (ELISA) kits for mouse FGF10, mouse IL-6, mouse IL-8, human IL-6, and human IL-8 were purchased from Boyun Biotechnology (Shanghai, China). The compound BAY11-7082 was purchased from Selleck (Houston, Texas, USA). N-acetylcysteine (NAC) and ML385 were purchased from Sigma-Aldrich (Shanghai, China).

Cells and animals

Human bronchial epithelial cells (HBECs) were purchased from the Chinese Academy of Sciences (Shanghai, China) and cultured at 37 ℃ inside the cell culture incubator with 5% carbon dioxide (CO_2) in Roswell Park Memorial Institute (RPMI)-1640 medium (GIBCO, Weihao, MA, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO), 50 U/mL penicillin, and streptomycin (GIBCO). Male C57BL/6J mice (6–8 weeks, 20–25 g) were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China) and housed in a specific pathogen-free facility at The First Affiliated Hospital of Wenzhou Medical University. Animal experiments were performed under a project license (No. 2021-0037) granted by the ethics committee for animal welfare at The First Affiliated Hospital of Wenzhou Medical University, in compliance with national guidelines for the care and use of animals. A protocol was prepared before the study without registration.

Experimental design

For *in vivo* animal experiments, the mice were randomly grouped (Vehicle group, FGF10 group, PM group, PM + FGF10 group; n=12 mice per group). The mouse model of PM-induced lung injury was established through intratracheal instillation of 4 mg/kg PM for 2 consecutive days. To explore the potential effect of FGF10, 5 mg/kg FGF10 was intratracheally instilled 1 h before PM exposure. The mice were euthanized with an intraperitoneal injection of pentobarbital sodium 24 h after the second PM exposure, and their bronchoalveolar lavage (BAL) and lung tissues were collected.

For *in vitro* cell culture experiments, PM was initially re-suspended in phosphate buffer saline (PBS) at a stock concentration of 4 mg/mL, and then HBECs were incubated with 200 μg/mL PM for the indicated time. FGF10 (10 ng/mL), NAC (2.5 mM), BAY 11-7082 (5 μM), or ML385 (10 μM) were added to the pretreated HBECs at 1 h before PM exposure for the indicated purpose.

Hematoxylin and eosin (H&E) and histological analysis

Firstly, the harvested lung tissues were fixed in 4% paraformaldehyde for 24 h. Next, the lung tissues were embedded in paraffin and precisely sectioned into 5 μm sections. Finally, the slides were de-paraffinized and separately stained with H&E for histological analysis. Three independent experienced investigators carried out the histological analysis. The histological analysis criteria were as follows: 0 meant that no apparent inflammation was

observed; 1 denoted occasional cuffing with inflammatory cells; 2 represented that the majority of bronchi or vessels were surrounded by a thin layer (1 to 5) of inflammatory cells; and 3 indicated that the majority of bronchi or vessels were surrounded by a thick layer (>5) of inflammatory cells (19).

Redox state measurement

For *in vitro* experiments, the HBECs were plated in 24-well plates and exposed to PM after pre-treatment with FGF10. Subsequently, the HBECs were incubated with 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM) in serum-free 1,640 media for 20 min at 37 ℃ in a 5% CO_2 culture chamber. Following this, the HBECs were washed thrice with PBS. The fluorescence intensity was measured with an Operetta High Content Screening System (PerkinElmer, USA), and Operetta Harmony 4.8 (PerkinElmer) analysis was conducted.

For the *in vivo* animal experiments, fresh lung tissues embedded in optimal cutting temperature compound (OCT) were precisely sliced and separately stained with dihydroethidium (DHE). DHE is naturally oxidized by superoxide, which emits red fluorescence and can be visualized through a fluorescence microscope. According to the manufacturer's instructions, the levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in the lung tissues were measured using a lipid peroxidation MDA kit and H_2O_2 kit, respectively.

Western blot

The harvested lung tissues and the cultured HBECs were lysed with Radio Immunoprecipitation Assay (RIPA) lysis buffer containing a chemical mixture including phenylmethanesulfonyl fluoride (PMSF), protease inhibitors, and a phosphatase inhibitor. An equal concentration of total protein was loaded onto the different lanes. The loaded proteins were separated based on their molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins in the SDS-PAGE gel were then transferred to polyvinylidene difluoride (PVDF) membranes and incubated with primary antibodies at a specific diluted concentration. The transferred PVDF membranes were kept at 4 ℃ overnight, followed by incubation for 1 h at room temperature with the secondary antibody at a specific dilution. The immune-reactive bands were measured with an Enhanced chemiluminescence (ECL) reagent on a Bio-Rad gel imaging system (Bio-Rad, USA).

Enzyme-linked immunosorbent assay (ELISA)

For ELISA, the BAL was carefully collected through a tracheal cannula, with 1 ml of PBS instilled into the lung. The collected BAL was then centrifuged at 12,000 rpm for 15 min at 4 ℃. The levels of IL-6 and IL-8 in the BAL supernatants and cell culture supernatants were quantified using the indicated ELISA kits, according to the manufacturer's instructions.

Statistical analysis

All of the data were expressed as mean ± SEM (Standard Error of Mean). *In vitro* experiments were conducted with n≥3 independent replicates, and the *in vivo* experiments were conducted with n=12 mice per group. Statistical analyses were performed using the GraphPad Prism program (GraphPad, San Diego, CA, USA). The Student's *t*-test was employed for statistical analysis of significance. One-way ANOVA (Analysis of Variance) followed by Tukey's post-hoc analysis was used to evaluate the statistical difference between multiple groups. A P value less than 0.05 was considered to be statistically significant.

Results

FGF10 is significantly upregulated upon PM exposure in vivo

To clarify the possible role of FGF10 in PM-induced lung injury, changes in FGF10 protein expression were evaluated after PM exposure *in vivo*. The schematic diagram of the *in vivo* experimental design is displayed in *Figure 1A*. We generated the mouse model of PM-induced lung injury. Western blot of the mouse lung tissues revealed upregulated expression of the FGF10 protein upon PM exposure (*Figure 1B,1C*). As FGF10 is typically a paracrine growth factor, the protein expression of FGF10 was consistently upregulated in BAL (*Figure 1D*). These findings suggested that the upregulated expression of the endogenous FGF10 protein represented a potential self-protection response against PM exposure. To confirm the potential role of FGF10 in PM-induced lung injury, intratracheal instillation of exogenous FGF10 was performed. A schematic diagram of the *in vivo* experimental design with intratracheal instillation of FGF10 is shown in *Figure 1E*.

