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Hydrogen ameliorates lung injury in a rat model of subacute exposure to concentrated ambient PM2.5 via Aryl hydrocarbon receptor

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ABSTRACT

Background: Ambient fine particulate matter (PM2.5) could induce lung injury. Aryl hydrocarbon receptor (AhR) is involved in the molecular mechanisms of prooxidative and pro-inflammatory effect of PM2.5. Molecular hydrogen has antioxidant properties. The protective effect and mechanism of hydrogen on PM2.5-induced lung injury remain unclear.

Objectives: This study aimed to determine whether hydrogen could alleviate lung injury in a rat model of subacute exposure to concentrated ambient PM2.5, and explore the mechanism related to AhR.

Methods: Male Wastar rats were exposed to either concentrated ambient particles (CAPs) (diameter: ≤2.5 μm, average concentration: 1328 ± 730 μg/m^3) or filtered air (FA) by nose-only inhalation (5 h/day, 5 days/week for 4 weeks). Hydrogen-treated rats inhaled 66.7% hydrogen from water electrolysis for 2 h after each exposure to CAPs or FA.

Results: CAPs inhalation induced lung injury, as demonstrated by pulmonary function decrease, histopathological damage, mucus hypersecretion [Periodic acid-Schiff (PAS) staining for mucins, immunohistochemistry and quantitative real-time PCR (RT-qPCR) for mucin 5AC (MUC5AC) expression], increased pro-inflammatory cytokines (TNF-α, IL-8 and IL-1β) and oxidative damage indexes [malondialdehyde (MDA) and 8-isoprostane F2α (8-iso-PG)]. While, hydrogen inhalation significantly alleviated the damages mentioned above. In addition, low expression of AhR in lung tissues determined by Western Blot was found after CAPs exposure, whereas hydrogen inhibited AhR decline induced by CAPs.

Conclusions: High concentrations of hydrogen could ameliorate pulmonary dysfunction, airway mucus hypersecretion, oxidation damage, and inflammation response in rats exposed to concentrated ambient PM2.5. Additionally, hydrogen alleviates lung injury induced by PM2.5 possibly through AhR-dependent mechanisms.

1. Introduction

In recent years, air pollution, especially the fine particulate matters (aerodynamic diameter < 2.5 μm, PM2.5), has posed a huge threat to public health. In some areas in China and other countries, people have been suffering from serious air pollution with a high concentration of PM2.5. Many epidemiological investigations have identified that the increase of PM2.5 concentration is significantly related to the increased number of outpatient visits in the department of respiration [1]. It is critical to develop therapeutic strategies preventing the respiratory system damages from PM2.5 exposure.

Previous studies have shown that PM2.5 could lead to lung injury, manifested by decreased lung function, infiltration of inflammatory cells in lung tissues, pulmonary edema, airway mucus hypersecretion, and so on. The effects of PM2.5 are related to oxidative stress and inflammatory responses. The exposure to PM2.5 could result in reactive oxygen species (ROS) production [2] and pro-inflammatory cytokines release in airway epithelial cells [3,4]. ROS include a wide variety of

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molecules and free radicals derived from molecular oxygen, such as superoxide anion radical (O_2^-•), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), etc [5]. The generation of ROS in turn may provoke inflammatory responses and induce the release of pro-inflammatory cytokines and chemokines, leading to inflammation and lung injury [5].

Hydrogen is a kind of noble antioxidant gas with the character of selectively neutralizing cytotoxic ROS [6]. Many researchers have demonstrated the therapeutic effects of molecular hydrogen on various diseases through its antioxidative capability [7]. A new study showed that hydrogen inhalation could significantly improve the lung inflammation and lung functions in a cigarette smoke-induced COPD rat model [8]. While, the effects of hydrogen on lung injury induced by PM2.5 are still uncertain. And the underlying mechanism of effects of hydrogen remains to be elucidated.

The specific activity of scavenging hydroxyl radical cannot fully explain the anti-oxidative stress and anti-inflammatory effects of hydrogen. Recently, some studies have shown that hydrogen reduces hyperoxic lung injury via the nuclear factor erythroid 2 p45-related factor 2 (Nrf2) pathway [9]. While, the activation of Nrf2 is mediated by Aryl hydrocarbon receptor (AhR) [10]. Polycyclic aromatic hydrocarbons (PAHs), which are organic components in PM2.5, act as ligands for AhR. AhR has an important role in oxidative stress and antioxidant defense [11]. Oxidative stress results from the imbalance of prooxidative and antioxidative mechanisms. Some evidences indicated that the activation of AhR can lead to oxidative stress [12]. And, it has been reported that AhR is involved into the molecular mechanisms of prooxidative and pro-inflammatory effect of PM2.5 [13,14]. However, protective functions of AhR against oxidative stress have also been proved in vitro and in vivo. For instance, down-regulation of AhR expression increased ROS production and amplified the hyperoxic toxicity [15]. AhR-/- mice are more susceptible to hyperoxia-mediated lung injury and inflammation [16–18]. Some AhR signaling pathways, such as the AhR-Nrf2 or AhR-cytochrome P450 1A1(CYP1A1) pathway, could play antioxidative responses [11]. If hydrogen could reduce PM2.5-induced lung injury, whether the protective effects of hydrogen is related to AhR needs to be investigated.

In this study, we used the CAPs exposure facility to conduct PM2.5 inhalation toxicology research, and the rats were exposed to high concentrations of real-time CAPs (Particle size distriction meets PM2.5) by nose-only inhalation for 4 weeks. We assessed the effect of PM2.5 inhalation on pulmonary function, airway mucus secretion, airway inflammation and oxidative stress indexes in healthy rats, and examined the effects of 66.7% hydrogen inhalation on alleviating lung injury caused by PM2.5. Concerning on the mechanisms about hydrogen protective role, we observed the expression of AhR in the lung of rats. Our hypothesis is that the PM2.5 could activate AhR, and hydrogen could affect the expression of pulmonary AhR protein after PM2.5 exposure and reduce the degree of lung injury. This study illustrated hydrogen could be a therapeutic agent to prevent pulmonary impairment from PM2.5 attack. And AhR could be a new target for further study on the mechanism of hydrogen actions.

2. Materials and methods

2.1. Animals

Thirty-five male Wistar rats (200 ± 20 g, laboratory Animal Center of Hebei Medical University, Shijiazhuang, China) were used in this study. All animal experiments were performed in a humane manner, and also in accordance with the Institutional Animal Care Instructions. Animal handling and experimental procedures described herein were approved by the Ethical Committee on Animal Use of the Second Hospital of Hebei Medical University.

Animals were housed in individually ventilated cages (maximum of 4 rats per cage) with filtered clean air, with a controlled temperature (22 ± 0.65 °C) and humidity (56 ± 0.08%) environment, and a 12-h light/dark cycle. The rats were fed a rodent diet and filtered water ad libitum.

