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Carbon black nanoparticles induce pulmonary fibrosis through NLRP3 inflammasome pathway modulated by miR-96 targeted FOXO3a

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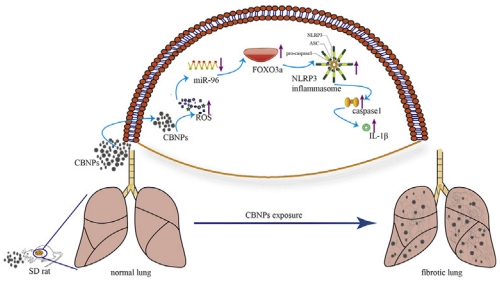
HIGHLIGHTS

- CBNPs-induced pulmonary fibrosis might be linked with persistent lung inflammation.
- The potential mechanism might relate with NLRP3 inflammasome activation.
- NLRP3 inflammasome activation might be modulated by miR-96 targeted FOXO3a.

GRAPHICAL ABSTRACT



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ABSTRACT

Carbon black nanoparticle (CBNP) is a core constituent of air pollutants like fine particulate matter (PM2.5) as well as a common manufactural material. It was proved to pose adverse effects on lung function and even provoke pulmonary fibrosis. However, the underlying mechanisms of CBNPs-induced pulmonary fibrosis remain unclear. The present study aimed to investigate the mechanism of fibrotic effects caused by CBNPs in rat lung and human bronchial epithelial (16HBE) cells. Forty-nine male rats were randomly subjected to 7 groups, means the 14-day exposure group (30 mg/m^3), the 28-day exposure groups (5 mg/m^3 and 30 mg/m^3), the 90-day exposure group (30 mg/m^3) and their respective controls. Rats were nose-only-inhaled CBNPs. 16HBE cells were treated with 0, 50, 100 and 200 μg/mL CBNPs respectively for 24 h. Besides, Forkhead transcription factor class O (FOXO)3a and miR-96 overexpression or suppression 16HBE cells were established to reveal relative mechanisms. Our results suggested CBNPs induced pulmonary fibrosis in time- and dose-dependent manners. CBNPs induced persisting inflammation in rat lung as observed by histopathology and cytology analyses in whole lung lavage fluid (WLL). Both in vivo and in vitro, CBNPs exposure significantly increased the expression of NLRP3 inflammasome, accompanied by the increased reactive oxygen species (ROS), decreased miR-96 and increased FOXO3a expressions dose -and time-dependently. MiR-96 overexpression or FOXO3a suppression could partially rescue the fibrotic effects through inhibiting NLRP3 inflammasome.

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Conclusively, our research show that CBNPs-induced pulmonary fibrosis was at least partially depended on activation of NLRP3 inflammasome which modulated by miR-96 targeting FOXO3a.
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1. Introduction

Carbon black nanoparticle (CBNP) is a type of ultrafine carbon particle, which is a major component of the soot generated by incomplete fuel combustion (Grahame et al., 2014). It was analyzed to be a core constituent of some air pollutants like diesel exhaust and fine particulate matter (PM2.5) (Janssen et al., 2011). Besides, carbon black (CB) is a widely-used manufactural material which ranks one of the top 50 industrial chemicals worldwide. Based on the poorly soluble and low toxic characteristics, CB was commonly used as negative control to study the toxicity of nanomaterials (Martinon et al., 2006). However, emerging findings showed evidences that carbon black exposure could have adverse effects on human health (Lin et al., 2011; Wellmann et al., 2006). Our previous results showed nanoscale CB exposure might contribute to reduce lung function and positively relate with secretion of pro-inflammatory cytokines in CB workers (Zhang et al., 2014). In chronic obstructive pulmonary disease (COPD) patients, CB was identified as a trigger for systemic inflammation (Garshick et al., 2018). In schoolchildren, acute respiratory inflammation was associated with CB exposure when using exhaled nitric oxide (eNO) as a biomarker (Lin et al., 2011).