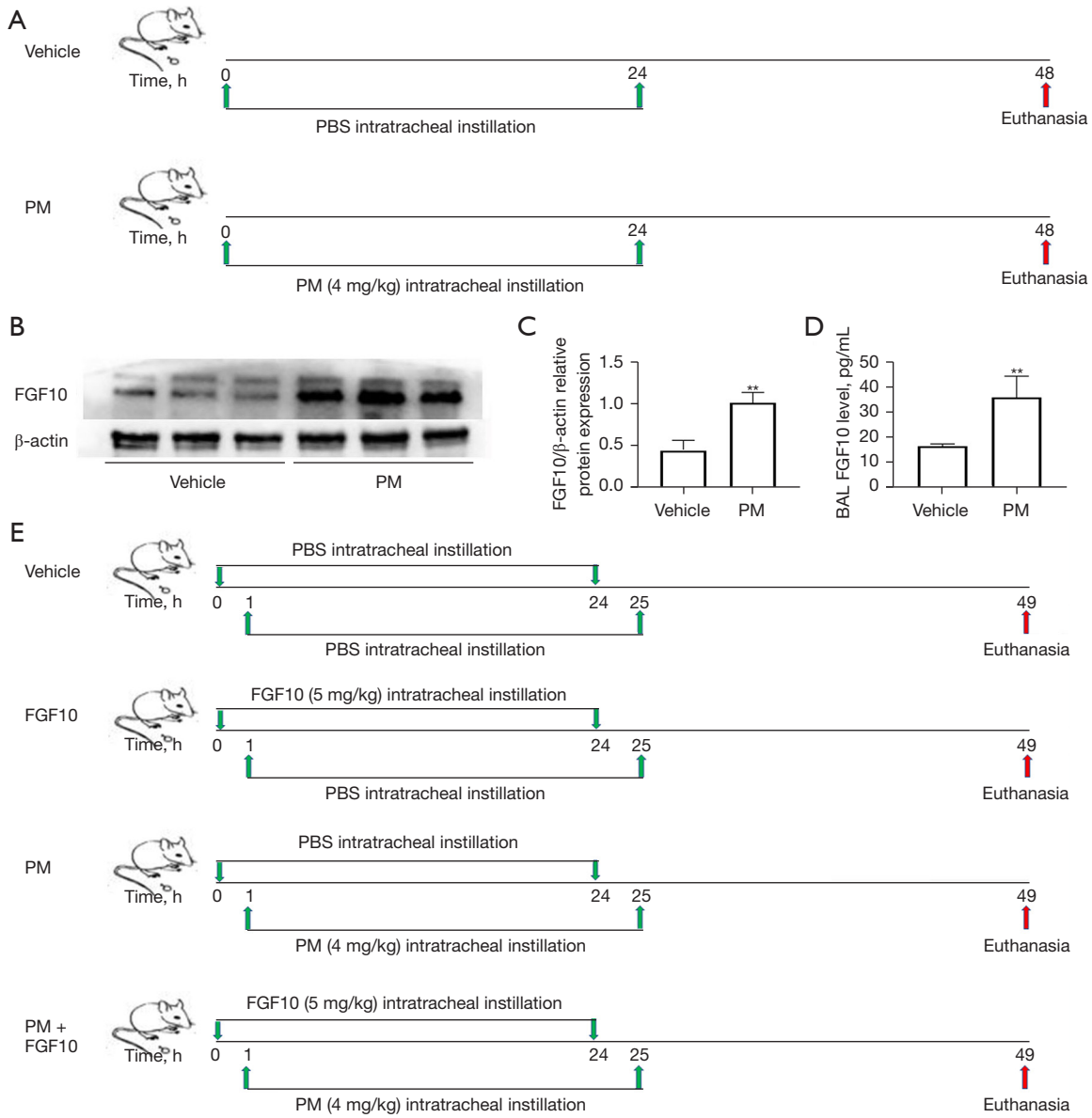

Figure 1 FGF10 was significantly upregulated upon PM exposure *in vivo*. (A) A schematic diagram showing the *in vivo* experimental design. Mice were intratracheally administered with a dosage of 4 mg/kg of PM for 2 consecutive days (n=12 per group). (B) The protein expression of FGF10 in the lung tissues was quantified by Western blot. For the quantitative analysis, the optical densities of the protein bands are shown in (C). (D) The protein expression of FGF10 in the BAL was measured by ELISA. (E) A schematic diagram of the *in vivo* experimental design with intratracheally administered FGF10. Values are presented as mean ± SEM; **P<0.01 *vs.* Vehicle group. PBS, phosphate buffer saline; PM, particulate matter; FGF10, fibroblast growth factor 10; BAL, bronchoalveolar lavage; ELISA, Enzyme-linked immunosorbent assay; SEM, standard error of mean.

FGF10 attenuates PM-induced lung injury and suppresses NF-κB signaling

Exogenous FGF10 undoubtedly supplements the effect of endogenous FGF10. The *in vivo* experimental plan with intratracheal administration of FGF10 was carried out. The histopathological analyses of lung sections stained with H&E revealed that PM exposure led to a marked infiltration of inflammatory cells clustered around the PM deposits,