2.2. Concentrated ambient PM2.5 exposure system

The PM2.5 exposure laboratory is located in the Second Hospital of Hebei Medical University (Shijiazhuang, China), with a 500 m distance from the busy traffic road.

The PM2.5 exposure system mainly contains a ambient fine particle concentrator [PM2.5 versatile aerosol concentration enrichment system (VACES), HRH-PM186, Hui ronghe Technology Co., Beijing] and a separate 30-port nose-only inhalation exposure chambers (PM2.5 Nose-only inhalation exposure system, HRH-MNE3026, Hui ronghe Technology Co., Beijing) for rat toxicology studies. The concentrator utilizes the two-stage impact cutters to increase the concentration of particles, which can effectively concentrate the ambient fine particulate matters (aerodynamic diameters ≤ 2.5 μm) from urban atmosphere without significantly changing its major physicochemical features and with a maximal concentrated factor of 10–30 folds. Then the rats were exposed to CAPs through the nose-only inhalation exposure chambers. During the exposure time, chamber environmental conditions (temperature, humidity, airflow, and chamber pressure) were monitored continuously, and real-time CAPs concentrations were estimated with an aerosol mass concentration monitor (CEL-712 Microdust pro, CASELLA, U.K.) on a nose-only chamber exhaust. Meanwhile an aerosol particles distribution monitor (GRIMM Aerosol Technik, 11-A, GmbH & Co.KG Germany) was operated to monitor detailed information on the size-fractionated aerosol counts. The GRIMM, which contains 31 size channels, can provide real-time aerodynamic diameter measurements in the range from 0.25 μm to 32 μm. It is enough to illustrate the particle size range of PM2.5.

The data of ambient PM2.5 components analysis during the exposure were got from Shijiazhuang Environmental Monitoring Center. Elemental composition, anion and NH4 + ions, elemental carbon (EC) and organic carbon (OC) were measured.

The data about component analysis of PAHs in ambient PM2.5 during the exposure period were got from Hebei University of Science and Technology, and PAHs components were analyzed by the gas chromatography and mass spectrometry [19].

2.3. Hydrogen administration

The mixed gas containing 66.7% hydrogen gas (H_2) and 33.3% oxygen (O_2) was produced by the hydrogen oxygen nebulizer (AMS-H-01, Asclepius, Shanghai, China), which was specifically designed to extract the hydrogen and O_2 from water by electrolysis, as described in previous studies [20–22]. Meanwhile, the oxygenerator (Asclepius Meditec Co.) was used as control, which could produce 66.7% nitrogen (N_2) and 33.3% O_2 mixture gas. All of the gas was inhaled in a transparent closed chamber. During exposure, a hydrogen concentration detector (XP-3140, Cosmos electric KK, Japan) and an oxygen concentration detector (CY-12C, Xin'An analytical instrument Co. Zhejiang, China) were used to monitor the concentration of hydrogen and O_2 in the chamber respectively.

2.4. Animal grouping and CAPs exposure protocol

After acclimating in IVC cages for 1 week, all rats were trained to adapt to the nose-only restraint cones for 7 days: the fixed time was 1 h on the first day, and the following fixed time was increased by one hour per day until 5 h/d.

The CAPs exposure period was from late February to late March in 2016 when the air pollution was usually very high in Shijiazhuang. The rats were randomly divided into five groups with 7 animals each: (1) FA (filtered air): the rats received filtered fresh air instead of CAPs in the exposure chambers, 5 consecutive hours/day, 5 days/week for 4 weeks;

(2) PM: the rats were exposed to CAPs, 5 consecutive hours/day, 5 days/week for 4 weeks; (3) PM + N (PM + 67% N_2, 33% O_2): the rats inhaled the 67% N_2 and 33% O_2 mixture gas for 2 h after each CAPs exposure (set as a negative control of PM + H group to remove the bias of 33% O_2); (4) PM + H (PM + 67% H_2, 33% O_2): the rats inhaled the 67% H_2 and 33% O_2 mixture for 2 h after each CAPs exposure; (5) FA + H (FA + 67% H_2, 33% O_2): the rats inhaled the 67% H_2 and 33% O_2 mixture for 2 h after each FA exposure.

2.5. Lung mechanics and pulmonary function measurement

Lung mechanics and pulmonary function were performed with a computer-controlled small-animal ventilator (FlexiVent; SCIREQ, Montreal, QC, Canada), as described previously [23]. 24 h after the last FA or CAPs exposure, rats were endotracheally intubated under pentobarbital anesthesia (70 mg/kg, intraperitoneally). Then paralysis was induced with 0.1 mg/kg Vecuronium Bromide injected intraperitoneally. Briefly, total respiratory system elastance [Ers] and total respiratory system resistance [Rrs] were measured by the snap shot and forced oscillation technique. Determination of forced vital capacity [FVC] and peak expiratory flow [PEF] were performed with the same computer-controlled small-animal ventilator connected to a negative pressure reservoir (SCIREQ), as described previously [23].

2.6. Bronchoalveolar lavage fluid (BALF) and tissue samples collection

After measurement of pulmonary function, rats were sacrificed by exsanguination from femoral arteries under deeply anesthetized. Immediately after death, the right extrapulmonary bronchus was ligated and the right lung lobes were removed. The right cranial lobe and middle lobe were stored at −80 °C until use for PCR or Western Blot analysis. The right caudal lobe was removed and fixed in 10% formalin neutral buffer solution for 24 h, After that, paraffin embedding was performed for histological analysis. Bronchoalveolar lavage fluid (BALF) was isolated by instilling and recollecting 3 ml of sterile PBS in the left lung, and this procedure was repeated three times, and the recovery volume was over 80% that of instilled. After centrifugation, the supernatant of BALF samples were stored at −80 °C for further analysis.

2.7. Histopathology

2.7.1. H&E staining

The paraffin blocks were cut into 4 μm-thick sections from mid-sagittal and para-hilar regions (perpendicularly to the main axial airway) which were stained with hematoxylin and eosin (H&E) to evaluate general morphology. The lung inflammation was evaluated by two analysts blinded to the groups using the pulmonary inflammation scoring method (see Table 1).

2.7.2. PAS staining

Periodic acid-Schiff (PAS) staining was applied to detect mucosubstances, using an PAS staining kit (Baso Biological Technology Co. Zhuhai, China). PAS-positive area and total area of corresponding bronchial epithelium area were measured by using the Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA), at a magnification of 200× by examining at least 50 consecutive fields for each group. And the data were presented as the ratio of PAS-positive area to the total area.

2.7.3. Immunohistochemistry (IHC)

For IHC, the sections were immunostained with anti-MUC5AC mouse monoclonal antibody (clone 45M1, Santa Cruz) according to the manufacturer's instructions. The sections were developed by diaminobenzidine solution (Yi Sheng Biotechnology Co. Shanghai, China) according to the manufacturer's instructions. Semi-quantitative analyses

Table 1
The pulmonary inflammation scoring method.