Several studies provide evidences that certain forms of nanomaterials such as muti-walled carbon nanotubes (MWCNTs) was a potently fibrogenic inducer in the lungs. Though numbers of mechanisms might be involved, sustained pulmonary inflammation was a key mediator closely associated with this fibrotic lesion (Dong and Ma, 2016; Nikota et al., 2017). However, it is noteworthy that although sharing the same chemical composition, heterogeneities in physiochemical properties like surface area, surface chemistry or particle aggregation between MWCNTs and CBNPs driving different toxicological potencies of them (Tian et al., 2006). Up to date, occasional report focused on the fibrotic effect of CBNPs and suggested a possible relationship between carbon black and pulmonary fibrosis (De et al., 2007). Whereas some other literatures showed the opposing findings and the molecular basis involved in the process remains poorly understood (Dong and Ma, 2016). Since then, further efforts were still required to decipher the mechanical events.

Inflammation is crucial for pathogenesis of pulmonary fibrosis. Multiple pre-existing findings described inflammatory reactions caused by CBNPs (Bourdon et al., 2012; Vesterdal et al., 2010). As an important part of inflammation, the NLRP3 (NOD-, LRR- and pyrindomain-containing 3) inflammasome pathway was considered an evolutionarily conserved component of innate effector mechanisms of host-defense against infectious or sterile stimuli such as irritant particles (Martinon et al., 2006; Nica et al., 2017; Sarvestani and McAuley, 2017). It's identified the ongoing NLRP3 inflammasome activation enables immune cells to produce potent inflammatory mediators (Prochnicki and Latz, 2017), thus linking with the development of chronic airway diseases (Dostert et al., 2015; Tate et al., 2016). Forkhead transcription factor class O (FOXO)3a proteins is a member of the Fork frame transcription factor family (Hannenhalli and Kaestner, 2009). It has been extensively studied in recent years because of its critical role in a wide spectrum of cellular processes including regulates proinflammatory cytokine expression (Lin and Peng, 2004). Significant FOXO3a variations were observed after nanoparticles treatment like silica nanoparticles (SiO2-NP) and nanoparticulate titanium dioxide (nano TiO2) (Mytych and Rattan, 2016; Zhou et al., 2019). Knockdown of FOXO3a in normal fibroblasts could reproduce idiopathic pulmonary fibrosis (IPF) fibroblast phenotype (Al-Tamari et al., 2018). Whereas FOXO3a overexpression in mouse lung epithelial cell could induce unsuccessful repair and renewal of epithelial cell subsequently and thereafter fail to prohibit the accumulation of myofibroblasts in pulmonary fibrosis (Xie et al., 2017a). Interestingly, it's asserted that up-regulated activity of FOXO3a could dampen the activation of the NLRP3 inflammasome in Kupffer cells (Liu et al., 2017), while FOXO3a dysfunction was found to exacerbate pulmonary fibrotic pathogenesis through inducing inflammatory responses (Nie et al., 2017). Therefore, it is hypotheses that FOXO3a-modulated NLRP3 inflammasome contributes to the pulmonary fibrosis induced by CBNPs.

FOXO3a protein is modulated by multiple regulatory factors with lately observations manifest a critical role played by miRNAs (Ching et al., 2016; Li et al., 2016; Spinetti et al., 2013). MiR-96 has been found to directly bind to 3′-untranslated region of FOXO3a mRNA, which subsequently inhibits its function (Lin et al., 2010). Alternation of miR-96 expression was detected to be induced by PM2.5, titanium dioxide nanoparticles or ambient black carbon particles in epithelial cells, animals and human (Colicino et al., 2016; Mao et al., 2018; Wang et al., 2019a). Aberrant expression of miR-96 was significantly associated with inflammation and fibrosis (Yu et al., 2018). Down-regulated miR-96 expression could strongly activate platelet and up-regulate inflammation (Wang and Cheng, 2015). In vitro transfection of miR-96 mimic into human hepatic stellate cell could decrease expressions of alpha smooth muscle actin (α-SMA) and collagen type I alpha 1 (CollA1), thus indicating an anti-inflammatory and anti-fibrotic role mediated by miR-96 (Luo et al., 2018). Therefore, we hypothesize that CBNPs might induce miR-96 dysregulation and subsequently affect FOXO3a activity, which drove NLRP3 inflammasome assembling and contribute to pulmonary fibrosis.