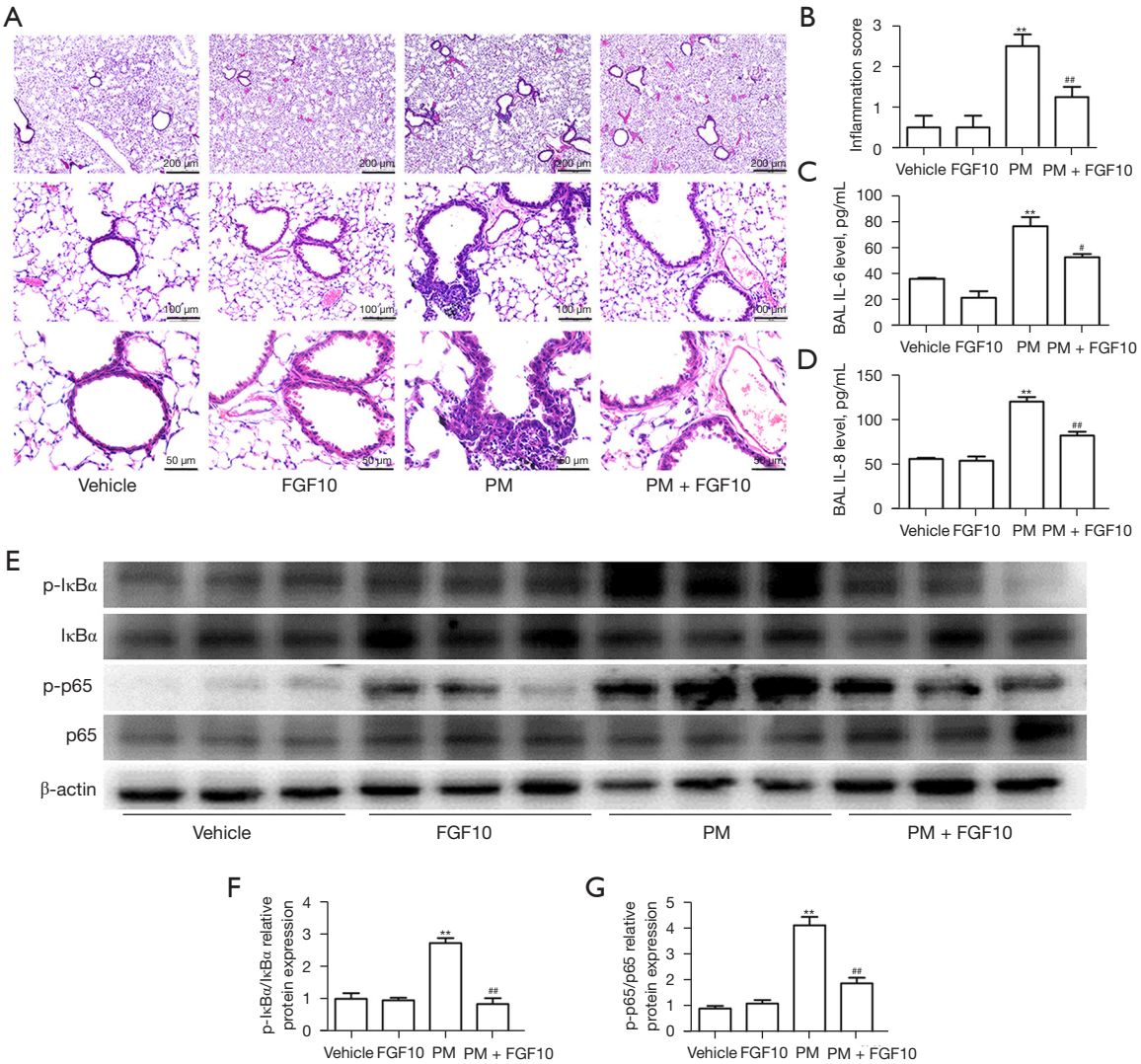

Figure 2 FGF10 attenuated PM-induced lung injury and suppressed NF-κB signaling *in vivo*. Mice were intratracheally administered with FGF10 at a dose of 5 mg/kg, 1 hour prior to PM exposure at a dose of 4 mg/kg, for 2 consecutive days (n=12 per group). (A) Representative images of H&E stained lung sections from the mice. (B) The semi-quantified inflammation score for H&E-stained lung sections from the mice. (C,D) The protein expression of IL-6 and IL-8 in the BAL were measured by ELISA. (E) The protein expressions of p-IκBα, IκBα, p-p65 and p65 in the lung tissues were quantified by Western blot. For quantitative analyses, the optical densities of the protein bands are displayed in (F,G). Values are presented as mean ± SEM; **P<0.01 *vs.* Vehicle group; #P<0.05, ##P<0.01 *vs.* PM group. PM, particulate matter; FGF10, fibroblast growth factor 10; H&E, hematoxylin-eosin staining; BAL, bronchoalveolar lavage; ELISA, Enzyme-linked immunosorbent assay; SEM, standard error of mean.

mainly restricted to the peripheral bronchi. However, the intratracheal instillation of FGF10 exhibited a pronounced reduction in inflammatory cells around the PM deposits (*Figure 2A*).

Next, the semi-quantified inflammation score for the lung sections stained with H&E was evaluated by three independent investigators. Consistent with the histopathological analyses, the intratracheal instillation of FGF10 reversed the inflammation score in the group exposed to PM. Also, no significant difference was observed between the vehicle and FGF10 groups (*Figure 2B*).

The protein expression of inflammatory cytokines, IL-6 and IL-8, from the BAL were quantified by ELISA. Compared to the vehicle group, PM exposure notably

upregulated the protein expression of IL-6 and IL-8, whereas FGF10 pre-treatment significantly reduced the protein expression of IL-6 and IL-8 (*Figure 2C*,*2D*). The redox-sensitive NF-κB signaling is also known to participate in PM-induced lung injury, which is closely related to the production of inflammatory cytokines such as IL-6 and IL-8. Western blot of lung tissues showed that PM exposure resulted in the phosphorylation of IκBαof lung tissues showed pre-treatment significantly inhibited NF-κo activation (*Figure 2E-2G*). These findings suggested that FGF10 played a suppressive role in oxidative stress and NF-κB signaling. Collectively, these results indicated that FGF10 played a protective role in PM-induced lung injury.

FGF10 suppresses oxidative stress and activates Nrf2 signaling in vivo

Oxidative stress is the critical regulator of PM-induced lung injury. However, the antioxidant role of FGF10 remains to be demonstrated. The DHE staining of lung sections illustrated that ROS production was markedly higher in the PM group than in the vehicle group. However, FGF10 pre-treatment effectively blocked ROS production (*Figure 3A*,*3B*). Simultaneously, the levels of MDA and H_2O_2, which are sensitive biomarkers that reflect the cellular redox status, were reversed with FGF10 pre-treatment (*Figure 3C*,*3D*), highlighting the antioxidant effect of FGF10 in PM-induced lung injury.

Nrf2 signaling represents an intrinsic cytoprotective mechanism against oxidative stress. Western blot of the lung tissues showed that FGF10 pre-treatment significantly activated Nrf2 signaling, as evidenced by the upregulation of the downstream mediators of Nrf2 signaling, namely, HO-1 and NQO1 (*Figure 3E-3I*). These findings suggested that FGF10 suppressed oxidative stress, which was reflected by the activation of Nrf2 signaling.