Parameter	Score				
	0	1	2	3	4
Capillary congestion	None		Only a few red blood cells	More red blood cells	Red blood cells almost fill the lumen
Proteinaceous debris filling the airspaces	None		Only a little fibrin exudation	More fibrin exudation	Fibrin exudation almost fills the lumen
Neutrophil exudation	None	Suspicious neutrophil exudation	Dispersed neutrophil exudation	Focal exudation of neutrophils	Large area exudation of neutrophils
Airway epithelial cells exfoliation	None	Suspicious epithelial cells exfoliation		Local epithelial cell exfoliation in lumen	Extensive exfoliation of epithelial cells
Alveolar septal thickening	Relatively normal	Slightly thickened		Significant thickened	Loss of normal alveolar structure

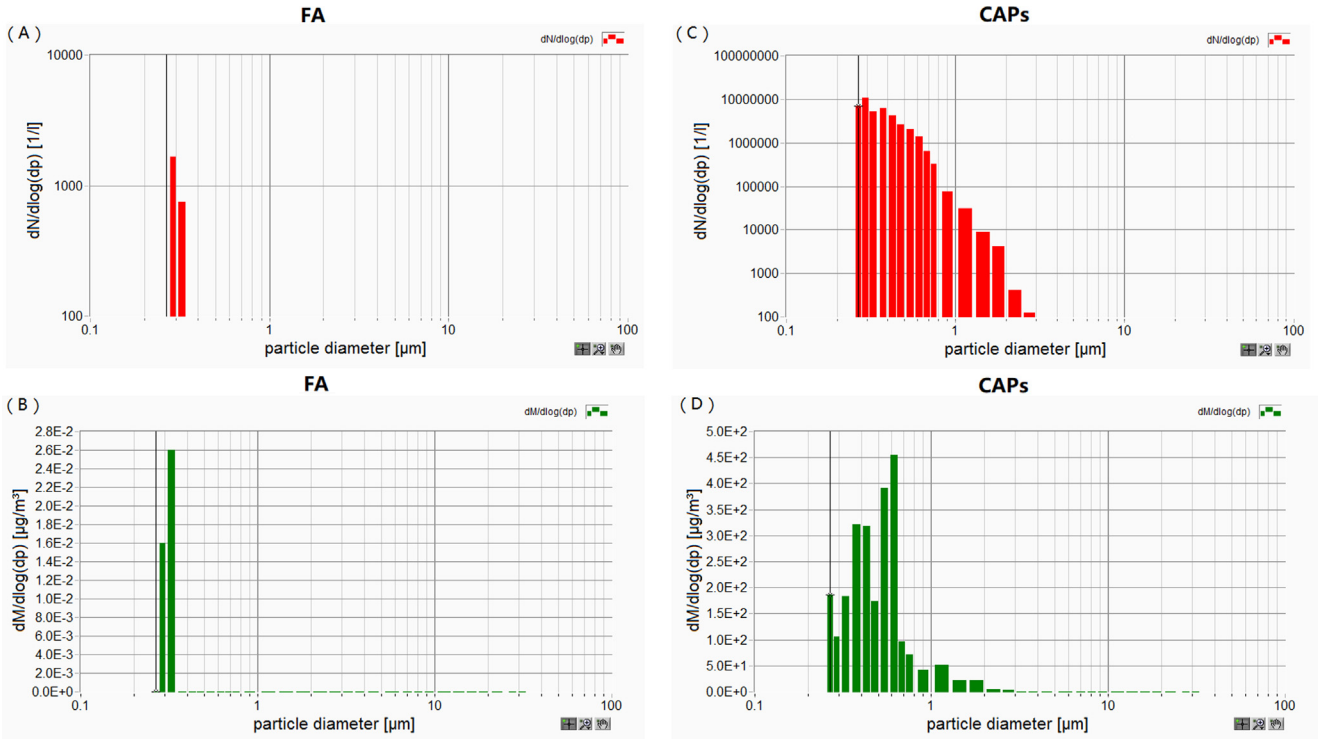

Fig. 1. The size distribution of CAPs. Particulate matter spectrometer Grimm11-A (GRIMM Aerosol Technik GmbH & Co. KG Germany) was operated to monitor detailed information on the size-fractionated aerosol counts. (A) the number of different size particles of FA (B) the mass of different size particles of FA (C) the number of different size particles of CAPs (D) the mass of different size particles of CAPs.

of the area of muc5ac-positive staining in the airway epithelium were defined by two independent investigators using the Image-Pro Plus 6.0, at a magnification of 200× by examining at least 50 consecutive fields for each group. And the data were presented as the ratio of IOD (integrated optical density, IOD = area × density) of MUC5AC-positive area to the total IOD of corresponding bronchial epithelium. All the images were captured with a light upright microscope (Zeiss, Germany).

2.8. Oxidative stress detection

To determine whether PM2.5 could induce the production of reactive oxygen species (ROS) in the lung, malondialdehyde (MDA) was measured after the lung tissues were homogenized in ice-cold KCl buffer (pH 7.6). The homogenate was then centrifuged to remove debris, and the clear upper supernatant fluid was taken for evaluating MDA concentration by MDA detection kit (Nanjing Jiancheng, Nanjing, China). The results were expressed as nmol/mg. 8-isoprostane F2α(8-iso-PG) in BALF supernatants was measured using a ELISA kits (Neobioscience biological Technology Co. Shanghai, China) following the manufacturer's instruction.

2.9. Measurement of inflammatory cytokines in BALF

The concentrations of TNF-a, IL-8 and IL-1β in BALF supernatants were determined by ELISA kits (Neobioscience biological Technology Co. Shanghai, China) following the manufacturer's instruction. The optic density was measured at 450 nm with a microplate photometer Elx800 (Biotek, Colmar, France).

2.10. RNA isolation and quantitative real-time PCR (RT-qPCR) analysis

Total RNA from the lung tissue homogenate was extracted using Trizol reagent (Tiangen biochemical Technology Co., Ltd. Beijing, China) as instructed by the manufacturer.

Total RNA was reverse transcribed to cDNA using Reverse Transcription Reagents (ABM, Canada). The expression of MUC5AC was measured by quantitative real-time PCR using SYBR Green Master Mix (ABM, Canada) on a StepOne real-time PCR system (Bio-Red, USA). All protocols were performed according to the manufacturer's instructions. Fold changes of mRNA levels were determined after normalization to internal control (GAPDH mRNA levels). The primer sequences of MUC5AC were as follows: Forward:5′-CTGTGTCCATCTTCAACCTTA-3′, Reverse: 5′-GCTCCATCTATCCAATCA-3′.

