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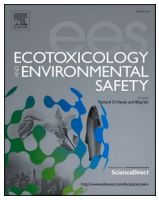



N^6-methyladenosine mediates Nrf2 protein expression involved in PM2.5-induced pulmonary fibrosis

Ding Ji [a,d,1], Chenxi Hu [a,e,1], Jie Ning [a,1], Xiaoling Ying [b], Haiqing Zhang [b], Bohan Zhang [a], Bixia Liu [b], Qingping Liu [a], Weidong Ji [b,*], Rong Zhang [a,c,**]

[a] Department of Toxicology, School of Public Health, Hebei Medical University, Shijiazhuang 050017, PR China
[b] Center for Translational Medicine, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, PR China
[c] Hebei Key Laboratory of Environment and Human Health, Shijiazhuang 050017, PR China
[d] Department of Otolaryngology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, PR China
[e] Department of Urology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, PR China

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ABSTRACT

It has been reported that particulate matter with an aerodynamic diameter of <2.5 μm (PM2.5) could induce epithelial–mesenchymal transition (EMT)- and extracellular matrix (ECM)-related pulmonary fibrosis (PF). The transcription factor Nrf2 alleviated PM2.5-induced PF by antagonizing oxidative stress. The N^6-methyladenosine (m^6A) modification plays a significant role in the stress response. However, the effect of m^6A modification on the mechanisms of Nrf2-mediated defense against PM2.5-induced PF remained unknown. Here, we explored the role and the underlying molecular mechanisms of m^6A methylation of Nrf2 mRNA in PM2.5-induced PF. We established filtered air (FA), unfiltered air (UA), and concentrated PM2.5 air (CA) group mice model and 0, 50, and 100 μg/mL PM2.5-treated 16HBE cell models. The extent of lung fibrosis in mice and fibrosis indicators were detected by histopathological analysis, immunohistochemical staining and western blotting. The molecular mechanism of m^6A-modified Nrf2 was demonstrated by m^6A-methylated RNA immunoprecipitation (MeRIP), RNA immunoprecipitation (RIP), qRT-PCR and T3 ligase-based PCR. Our data showed that PM2.5 exposure for 16 weeks could induce pulmonary fibrosis and activate Nrf2 signaling pathway. m^6A methyltransferase METTL3 was upregulated after PM2.5 treatment in *vivo* and in *vitro*. Moreover, METTL3 mediated m^6A modification of Nrf2 mRNA and promoted Nrf2 translation in mice and 16HBE cells after PM2.5 exposure. Mechanistically, three m^6A-modified sites (1317, 1376 and 935; numbered relative to the first nucleotide of 3'UTR) of Nrf2 mRNA were identified in PM2.5-treatment 16HBE cells. Furthermore, the m^6A binding proteins YTHDF1/IGF2BP1 promoted Nrf2 translation by binding to m^6A residues of Nrf2 mRNA. Our results revealed the mechanism of m^6A mediated Nrf2 signaling pathway against oxidative stress, which affected the development of PM2.5-induced PF.

1. Introduction

Particulate matters can potentially exacerbate preexisting pulmonary disorders such as asthma, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, and even cancer (Falcon-Rodriguez et al., 2016). Statistical analysis demonstrated that particulate matters with an aerodynamic diameter of < 2.5 μm (PM2.5) levels were correlated with respiratory symptoms in children (Drago et al., 2018). Long-term exposure to elevated levels of PM10 and PM2.5 has positive relation with mortality in patients with idiopathic pulmonary fibrosis (Sesé et al., 2018). Pulmonary Fibrosis (PF) is associated with epithelial–mesenchymal transition (EMT) and extracellular matrix (ECM) (Nair et al., 2016; Z. Zhang et al., 2018). As an important pathway of stromal cell production and excess ECM deposition, EMT can promote

Abbreviations: ARE, antioxidant response element; CA, concentrated air; CDS, coding sequence; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; FA, filtered air; GSH, glutathione; MDA, malondialdehyde; m^6A, N6-methyladenosine; PF, pulmonary fibrosis; PM2.5, particulate matter with an aerodynamic diameter of < 2.5 μm; ROS, reactive oxygen species; SOD, superoxide dismutase; UA, unfiltered air.