FGF10 exerts anti-inflammatory effects via modulation of NF-κB signaling in HBECs

To further validate the cytoprotective role of FGF10, the HBECs were subjected to 200 μg/mL PM alone or in combination with 10 ng/mL FGF10. The ELISA results for cell culture supernatants showed elevated protein expression of IL-6 and IL-8 with PM exposure, which was reversed by FGF10 pre-treatment (*Figure 4A*,*4B*). It is known that NF-κB signaling is the central inflammatory signaling responsible for the expression and secretion of IL-6 and IL-8 in the PM-induced inflammatory response. Therefore, to determine the effect of FGF10 on NF-κB signaling in the PM-induced inflammatory response, BAY11-7082, a synthetic chemical inhibitor of NF-κB, was selected for further experiments. This NF-κB inhibitor was found to exert similar effects as FGF10 (*Figure 4C*,*4D*). Moreover, pre-treatment with FGF10 reversed the effects on NF-κB signaling induced by PM exposure (*Figure 4E-4G*). Collectively, these results suggested that PM exposure promoted the NF-κB signaling-dependent inflammatory response; meanwhile, FGF10 reversed the inflammatory effects of PM exposure on NF-κB signaling.

The anti-oxidative effect of FGF10 on ROS production and the modulation of NF-κB signaling by the antioxidant NAC exert anti-inflammatory effects in HBECs

The regulatory mechanism of FGF10 on NF-κB signaling remains to be elucidated. ROS, as the representative biomarker of oxidative stress, promotes various cellular processes including the activation of NF-κB signaling. Fluorescence staining of ROS in the HBECs showed that PM exposure caused an elevation in ROS levels, while FGF10 pre-treatment reduced ROS production upon PM exposure (*Figure 5A*,*5B*).

To outline the direct phosphorylation of proteins involved in NF-κB signaling by ROS, the antioxidant NAC was utilized as an intervention upon PM exposure. Western blot analysis pointed out that the phosphorylation of p65 and IκBα was reduced after NAC pre-treatment compared to the PM group (*Figure 5C-5E*). PM exposure led to significant upregulation of p-p65 and p-IκBα and NAC effectively reversed upregulated p-p65 and p-IκBα. Consistent with the suppression of NF-κB signaling, NAC pre-treatment also suppressed the expression of IL-6 and IL-8 (*Figure 5F*,*5G*). These findings implied that ROS partially mediated the activation of NF-κB signaling in the PM-induced inflammatory response, and FGF10 potentially functioned as a scavenger of ROS to mediate its anti-inflammatory effects.

Effect of FGF10 on the activation and expression of Nrf2 signaling in HBECs

Considering that FGF10 is not a direct antioxidant like NAC, the anti-oxidative effect of FGF10 needs to be further explored. The anti-oxidative effect of Nrf2 signaling is reportedly the critical pathway protecting against

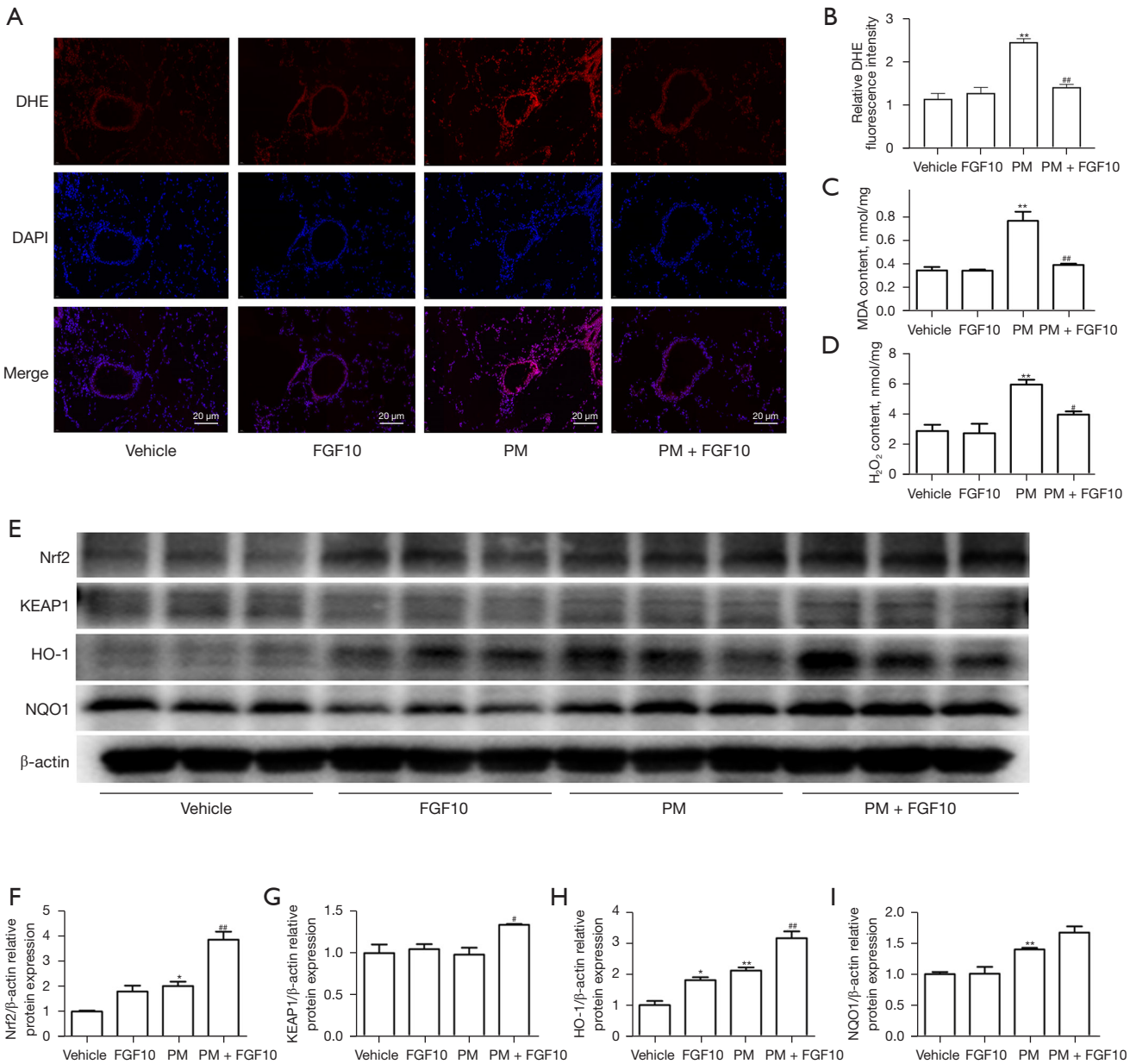

Figure 3 FGF10 suppressed oxidative stress and activated Nrf2 signaling *in vivo*. The mice were intratracheally administered with FGF10 at a dose of 5 mg/kg, 1 hour prior to PM exposure at a dose of 4 mg/kg, for 2 consecutive days (n=12 per group). (A) Representative images of DHE-stained lung sections from the mice. (B) The semi-quantified fluorescence intensity of the lung sections stained with DHE. (C,D) The MDA content and corresponding oxidative stress marker (H_2O_2) were measured in the lung tissues from mice. (E) The protein expression of Nrf2, KEAP1, HO-1, and NQO1 in lung tissues from mice were quantified by Western blot. The optical densities of the protein bands are illustrated in (F-I). Values are presented as mean ± SEM. *P<0.05, **P<0.01 *vs.* Vehicle group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 *vs.* PM group. DHE, dihydroethidium; DAPI, 4, 6'-diamidino-2-phenylindole; PM, particulate matter; FGF10, fibroblast growth factor 10; MDA, malondialdehyde; SEM, standard error of mean.