2.11. Western blot analysis

Briefly, samples (50ug of protein) were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes with 0.45um aperture. The membranes for western blotting were incubated at 4 °C overnight with the primary antibodies as follows: AhR polyclonal antibody (Affinity Biosciences, USA; AF6278; dilution: 1:500), and GAPDH antibody (Abways TECHNOLOGY, Shanghai; AB0037; dilution: 1:3000). Blotting bands were washed in three changes of TBST for a total time of 45 min. The bands were then incubated with the matched horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG HRP matching for AhR and GAPDH, dilution: 1:5000) for 1 h at 22 °C, and washed with TBST and TBS for 10 min. The immunoreactive bands were detected with the enhanced chemiluminescence (ECL) kit and the band density was analyzed by Image J 1.48 V software (National Institutes of Health, USA).

2.12. Statistical analysis

All data were analyzed by SPSS 21.0 or GraphPad Prism 6.0 statistical software and presented as mean ± standard deviation (SD) or Median [25% Percentile, 75% Percentile] (Non-normal distribution data). Comparison of the means among groups was made by one-way

Table 2
Composition of Ambient PM2.5 during exposure.

Components	Median [25% Percentile, 75% Percentile]
Element (μg/m³)	
Fe	0.5350 [0.2275, 0.7300]
Cu	0.0100 [0.0100, 0.0200]
Zn	0.1800 [0.0700, 0.4000]
Pb	0.0800 [0.0600, 0.1700]
Al	0.7500 [0.5950, 0.8600]
Si	1.550 [1.165, 1.945]
Na	1.300 [0.8500, 2.080]
K	1.430 [1.030, 2.400]
Ca	0.6300 [0.2600, 0.8300]
Ions (μg/m³)	
NO_3^-	14.24 [8.080, 32.62]
SO_4^{2-}	8.310 [5.620, 17.03]
NH_4^+	9.500 [5.110, 20.93]
EC and OC (μg/m³)	
OC	21.22 [18.25, 29.38]
EC	4.845 [3.373, 5.888]

analysis of variance (ANOVA). Pairwise comparisons with homogeneity of variance were performed using the Student–Newman–Keuls (SNK) method. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Mass concentrations and particle size distribution of the CAPs

The average ambient daily PM2.5 concentration during the exposure period was 122.4 ± 96.9 μg/m³. The concentrations of CAPs were between 452 μg/m³ (minimum) and 2786 μg/m³ (maximum) in the CAP chambers. The mean mass concentration of CAPs inside the exposure chamber was 1328 ± 730 μg/m³. The CAPs particle size distribution meets PM2.5 as shown in Fig. 1.

3.2. Composition of ambient PM2.5

The composition of ambient PM2.5 during exposure period in this study is presented in Table 2. The concentrations of PAHs in ambient PM2.5 are shown in Table 3.

3.3. Concentrated ambient PM2.5 decreased lung mechanics and pulmonary function, and hydrogen played a protective role in this process

Exposure to PM2.5 was associated with a significant reduction in FVC and PEF in the PM group compared with the FA group ($p < 0.01$, Fig. 2A and B). In contrast, treating PM2.5-exposed rats with hydrogen significantly reversed the changes on FVC ($p < 0.01$, Fig. 2A) and PEF ($p < 0.05$, Fig. 2B) induced by PM2.5. Ers and Rrs were both significantly increased after the rats were exposed to PM2.5 ($p < 0.01$, Fig. 2 C and D), however, the indices rebounded after hydrogen treatment, showing values almost close to that of the FA ($p < 0.01$, Fig. 2C and D). Hydrogen showed a significant protective effect against the PM2.5-induced lung function and Lung Mechanics changes. And there was no difference between the PM group and PM + N group in the indexes, which means simple inhalation of oxygen had no effect on reducing the harm. In addition, the results showed no significant difference between the FA + H group and the FA group.

Table 3
Concentrations of PAHs in Ambient PM2.5 during exposure.

PAHs (ng/m³)	Median [25% Percentile, 75% Percentile]
Naphthalene	12.56 [8.678, 19.16]
Acenaphthylene	0.3100 [0.1725, 1.685]
Fluorene	0.8800 [0.3300, 1.395]
Anthracene	5.120 [3.095, 7.460]
Fluoranthracene	8.160 [3.460, 14.28]
Pyrene	4.870 [1.940, 8.255]
Benzo[a]anthracene	14.26 [4.883, 27.26]
Benzo[b]fluoranthene	29.46 [19.87, 36.05]
Benzo[a]pyrene	15.26 [9.850, 21.18]
Benzo[g,h,i]perylene	17.23 [11.88, 24.49]
Indeno[1,2,3-cd]pyrene	10.01 [7.220, 11.78]
Total PAHs	110.2 [68.22, 158.7]

3.4. Hydrogen inhibited PM2.5-induced mucus hypersecretion and MUC5AC expression in the airway epithelium

As shown in Fig. 3A, PAS-positive staining was sporadically seen in the epithelium of the FA or the FA + H group rats, while the airway epithelium of the PM and PM + N group contained many large granular stores of positive substances, and the PM2.5 exposure significantly increased PAS-positive rate as compared with that in the FA ($p < 0.01$). As expected, much lighter positive staining areas were observed in the PM + H group as compared with the PM or PM + N group ($p < 0.01$). In other words, hydrogen significantly decreased PM2.5-induced positive staining rate. The positive rates of airway epithelium were 1.98 ± 0.22%,13.89 ± 0.46%,13.35 ± 1.04%,5.94 ± 0.90% and 2.22 ± 0.33% in the FA group, PM group, PM + N group, PM + H group and FA + H group, respectively.

To determine the effect of hydrogen on PM2.5-induced expression of MUC5AC, IHC was applied to detect the MUC5AC protein in the airway epithelium, and RT-qPCR was applied to detect the mRNA levels in the lung tissues of rats. As shown in Fig. 3B, positive immunoreactivity for MUC5AC antibody was characterized by brown staining, and the results of immunohistochemical analysis showed that the airway epithelium of the PM and PM + N groups had a large number of brown areas compared with controls. While hydrogen significantly alleviated the positive staining of MUC5AC in the airway epithelium in the PM + H group as compared with the PM or PM + N group. The ratio of IOD of MUC5AC-positive area to the total IOD of corresponding bronchial epithelium was significantly increased (nearly 8-fold higher) in the PM group as compared with the FA group ($p < 0.01$), and this increase was abrogated by hydrogen ($p < 0.01$). What's more, as shown in Fig. 3C, the level of MUC5AC mRNA in rats lung tissues increased by about 6-fold in the PM group as compared with the FA group ($p < 0.01$). Hydrogen significantly decreased PM2.5-induced MUC5AC mRNA levels ($p < 0.01$).

In addition to bronchi and conducting bronchioles, MUC5AC-positive staining was also observed in the epithelial lining of some respiratory bronchioles following CAPs exposure (see Fig. 4). But this phenomenon occured in some individual animals in the PM group and the number was not enough for statistical analysis.