In the current study, we established a nose-only CBNPs exposure rat model and an in vitro study with human bronchial epithelial cell line (16HBE) to evaluate the pulmonary fibrosis and its mechanism related with inflammation. FOXO3a suppression and overexpression 16HBE cells were applied to elucidate the potential role of FOXO3a on mediating inflammasome after CBNPs treatment. MiR-96 on the pulmonary fibrosis pathogenesis via modulating FOXO3a was studied by using miR-96 mimic and inhibitor. This study will provide valuable insights into the potential pathological mechanisms underlying pulmonary fibrosis induced by CBNPs exposure and shed more light on preventive and therapeutic strategies in cope with such lesions.

2. Materials and methods

2.1. Characterization of CB particles

Carbon black power (>99.8% purity) was purchased from the CB manufacturing factory (Jiaozuo Chemical Industry Limited Company, Henan, China) and its physiochemical characteristics were the same as previous reports (Zhang et al., 2014). In brief, Tecnai

G220 transmission electron microscope (TEM, FEI, USA) and Sirion 200 scanning electron microscope (SEM, FEI, Holland) were applied to evaluate the size and morphology of CB particles. Brunauer-Emmett-Teller (BET) method was used to assess the specific surface characteristics of CB. Distribution of the size of CB nanoparticles in exposure chambers was measured by ultraviolet aerodynamic particle sizer (TSI instrument Co., Ltd, Minneapolis, USA). In consideration of the in vitro assay, CB was suspended in Modified Eagle's medium (MEM) and the size and zeta potential of CB particles were performed on DelsaTM Nano particle analysis instrument (Beckman coulter, CA, USA). Detection for endotoxin of the CB particles was carried out with Toxin SensorTM Chromogenic LAL Endotoxin Assay Kit (Genscript, NJ, USA) following the manufacturer's protocol. It based on the principle that endotoxin could catalyze the activation of a proenzyme in Limulus amoebocyte lysate (LAL), which could subsequently cleave a substrate to produce a colored end-product. The end-product was measured spectrophotometrically and compared to the standard curve to calculate endotoxin content in CB particles. Three separate tests were conducted.

2.2. Animals and experimental design

Forty-nine male specific pathogen-free Soragues-Dawley rats purchased from Center of Experiment Animal of Hebei Medical University at 6 weeks of age were subjected to the study. Before the commencement of the CBNPs inhalation exposure, rats remained without manipulation for a week to eliminate distress from transportation. Then rats were randomly divided into 7 body weight-matched groups, means the 14-day exposure group (30 mg/m^3), the 28-day exposure groups (including 5 mg/m^3 and 30 mg/m^3), the 90-day exposure group (30 mg/m^3) and their respective controls. Rats in exposure groups were placed in polycarbonate chambers and exposed to CBNPs by nose-only inhalation. The control rats were exposed to clean air only in same-sized chambers equipped with HEPA-filters located in the same air conditioned room. CBNPs exposure time was set as 6 h a day and 7 days per week. Rats were kept access to free-feeding of commercial diet and water except the exposure period. Twenty-four hours after the last exposure, rats were anesthetized by pentobarbital sodium and sacrificed. Lungs of rats were separated for further study. The present protocol was approved by the Committee of the Ethics Animal Experiments of Hebei Medical University (IACUC-Hebmu-20160047) and carried out under the Hebei Medical University institutional guidelines for ethical animal use.