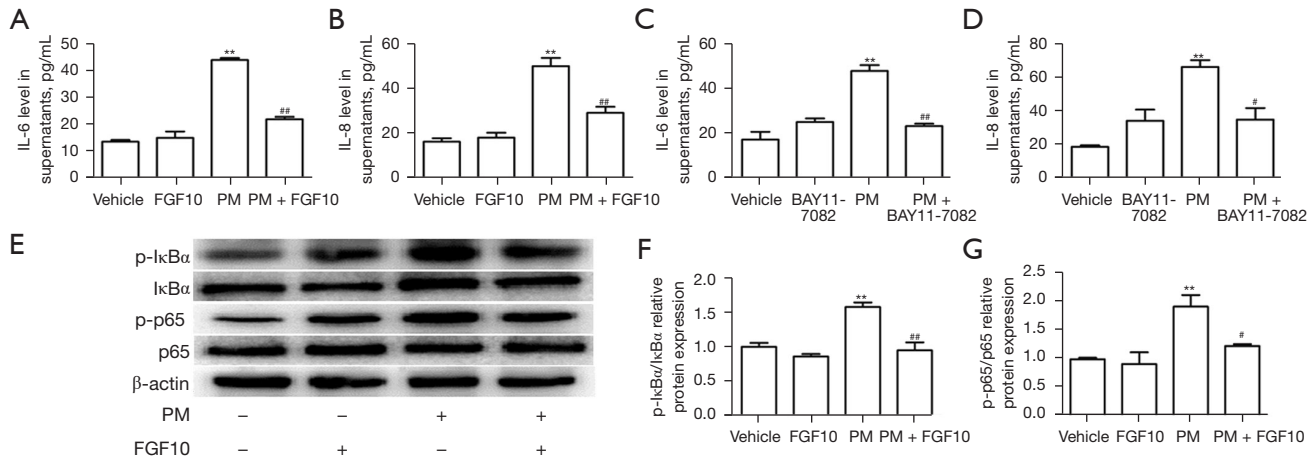

Figure 4 FGF10 exerted anti-inflammatory effects through the modulation of NF-κo signaling in HBECs. (A,B) The HBECs were pre-treated with 10 ng/mL FGF10 for 1 hour, then exposed to 200 μg/mL PM for 24 hours. The protein expressions of IL-6 and IL-8 in the culture supernatants harvested from the indicated groups were measured by ELISA. (C,D) The HBECs were pre-treated with 5 μM BAY11-7082 for 1 hour, then exposed to 200 μg/mL PM for 24 hours. The protein expressions of IL-6 and IL-8 in the culture supernatants were measured by ELISA. (E) The HBECs were pretreated with 10 ng/mL FGF10 for 1 hour, then exposed to 200 μg/mL PM for 1 hour. The protein expression of p-IκBα, IκBα, p-p65 and p65 were quantified by Western blot. For quantitative analysis, the optical densities of the protein bands are illustrated in (F,G). Values are presented as mean ± SEM. **P<0.01 *vs.* Vehicle group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 *vs.* PM group. IL-6, interleikin-6; IL-8, interleikin-8; PM, particulate matter; FGF10, fibroblast growth factor 10; HBECs, human bronchial epithelial cells; ELISA, Enzyme-linked immunosorbent assay; SEM, standard error of mean.

oxidative stress. Therefore, we explored the relationship between FGF10 and Nrf2 signaling. As illustrated in *Figure 6A-6E*, PM exposure resulted in elevated expression of Nrf2, reflecting a self-protecting cellular mechanism. However, the protein expression of Nrf2 was highest upon PM exposure when combined with FGF10 pre-treatment. Similarly, the expression of HO-1 and NQO1 were also highest when PM exposure was combined with FGF10 pre-treatment.

We utilized the specific Nrf2 inhibitor, ML385, to further validate the regulatory effects of FGF10 on Nrf2 signaling. It was found that ML385 pre-treatment abolished the rescue of IL-6 and IL-8 expression by FGF10 upon PM exposure (*Figure 6F,6G*). These findings demonstrated that Nrf2 signaling was critical for the protective effect of FGF10 in the PM-induced inflammatory response.

Discussion

As a consequence of the widespread health-related concerns associated with PM, numerous efforts have been deployed to determine the molecular mechanism of PM-induced lung injury to identify potential therapeutic targets. PM exposure leads to lung injury primarily through the inflammatory mechanism mediated by oxidative stress. It is recognized that oxidative stress, owing to the excess production of ROS and insufficient synthesis of the antioxidant enzymes, is a pivotal pathophysiological characteristic of PM-induced lung injury (20). NF-κB signaling is a well-known redox-sensitive signaling that mediates inflammation. Under oxidative stress conditions, elevated levels of ROS stimulate the redox-sensitive NF-κB signaling, which promotes irreversible cellular injury and the secretion of inflammatory mediators (21,22). In contrast to the inflammatory NF-κB signaling, the redox-sensitive Nrf2 signaling represents a finitely self-protective mechanism (23).