* Corresponding author.
** Correspondence to: Dept. Toxicology, Hebei Medical University, 361 Zhongshan east Rd., Shijiazhuang, Hebei 050017, PR China.
E-mail addresses: jiweidong@mail.sysu.edu.cn (W. Ji), rongzhang@hebmu.edu.cn (R. Zhang).
[1] These authors contributed equally to this work.

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scar tissue formation in the lungs, eventually leading to the development of PF (Kim et al., 2006).

NLRP3 inflammasome triggered by PM2.5 was associated with lung fibrosis (Zheng et al., 2018). PM2.5 increases ROS production, therefore, aggravating the pulmonary inflammatory response in mice (Valavanidis et al., 2013; L. Wang et al., 2019). Nuclear factor erythroid-2-related factor 2 (Nrf2) signals regulate reduction-oxidation balance and play important roles during the development of PF (Borcherding et al., 2019). A previous study found that *Trametes orientalis* polysaccharide (TOP-2) could alleviate PM2.5-induced lung injury in mice by activating the Nrf2/HMOX-1 pathway and inhibiting the NLRP3 inflammasome (Zheng et al., 2019). Nrf2 attenuates the EMT and PF development processes by regulating the expression of the transcription factor Snail in bleomycin-induced PF (Zhang, Z. et al., 2018; Zhou et al., 2016). These results indicate Nrf2 signal activation might be involved in PM2.5-induced PF.

Environmental pollutants can alter global N^6-methyladenosine (m^6A) levels and the expression of RNA methylation modulator genes, thereby inducing injury (Cayir et al., 2019). Our previous study found that m^6A-regulated miRNA-126 processing activated the PI3K-AKT-mTOR pathway and exacerbated PF in rats after nanoscale carbon black particle inhalation (Han et al., 2020). It has been reported that m^6A-regulated miR-873–5p can mediate the Keap1-Nrf2 pathway to resist colistin-induced oxidative stress (J. Wang et al., 2019). Furthermore, m^6A-mediated nuclear receptor PPaRα mRNA stability to regulate lipid metabolism during oxidative stress, which was induced by circadian clock change in mice liver (Zhong et al., 2018). In our previous study, m^6A on Nrf2 mRNA was concentrated in 3′-untranslated region (UTR) in human bronchial epithelium cell line (16HBE cells) (Yang et al., 2019). Zhao et al. found Nrf2 mRNA m^6A methylation in prepubertal testicular tissue of rats exposed to di-(2-ethylhexyl) phthalate (DEHP) (Zhao et al., 2020), suggesting the presence of potential m^6A methylation sites in the 3′-UTR of Nrf2 mRNA. Therefore, it is hypothesized that m^6A-modification of Nrf2 mRNA regulates the Nrf2 signal activation and then could defend the oxidative stress which affects the development of EMT and PF after PM2.5 exposure.

In this study, we established PM2.5 exposure models in mice and 16HBE cells. The PM2.5 was collected from Shijiazhuang, Hebei province, China. Our data identified that altered m^6A modification of Nrf2 mRNA could affect the development of PF induced by PM2.5. Furthermore, we determined the exact Nrf2 mRNA m^6A sites (1317, 1376, and 935) which enhanced Nrf2 translation by binding the m^6A-binding proteins, such as YTH domain-containing proteins (YTHDFs) and insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs). Our investigation indicates that the m^6A-mediated Nrf2 signaling pathway might be as a potential target in preventing PM2.5-induced PF.

2. Materials and methods

2.1. PM2.5 collection and extraction

The collection of PM2.5 come from Shijiazhuang, Hebei province, China, which was the same as the time with mice exposure. Airborne PM2.5 was collected on a Teflon® filter by a high-volume air sampler (Thermo Scientific, Franklin, MA, USA) operating at ventilation frequency (18–20/h). The retrieved filter membranes were divided into small pieces, ultrasonicated in iced water for 30 min. The PM2.5 extracts were collected, freeze-dried, packed in tinfoil, and preserved at −20 °C. The PM2.5 extracts were resuspended in deionized water until use.