2.3. Inhalation system and CBNPs suspension preparation

The nose-only exposure mode could ensure maximum reproducibility of the test substance uptake by inhalation, thus minimizing the substance deposition on the fur that could be subsequently taken by grooming. Here the inhalation exposure system (Beijing Huironghe Technology Co., Ltd. Beijing, China) was used to exposure rats to different doses of CBNPs suspended in the air as our previous description (Chu et al., 2019). The exposure system mainly consisted of an aerosol generator, an exposure column, a gas control system, a sampler, a detector for exposure parameters (temperature, humidity, oxygen and carbon dioxide concentrations) and multiple independent polycarbonate chambers that only permit the tip of the rat's nose out of the opening in front of the chamber (Fig. S1). Briefly, CB powder was baked at 80 °C for 12 h before packed in an aerosol generator column. Then the CB powder was transported onto a rotating brush at 13 L/min feed rate and being loosened into practically individual particle at 23 L/min speed in the dispersion head by dispersion air at 10 L/min. The concentration of CB was precisely tuned by the aerosol generator via a sampling pump. The relative humidity, temperatures, oxygen concentration and carbon dioxide concentration of the exposure chambers during exposure period were monitored and recorded continuously. Besides, the concentration of CBNPs in exposure chambers was calibrated by filter weighting method once per hour throughout all exposure durations using a sampler. In attempt to assess the deposition quality of CBNPs in lung tissue of rats, we applied a MPPD model (version 3.04) and calculated the deposition fraction was 0.1146.

As for the in vitro assay, after heating at 80 °C for 12 h, CBNPs was freshly suspended into PBS (supplemented with 0.04% Tween80) to achieve a final concentration of 10 mg/mL. Before use, the stock suspension was vigorous stirred and then sonicated for 30 min at 60 W with an ultrasonic disintegrator (KQ3200E, Kunshan Ultrasonic Instrument Co., Ltd.).

2.4. HE, masson, immunohistochemistry and immunofluorescence analysis

After deeply euthanized by an overdose of pentobarbital (>100 mg kg-1 body weight, i.p.), the lungs were rapidly removed and tissue sections from the left lung lobe of 3 rats in each group were fixed by intratracheal perfusion with 4% paraformaldehyde for 48 h, dehydrated in a graded ethanol series and embedded in paraffin. For pathological analysis, five-micron-thick sections were stained with hematoxylin and eosin and examined under light microscope (Olympus Optical Co., Ltd, Tokyo, Japan).

The distribution of fibrous collagen in lung tissue was determined by morphometric evaluation of specimens via Masson trichrome staining according to manufacturer's protocol (Sigma Aldrich, Missouri, USA). Briefly, the sections were stained with iron hematoxylin, Ponceau-acid fuchsin solution and phosphomolybdic acid solution sequentially, and then stained with aniline blue. Sections were examined microscopically with × 200 and × 400 magnification thereafter.

Immunohistochemistry (IHC) was carried out for evaluating α-SMA and FOXO3a expressions in lung tissues of rats. The paraffin sections were dewaxed in xylene, rehydrated with distilled water and subjected to antigen retrieval at 95 °C for 30 min. Subsequently, slides were incubated with α-SMA antibody (1:100; EarthOx, LLC, San Francisco, USA) or FOXO3a antibody (1:1000; Cell Signaling Technology, Boston, USA) overnight at 4 °C. Then the excess primary antibody was washed away by PBS washing and slides were incubated with an anti-rabbit secondary antibody (ZSGB-Biology, Beijing, China) and developed using avidin-conjugated HRP with diaminobenzidine (DAB) as a substrate (ZSGB-Biology), followed by hematoxylin counterstaining. Images were visualized and photographs were taken using a light microscope (Olympus Ltd., Japan).

For detection of FOXO3a expression in 16HBE cells under CBNPs treatment, cells were cultured on coverslips and treated with various concentrations of CBNPs for 24 h. Thereafter, cells were washed carefully with PBS for three times and fixed with 4% paraformaldehyde for 15 min followed by another three-times washing. Then cells were treated with 0.5% Triton X-100 and incubated in FOXO3a primary antibody (1:1000; Cell Signaling Technology, Boston, USA) at 4 °C overnight. Next, Dylight549-conjugated goat anti-rabbit IgG (MultiScience Biotechnology Co., Ltd, Hangzhou, China, 1:200 with 1% BSA/PBS) were incubated for 60 min at room temperature in dark. Subsequently, 2 μg/mL DNA-specific fluorochrome 4, 6-diamino-2-phenylindole (DAPI) was given for nuclei staining. Slides were observed and images were taken under fluorescence using an Olympus BX51T microscope.