As for the significant role of oxidative stress, efforts toward the reversal of oxidative stress potentially provide an effective strategy against PM-induced lung injury. Although the ROS scavenger NAC is a promising therapeutic agent, the elevation in mesenchyme-derived FGF10 upon PM exposure is more intriguing. FGF10 plays a pivotal role in organogenesis, repair after lung injury and regarded as a therapy to repair impaired epithelium. Considering the multifunctional effects of FGF10 in the repair process associated with lung injury (24,25), the elevated levels of

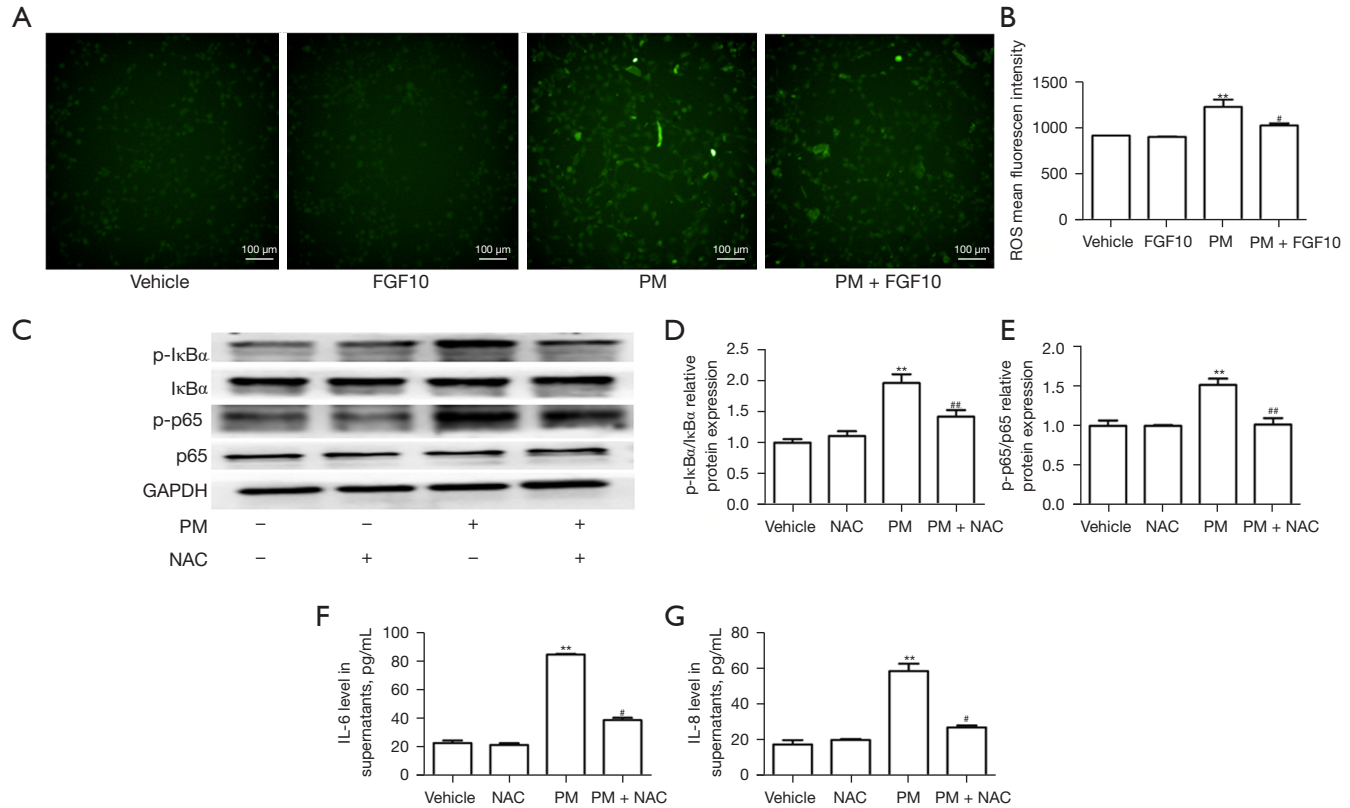

Figure 5 Anti-oxidative effect of FGF10 on ROS production and modulation of NF-κB signaling by the antioxidant NAC in HBECs. (A,B) HBECs were pre-treated with 10 ng/mL FGF10 for 1 hour, and then exposed to 200 μg/mL PM for 1 hour. The HBECs were labeled with DCFH-DA to measure the ROS levels. (C) HBECs were pre-treated with 2.5 mM NAC for 1 hour, then exposed to 200 μg/mL PM for 1 hour. The protein expressions of p-IκBα, IκBα, p-p65 and p65 were quantified by Western blot. The optical densities of the protein bands are illustrated in (D,E). (F,G) The HBECs were pre-treated with 2.5 mM NAC for 1 hour, then exposed to 200 μg/mL PM for 24 hours. The protein expressions of IL-6 and IL-8 in the culture supernatants were measured by ELISA. Values are presented as mean ± SEM. **P<0.01 *vs*. Vehicle group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 *vs*. PM group. PM, particulate matter; FGF10, fibroblast growth factor 10; ROS, reactive oxygen species; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NAC, N-acetylcysteine; HBECs, human bronchial epithelial cells; DCFH-DA, 2,7-Dichlorodihydrofluorescein diacetate; SEM, standard error of mean.

FGF10 are consistent with the body's self-repair mechanism against PM exposure. As a complement to the reported regulatory effect on HMGB1-TLR4 signaling and reversal of endoplasmic reticulum stress (15,16), further investigation of the anti-oxidative effects of FGF10 and the underlying molecular mechanism would certainly broaden our current understanding of the multifunctional effects of FGF10 and validate FGF10 as a novel therapeutic target for treating PM-induced lung injury.

For the *in vivo* studies, intratracheal instillation of PM was performed for 2 consecutive days to establish a model of short-term PM exposure, as previously reported (26,27). As PM is a chemical mixture and the composition varies with the geographical area and season, commercial PM with its settled constituents was purchased to guarantee experimental reproducibility. Consistent with previous findings, intratracheal instillation of PM promoted bronchitis but not alveolitis, with significant elevation of endogenous FGF10. This result contrasts with the observation in humans that PM exposure promotes both bronchitis and alveolitis. We speculated that the currently used method for the intratracheal instillation of PM might impair the intrinsic aerodynamics of PM. We also speculated that the levels of available FGF10 in the lung positively impact the outcome of PM-induced lung injury (28).