3.5. Hydrogen decreased PM2.5-induced oxidative damage in the lung

PM2.5 exposure induced a significant increase in MDA levels in the lung tissues of rats ($p < 0.01$, Fig. 5A) as compared to the FA group, while hydrogen treatment significantly decreased the MDA levels relative to the PM group ($p < 0.01$, Fig. 5A). The measurement of 8-iso-PG levels in the BALF, another index reflecting oxidative stress, showed a significant increase after exposure to PM2.5, and hydrogen treatment restored the 8-iso-PG levels (Fig. 5B). It appeared that PM2.5 induced oxidative damage to the lung, and hydrogen could alleviate the PM2.5-induced damage, and oxygen inhalation had no protection, as there was no significant difference between the PM + N and the PM group.

3.6. Hydrogen suppressed PM2.5-induced inflammation in the lung

Histopathological examination by H&E staining showed that inhalation of PM2.5 resulted in marked thickening of alveolar walls and infiltration of inflammatory cells, featured with infiltration of the

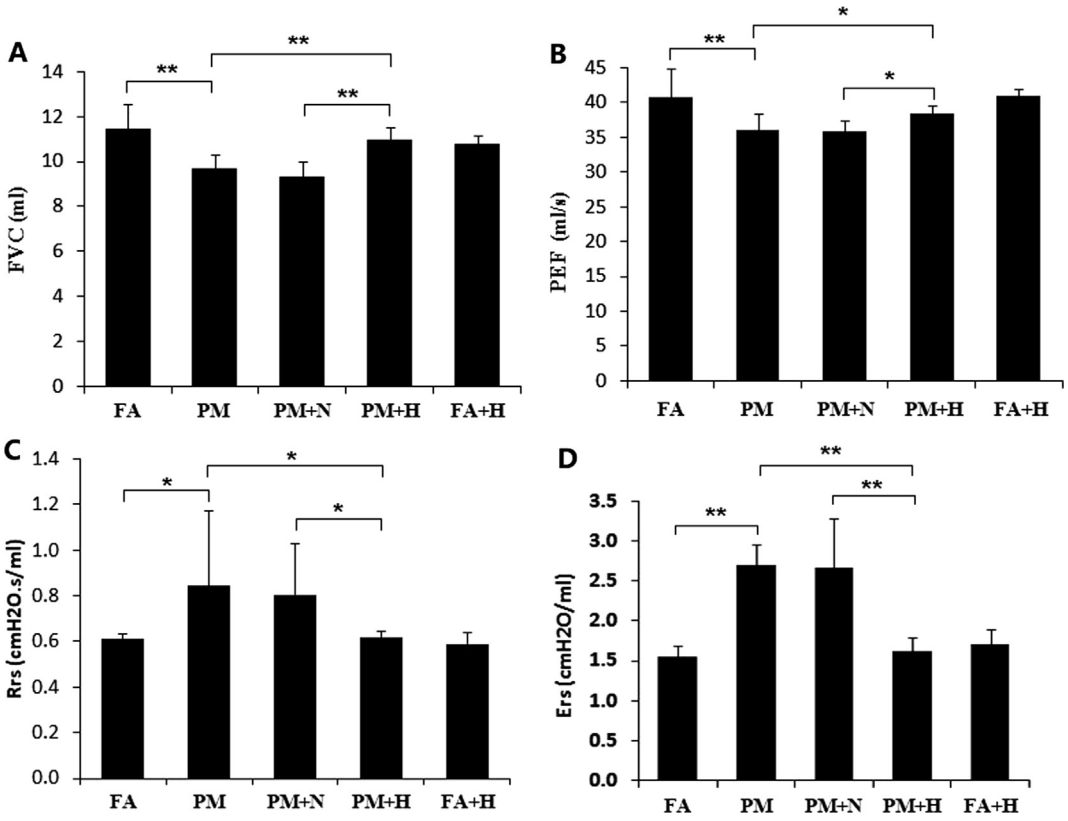

Fig. 2. Hydrogen protected rats from $PM_{2.5}$-induced lung mechanics and pulmonary function decrease. (A) Forced vital capacity (ml) [FVC] and (B) Peak expiratory flow (ml/s)[PEF]. (C) total respiratory system resistance (cmH_2O.s/ml) [Rrs] and (D) total respiratory system elastance (cmH_2O/ml) [Ers]. Data are expressed as mean ± SD. $^{**}p < 0.01$, $^{*}p < 0.05$.

macrophages and lymphocytes, and red blood cells scattered in the alveolar areas (Fig. 6A). Meanwhile, the mucus and cell debris could be seen in the bronchiolar lumen, and inflammatory cells infiltration and bronchial epithelial hyperplasia were significantly observed in the bronchial wall from rats in the PM and PM + N group (Fig. 6B). While the pathological damage was milder in the PM + H group than that in the PM or PM + N group (Fig. 6A and B). The inflammatory scores of lungs from the PM group (13.14 ± 1.35) was significantly higher than that from the FA (1.57 ± 0.79), ($p < 0.01$), but hydrogen treatment effectively reduced PM2.5-induced histopathological damages (5.57 ± 0.98), while the nitrogen and oxygen mixture inhalation had no protection (PM + N group: 13.14 ± 1.35) (Fig. 6C).

To further characterize the inflammatory response in vivo, we evaluated the pro-inflammatory cytokines in the BALF. As Fig. 6D, E and F displayed, the levels of TNF-α, IL-1β and IL-8 in the BALF showed an obvious increase in the PM group compared with the FA group ($p < 0.01$). Hydrogen inhalation resulted in a significant decrease of the pro-inflammatory cytokines mentioned above ($p < 0.01$). It appears that hydrogen could inhibit the inflammation provoked by PM2.5.

3.7. Concentrated ambient PM2.5 exposure could down-regulate pulmonary AhR protein level, while hydrogen could inhibit the AhR decline

As shown in Fig. 7A and B, the expression of AhR protein was decreased in the PM group compared with the FA group ($p < 0.01$). PM2.5 down-regulated the AhR level. While, the AhR level in PM + H group was higher than that in PM group or in PM + N group ($p < 0.01$). Nevertheless, hydrogen inhalation itself does not increase or affect AhR level because there was no significant difference between FA + H and FA group. So, AhR decline or down-regulation was inhibited when PM2.5 exposure was combined with hydrogen inhalation as shown in PM + H group. In other words, hydrogen could inhibit AhR decline induced by PM2.5.

4. Discussion

The concentrated ambient PM2.5 (or CAPs) were derived from the air in the urban community near a busy traffic road of Shijiazhuang during the coal burning season when particulate air pollution was usually severe. And the mean mass concentration of CAPs in this study was 1328 ± 730 μg/m^3, which is a relatively high concentration compared to the existing same type studies [24,25].