2.2. Animals and PM2.5 exposure

Twenty-four male C57BL/6 mice (six-week-old), weighing 18–20 g, were obtained from Vital River Laboratory (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China). During the acclimatization and exposure, distilled water and commercial standard pellet diet were provided to all the mice, which were housed in cages with a 12:12 light/dark cycle and standard conditions (22–24 °C and 50 % relative humidity). All mice were exposed to different air for 16 weeks, 6 h per day (from 9:00–15:00), 7 days per week and they were housed in an individually ventilated cage (IVC) with clean air during the remaining time.

Twenty-four mice were randomly divided into three groups (8 mice/group). Random number remainder grouping method was used for random grouping. The mice were whole-body exposed to filtered air (FA), unfiltered air (UA), and concentrated PM2.5 (CA) after 1 week of acclimatization. The FA group served as control. The FA and UA mice were housed in the chambers with or without high efficiency particulate air filters, respectively. CA mice were housed in the chamber with the concentrated PM2.5 using the PM2.5 concentration enrichment system (Beijing Huironghe Technology Co., Ltd, Beijing, China) according to our previously reported procedures (Su et al., 2020). After the last day of exposure, all mice survived and were anesthetized with 1 % pentobarbital sodium, then sacrificed, and lung tissues were separated and weighed. Six random samples every group was applied for in vivo study. All experimental protocols were approved in advance by the Committee of the Ethics Animal Experiments of Hebei Medical University (IACUC-Hebmu-20180163).

2.3. Cell culture and treatment

The human bronchial epithelial cell line (16HBE) was gifted by Dr. D. C. Gruenert (University of California, San Francisco, CA, USA) (Gross et al., 1993). The cells were cultured in basic Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) with 10 % fetal bovine serum (FBS), 100 U/mL Penicillin, and 100 μg/mL Streptomycin in a water-saturated atmosphere under 5 % CO_2 at 37 °C. All the mycoplasma contamination tests conducted using a polymerase chain reaction (PCR)-based universal mycoplasma detection kit were negative. The medium was replenished every day. A 0.05 % solution of pancreatin ethylenediaminetetraacetic acid (EDTA; Gibco) was used to dissociate the cells when they completely covered the bottom of the culture dish.

The cells were transferred to 6-well plates with DMEM, FBS and Penicellin-Streptomycin, then serum starved for 12 h when the cells covered 80–90 % of the board. The PM2.5 extracts were resuspended in deionized water and diluted freshly by DMEM. The cells were then treated with 0, 50, or 100 μg/mL PM2.5 for 24 h. 2.0 mL/well PM2.5 solution (50 and 100 μg/mL) was added in 6-well cell culture plates, which was equal to 10.42 μg/cm^2 and 20.83 μg/cm^2.

2.4. Histopathological analysis and immunohistochemical (IHC) staining

Lungs were fixed with 4 % paraformaldehyde (6 mice per group). The paraffin-embedded lung tissues were cut into 5-μm continuous sections then stained with hematoxylin and eosin (H&E). According to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA), Masson's trichrome staining was performed to evaluate the distribution of fibrous collagen in lung. Tissue sections were observed under a light microscope (Olympus Optical Co., Ltd, Tokyo, Japan) to evaluate changes of histopathology and fibrosis, respectively. Histological examination was performed by two experienced histologists in a blinded manner. Three continuous slices in each lung and six random fields were selected from each slice at 200 × magnification under microscopy. ImageJ software was used to quantify the results of Masson's trichrome. The collagen volume fraction was calculated as the mean ratio of fibrotic area to the whole area of the lung tissue from all the measurements from the section (Paul et al., 2017), the data were calculated and analyzed by SPSS software.

For IHC staining, serial tissue sections were dewaxed in xylene and rehydrated with distilled water. Then antigen was retrieved by the target antigen retrieval solution (Dako, Carpinteria CA, USA) for 30 min at 95 °C. After the primary antibodies against Nrf2 (BS1258, Bioworld,