To further clarify this, recombinant FGF10 was

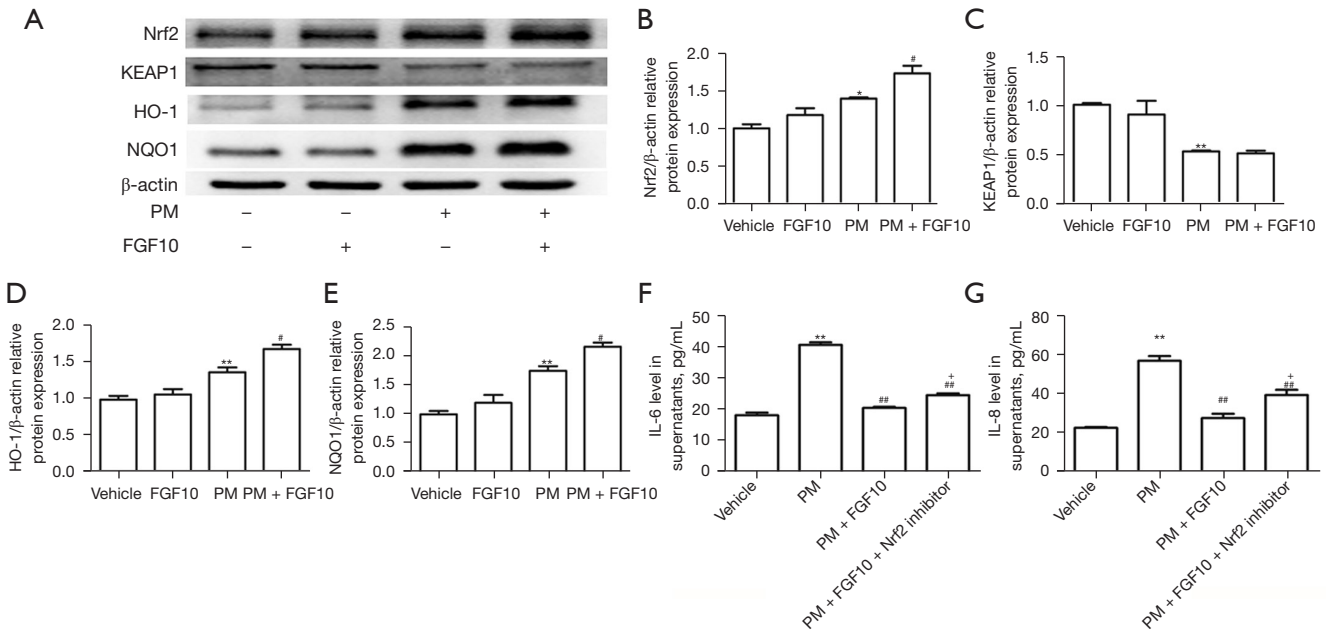

Figure 6 Effect of FGF10 on the activation of Nrf2 signaling, and the expression of the downstream mediators of this pathway in HBECs. (A) HBECs were pretreated with 10 ng/mL FGF10 for 1 hour, then exposed to 200 μg/mL PM for 24 hours. The protein expressions of Nrf2, KEAP1, HO-1, and NQO1 were quantified by Western blot. For quantitative analysis, the optical densities of the protein bands are illustrated in (B-E). (F,G) HBECs were pretreated with 10 μM ML385 for 1 hour, then exposed to 10 ng/mL FGF10 and 200 μg/mL PM for 24 hours. The protein expressions of IL-6 and IL-8 in the culture supernatants were measured by ELISA. Values are presented as mean ± SEM. *P<0.05, **P<0.01 *vs.* Vehicle group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 *vs.* PM group; $^{+}$P<0.05 *vs.* PM + FGF10 group. PM, particulate matter; FGF10, fibroblast growth factor 10; HBECs, human bronchial epithelial cells; SEM, standard error of mean.

employed as an exogenous supplementation. As expected, the exogenous FGF10 markedly relieved the degree of lung injury, as evidenced by the histopathological examinations and secreted inflammatory mediators. Simultaneously, the production of oxygen-free radicals and ROS as well as the synthesis of antioxidant entities were investigated to measure the redox state. Oxidative stress is considered one of the most prevalent mechanisms in the pathogenesis of lung injury, and PM is the primary inducer of excessive ROS, promoting mitochondrial dysfunction (29). It was shown that the exogenous FGF10 reversed ROS generation upon PM exposure, together with the downregulation of oxidative stress markers, such as MDA and H_2O_2.

NF-κB signaling is related to the secretion of inflammatory mediators such as IL-6 and IL-8 and contributes to the development of lung injury (8). As for the regulation of NF-κB signaling by FGF10, it has been reported that FGF10 derived from the neuron and microglia/macrophages inhibits the NF-κB-dependent neuroinflammation through FGFR2/PI3K signaling, which accelerates the recovery from spinal cord injury (30). Furthermore, neuro-derived FGF10 inhibits NF-κB-dependent neuro-inflammation by activating the PI3K survival signaling, which was reported to improve cerebral ischemia injury (31). A similar inhibitory effect of FGF10 on NF-κB signaling was observed in the current study.

Wang *et al.* (8) discovered the role of ROS-dependent NF-κB signaling in lung inflammation upon PM exposure. Consistent with the findings of Wang *et al.* (8), the inhibition of NF-κB signaling was related to the anti-oxidative effects of FGF10 on the activation of Nrf2 signaling. These findings synergistically revealed that FGF10 exerted protective anti-oxidative activity in response to PM exposure and anti-inflammatory effects through the inhibition of NF-κB signaling. However, it should be noted that the anti-oxidative effect of FGF10 is likely different from that elicited by the direct antioxidant, NAC. The molecular mechanism mediating the protective anti-oxidative property of FGF10 remains to be elucidated.

Nrf2 signaling is a crucial regulator of redox homeostasis,

which counterbalances excessive ROS production. Nrf2 is physically located in the cytoplasm through binding to KEAP1. Upon stimulation of Nrf2 signaling and with the degradation of KEAP1, Nrf2 spontaneously translocates into the nucleus to further bind to anti-oxidative response elements, thereby regulating the expression of genes encoding antioxidant enzymes such as HO-1 and NQO1 (32,33). The regulatory mechanism of Nrf2 signaling is complex, as redox-sensitive signaling is potently induced by oxidative stress.

Also, the regulatory effect of FGF10 on Nrf2 signaling has been discovered both in peripheral nerve regeneration and hepatic ischemia-reperfusion injury (17,18). It has been shown that the protein expressions of Nrf2, along with that of its downstream mediators, HO-1 and NQO1, were slightly upregulated with PM exposure, highlighting the regulatory effect of oxidative stress on Nrf2 signaling. Others have reported conflicting findings, namely, that PM exposure suppresses Nrf2 signaling and downregulates the expression of downstream anti-oxidative genes, thus leading to excessive intracellular ROS (34,35). The causal relationship between oxidative stress and Nrf2 signaling in the context of PM is not direct, and the contradiction might be dosage-dependent. The activated Nrf2 signaling is regarded as a compensatory mechanism. However, it was not sufficient to counteract the oxidative lung injury. Exogenous FGF10 further amplified Nrf2 signaling to the degree that led to reduced oxidative lung injury. It should be noted that the upregulated expression of KEAP1 was controversial to the activated Nrf2 signaling *in vivo*. The downstream HO-1 and NQO1 are more sensitive indicators of the state of Nrf2 signaling. These results highlighted that the lungs of PM-exposed mice were in a state of oxidative stress and that the protective role of FGF10 was at least partially attributed to the amplified Nrf2-mediated antioxidant effect.