We created a lung injury rat model of subacute exposure to CAPs inhalation by high mass concentration for 4 weeks duration and tested the model by pulmonary function, histopathology of lungs, mucus secretion, airway inflammation and oxidative stress indexes. The duration of CAPs exposure affects the degree of damage response. Some previous studies provided references for our CAPs exposure protocol. Rhoden, et al. reported that inhalating CAPs with high mass concentration (1060 ± 300 μg/m^3) for 5 h could lead to significant increases in the oxidative stress index but only slight bronchiolar inflammation [24]. Another 4-hour exposure of the fine particulate matter (PM) led to a slight increase in quasistatic elastance (Est), and a little but not significant increase in baseline Rrs in mice. And the airway responsiveness could be enhanced in the high mass concentration (> 350 μg/m^3) group of fine PM [25]. Clougherty, et al. [26] showed that 10 days (5hr/day) exposure of CAPs inhalation resulted in only one inflammatory factor elevated (TNF-α) and no change in respiratory function. Similarly, in our two-week CAPs exposure experiment, there was only mild histological airway inflammation and no significant change in lung function (data was not shown). However, when the exposure time was extended to 4 weeks, impaired pulmonary ventilation function including declined flows and volumes and increased

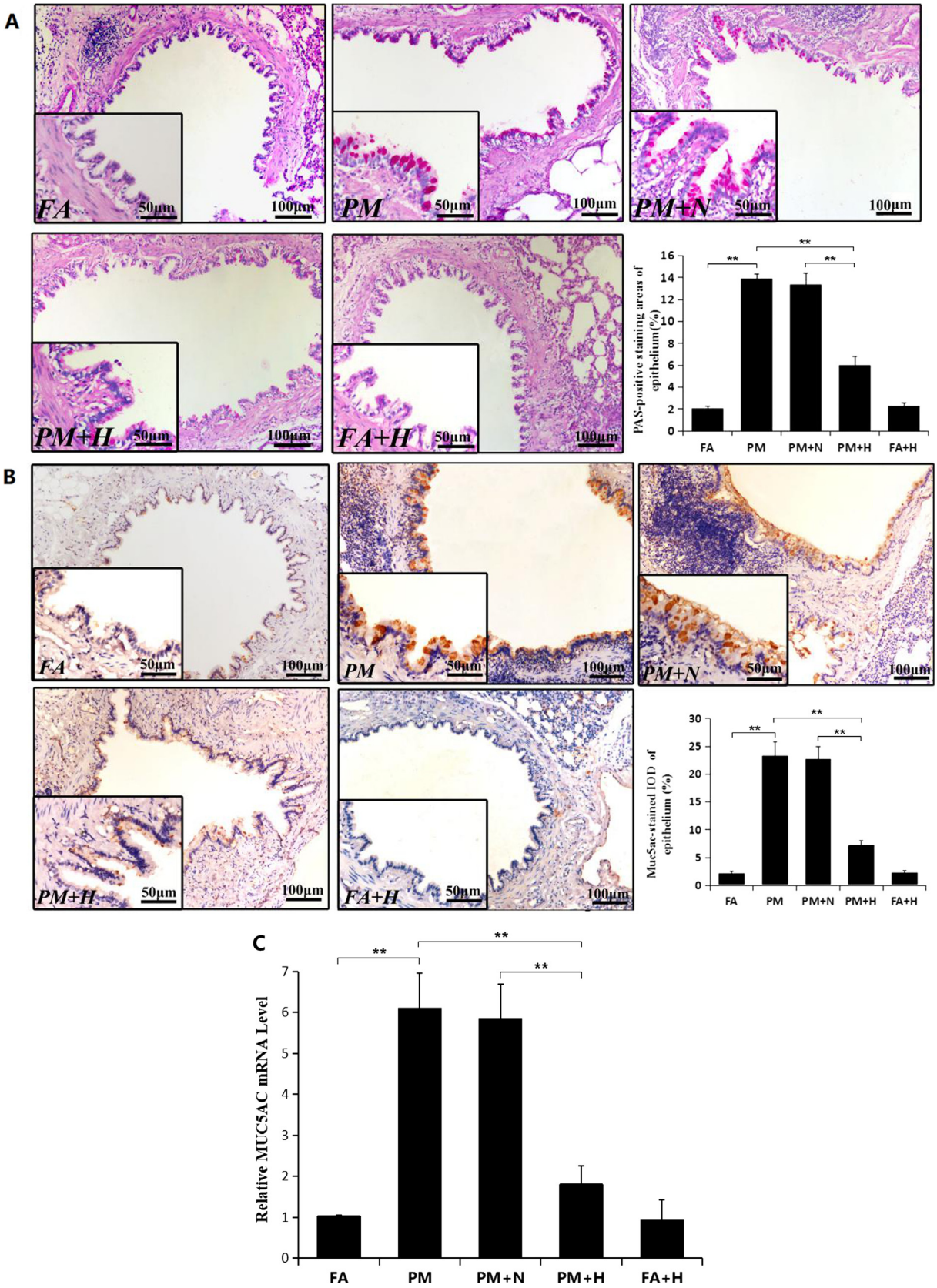

Fig. 3. Hydrogen inhibited PM2.5-induced mucus hypersecretion and MUC5AC expression in the airway epithelium. (A) Mucins in airway epithelium were determined by PAS staining. PAS-positive substance was characterized by purple staining, and PAS-positive rates were presented as the ratio of PAS-positive area to the total area that was used for quantification of PAS-positive area in the airway epithelium, which is shown in the right lower panel (B) The MUC5AC protein expression was detected by immunohistochemistry. Positive immunoreactivity for MUC5AC was characterized by brown staining, and the ratio of IOD of muc5ac-positive area to the total IOD of bronchial epithelium was applied for semi-quantitative analyses. The statistical result is shown in the right lower panel (C) Effects of hydrogen on PM2.5-induced MUC5AC mRNA expression was analyzed by RT-qPCR. The scale bars are shown in the figure. Data are expressed as mean ± SD. $^{**}p < 0.01$.