In the pathophysiology of PM-induced lung injury, notably bronchitis, the bronchial epithelial cells act as both the first line of defense against chemical and biological pathogens and also as the initiators of secondary inflammation (36). FGF10 is pivotal in regenerative medicine, and it binds to the specific epithelial receptor FGFR2b (37). As bronchial epithelial cells are the primary cells regulating FGF10 expression and also represent its principal cellular target, it is speculated that the protective effect of FGF10 is mediated through the regulation of bronchial epithelial cells (38). Based on the anti-oxidative effects of FGF10 *in vivo*, immortalized human bronchial epithelial cells were selected to validate the hypothesis *in vitro*. For the *in vitro* experiments, pre-treatment of bronchial epithelial cells with exogenous FGF10 was performed to mimic the *in vivo* mesenchymal-epithelial FGF10 signaling. In contrast to the *in vivo* setting, the ROS scavenger NAC and Nrf2 inhibitor were employed. Both FGF10 and NAC have an inhibitory effect on the PM-induced phosphorylation of NF-κB signaling and the subsequent secretion of Il-6 and IL-8. It has been reported that IL-6 and IL-8 are the target genes regulated by NF-κB signaling. NAC neutralizes excessive ROS to exert anti-inflammatory effects, while FGF10 is not a first-line antioxidant.

To validate the Nrf2-mediated anti-oxidative impact of FGF10 on NF-κB signaling, it was found that the anti-inflammatory effect of FGF10 was abolished when FGF10 and the Nrf2 inhibitor were combined. However, the mediators linking FGF10-FGFR2b and Nrf2 signaling require further exploration. It was reported that FGF10/Akt/Nrf2 signaling is critical in oxidative stress-mediated myocardial ischemia-reperfusion injury (39). Moreover, the protective effect of FGF10 in hepatic ischemia-reperfusion injury relies on PI3K/Akt-dependent Nrf2 activation (18). Therefore, it is speculated that PI3K/Akt signaling might be the mediator between FGF10 and Nrf2 signaling.

Although the current study identified the protective role of FGF10 and the anti-oxidative mechanism it promoted, there were several limitations that should be noted. Firstly, the PM-induced lung injury was confined to bronchitis. In the future, PM aerosolization will be performed instead of intratracheal instillation, as such a delivery route will likely also induce alveolitis. Secondly, while exogenous FGF10 was intratracheally instilled to magnify the effect of endogenous FGF10, the contribution of endogenous FGF10 signaling to the repair process remains unclear. Research on the regulation of FGF10 by lncRNA or miRNA would make the whole study more complete.

Further loss of function experiments to delete *Fgfr2b* expression in the bronchial epithelium or abrogate *Fgf10* mesenchymal expression will be conducted. Simultaneously, more oxidative stress and inflammatory indicators should be adopted to reveal the pathogenesis of PM-induced lung injury and pleiotropic effect of FGF10. Thirdly, this study lacks clinical specimens such as primary bronchial epithelial cells, which could be collected for cell-based experiments. Therefore, further studies must be carried out to determine the anti-oxidative mechanism associated with FGF10, and in particular to understand its complex regulatory network.

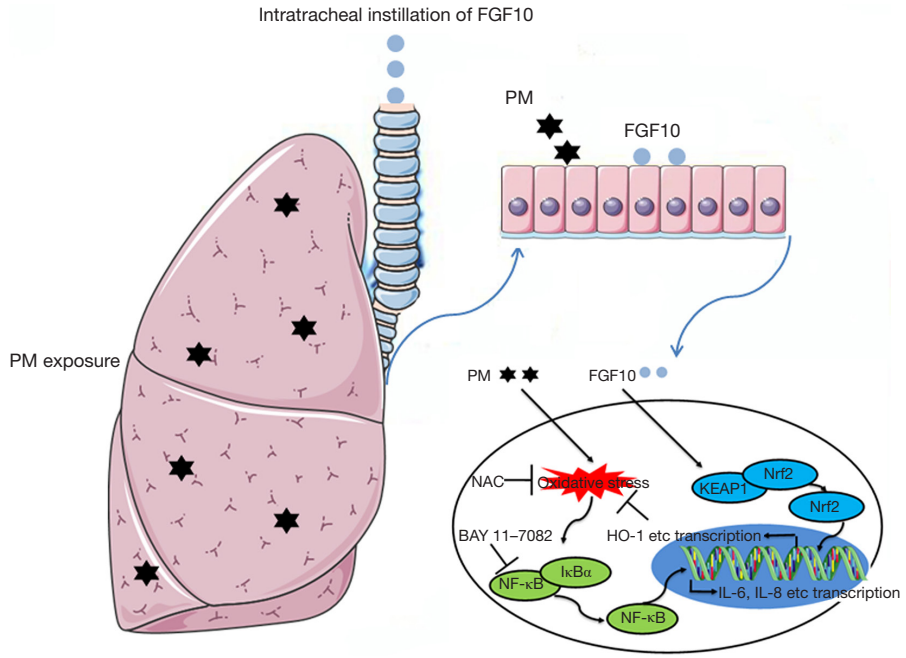

Figure 7 Schematic representation of the protective mechanism of FGF10 in PM-induced lung injury. PM exposure resulted in oxidative stress and promoted ROS formation, which then modulated NF-κB signaling to regulate the expression of the inflammatory cytokines, IL-6 and IL-8. FGF10 exerted anti-oxidative effects upon PM exposure by modulating Nrf2 signaling, which regulated the expression of the antioxidant mediators, HO-1 and NQO1. The anti-oxidative effect of FGF10 partially accounted for its inhibitory effect on NF-κB signaling. PM, particulate matter; FGF10, fibroblast growth factor 10; NAC, N-acetylcysteine; ROS, reactive oxygen species; NF-κB, nuclear factor-κB; IL-6, interleikin-6; IL-8, interleikin-8.

In conclusion, the current study demonstrated the protective effect of FGF10 in PM-induced lung injury. The Nrf2-mediated anti-oxidative effects of FGF10 were responsible for the inhibition of NF-κB signaling and contributed to confining PM-induced lung injury (*Figure* 7). The current study broadens our understanding of the multifunctional effects of FGF10 and identified FGF10 as a novel antioxidant with therapeutic potential against PM-induced lung injury.

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Footnote

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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. Animal experiments were performed under a project license (No. 2021-0037) granted by the ethics committee for animal welfare at The

First Affiliated Hospital of Wenzhou Medical University, in compliance with national guidelines for the care and use of animals.

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