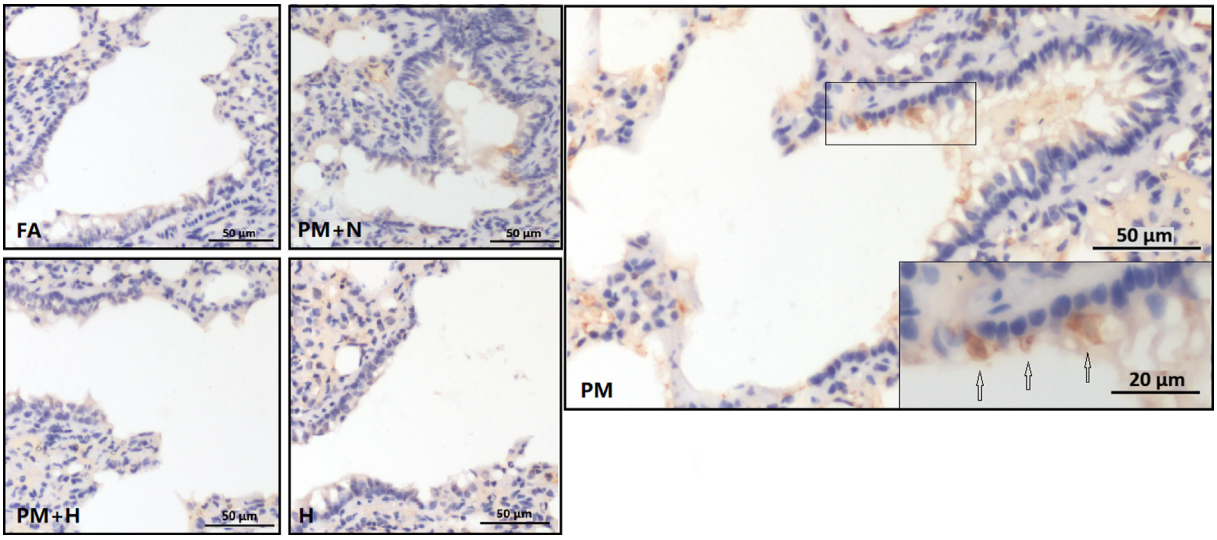

Fig. 4. MUC5AC-positive staining in the respiratory bronchioles of some rats exposed to CAPs. The arrows point to the MUC5AC-positive staining found in the respiratory bronchioles of individual rats in the PM group, but it was not found in the other four groups.

airway resistance were induced. And, serious histological changes and mucus hypersecretion were observed. The levels of pro-inflammatory cytokines and oxidative stress indicators were also augmented. It seems that 4 weeks could be a time point achieving functional, biochemical and histological changes induced by CAPs inhalation in our study.

Meanwhile, we investigated the protective effects of hydrogen inhalation against from CAPs-induced lung injury. Our results proved that hydrogen inhalation could improve lung function, alleviate pathological inflammation and injury of lung tissues, inhibit excessive secretion of airway mucus, reduce the levels of pro-inflammatory factors and oxidative stress in rats exposed to CAPs. Especially, the inhibition of mucus hypersecretion by hydrogen is of importance. Mucin hypersecretion is a key pathophysiological feature of inflammatory respiratory diseases. Mucus substances, which is an important part of innate immunity, play a protective effect on respiratory system by adhering the inhaled particles [27]. But the over-production of airway mucus would cause airflow obstruction and retention of inhaled allergens, which may be a trigger of airway inflammation and lung damage, and related to the pathophysiology of muco-obstructive lung diseases [28]. Some studies have confirmed that PM2.5 could induce airway mucus hypersecretion in *vivo* and in *vitro* [29–31]. In our study, using genetic (MUC5AC mRNA RT-qPCR), histological (H&E and PAS) and immunohistochemical (MUC5AC) analyses, we showed that hydrogen could alleviate the airway mucus hypersecretion induced by PM2.5 inhalation.

MUC5AC is the main mucin secreted by goblet cells which are found in bronchi and conducting bronchioles. The identification of MUC5AC could be used for the localization to goblet cells [32]. However, in addition to bronchi and conducting bronchioles, MUC5AC-positive staining was also observed in the epithelial lining of some respiratory bronchioles following CAPs exposure in our study (Fig. 4). Goblet cells do not exist in respiratory bronchioles epithelium in general. The predominant secretory cell type of respiratory bronchioles are Clara cells [27], which could convert into goblet cells in response to chronic irritation [33]. We infer that the expression of MUC5AC in respiratory bronchioles shown in our results is due to goblet cell metaplasia and this need further research.

After Ohsawa et al. [6] proved that hydrogen could selectively scavenge cytotoxic ROS in 2007, many researches have demonstrated the anti-oxidative stress and anti-inflammatory effects of hydrogen. But, as stated in our introduction, the ability of hydrogen in scavenging hydroxyl radical cannot fully explain the exact molecular mechanisms of the protective effects of hydrogen. Our study also showed the activity of hydrogen in reducing inflammation and oxidative stress indexes. Furthermore, we preliminarily explored the effect the hydrogen on AhR expression.

AhR is a transcription factor which can be activated by ligands such as environmental pollutants including dioxins [eg. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)] and PAHs (eg. benzo[a]pyrene) in PM2.5. AhR-triggered signal pathways have an important role in

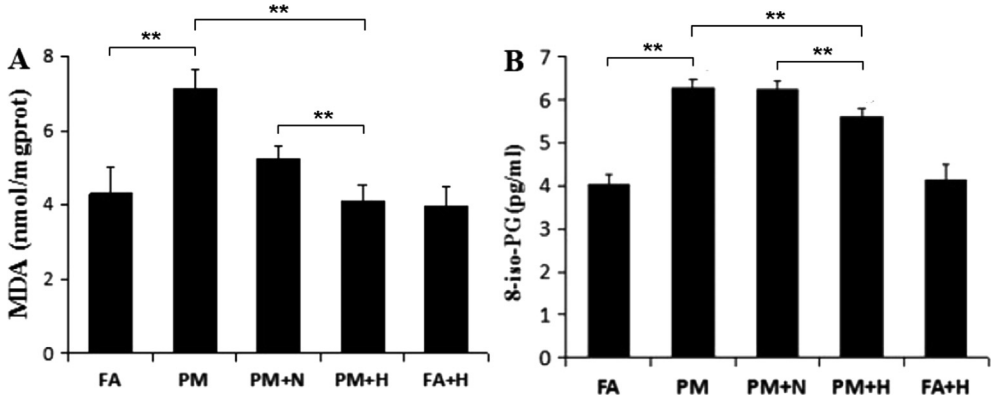

Fig. 5. PM2.5 induced oxidative damage in the lung of rats, whereas hydrogen protected against these effects. (A) MDA levels in the lung tissues (B) 8-iso-PG levels in the BALF. Data are presented as mean ± SD. $^{**}p < 0.01$.

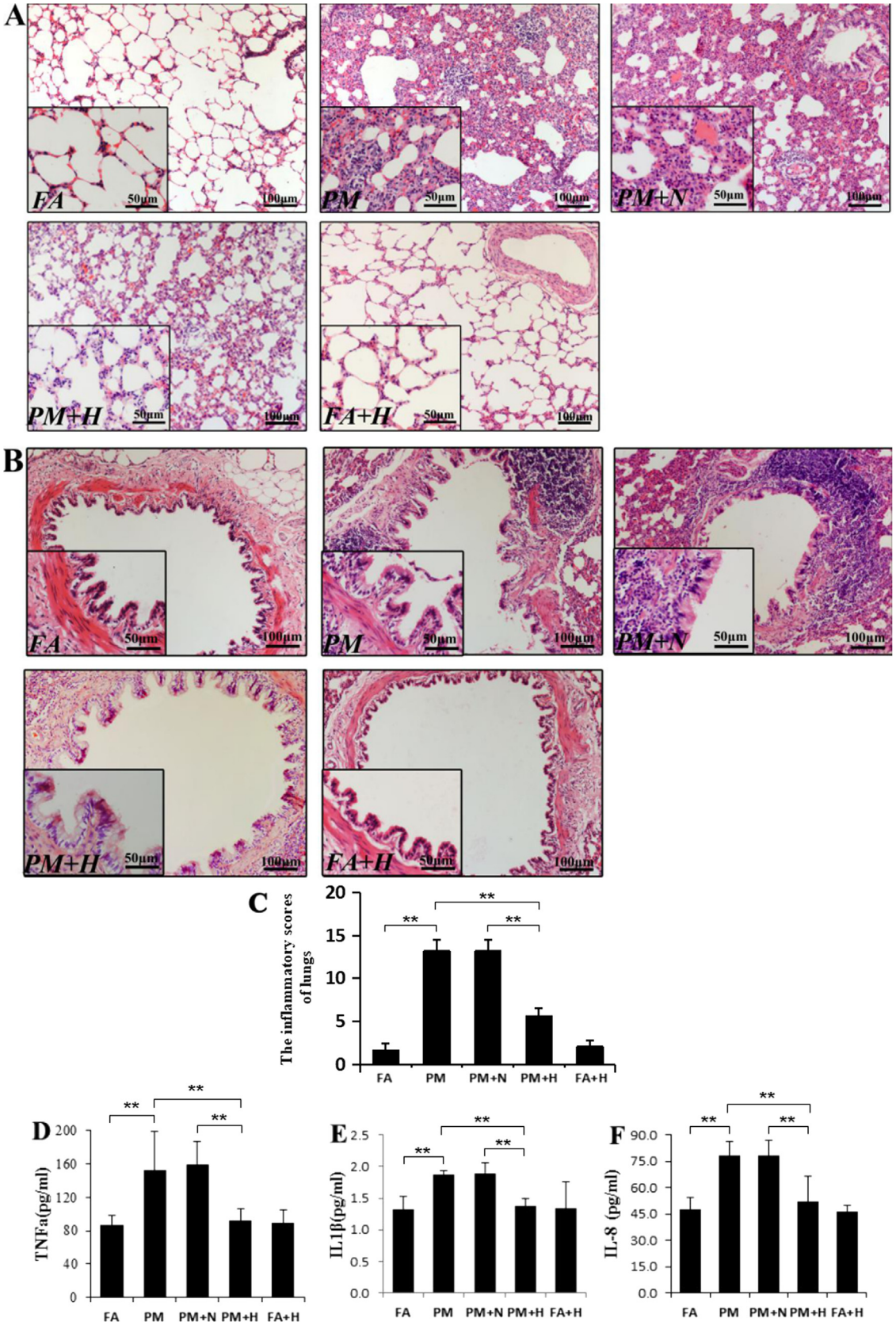

Fig. 6. Hydrogen suppressed PM2.5-induced inflammation in the lung. (A-B) H&E staining of alveolus and airways. Inflammatory scores of lung injury were shown in the figure (C). (C) Inflammation of rat lungs were scored. (D-F) Pro-inflammatory Cytokines (TNF-α, IL-1β and IL-8) in the BALF. The results are presented as mean ± SD. $^{**}p < 0.01$.

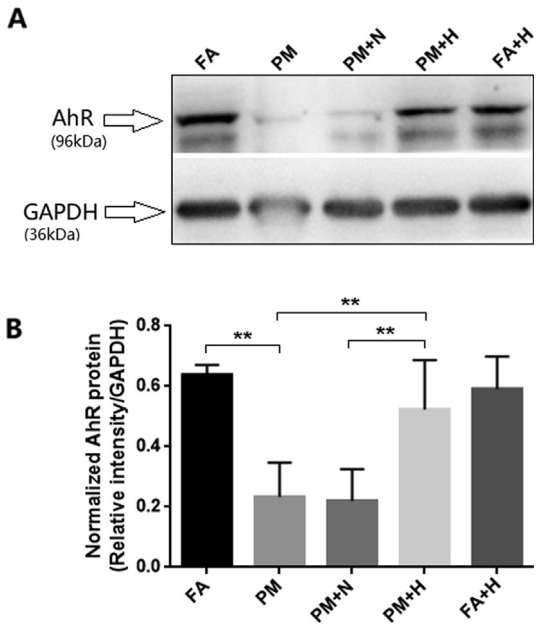

Fig. 7. Hydrogen inhibited pulmonary AhR protein decline in the lung tissues of rats when exposed to PM2.5. Pulmonary AhR protein expression was determined by western blotting (A). AhR band relative intensities were quantified and normalized to GAPDH (B). Values were means ± SD from 5 to 7 individual animals per group. Significant differences are indicated by $^{**}p < 0.01$.

keeping cellular homeostasis [34]. AhR regulates the balance of oxidative stress and antioxidant response. It has been reported that ambient particulate matters could induce the activation of AhR [35], which is involved into the molecular mechanisms of the inflammation effects of PM2.5 [13]. AhR protein will be down-regulated following activation by ligand exposure in *vivo* or in *vitro* [36]. For example, PAHs or TCDD could induce the degradation of AhR [37]. Pollens RS et al. reported that the AhR protein concentration in the lung of rats evaluated by western blotting was reduced by 70–90% following 3 h of TCDD exposure and returned to initial levels by nearly 2 weeks [38]. Low expression of AhR is related to inflammatory disease [39]. In our study, the down-regulation of AhR protein was induced by PM2.5 exposure. And lung injury was demonstrated by reduced pulmonary function, mucus hypersecretion, airway inflammation and oxidative damage. Maybe, during the high concentration of PM2.5 exposure for 4 weeks, the detoxification and antioxidant capacity of AhR were saturated. AhR was consumed by PM2.5 exposure.

While, we found that hydrogen inhalation could prevent the decline of AhR protein. Our results suggested that hydrogen could alleviate lung injury induced by PM2.5, possibly through AhR-related pathway. The detailed mechanisms of AhR involved in the treatment of hydrogen are still uncertain. Combining with previous studies and literatures, we propose some possible explanations for the role of AhR in hydrogen protection as follows.

PAHs are important components of concentrated ambient PM2.5 in our experiments. PAHs are ubiquitous pollutants, comprised of at least two fused benzene rings [40]. The cytotoxicity of PAHs depends on their special binding affinity with AhR, which is related to the molecular size and structure of the PAHs. Every phenyl ring of PAHs has a π-π bond interaction with the aromatic side chain of AhR [41]. These multi-ring PAHs differentially activate and consume the AhR in a structurally dependent manner, and induce cytotoxicity by AhR-dependent pathway [40]. Previous study has shown that the hydrogen molecule, which has a small molecular weight, can easily penetrate cell membranes and diffuse organelles [6]. The reaction of organic compounds with hydrogen molecules is called hydrogenation reaction. In our study, we speculate that the PAHs entering the body may react with

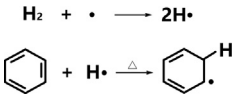

Fig. 8. Proposed model for the hydrogenation reaction of the benzene ring. Hydrogen molecules (H_2) could easily become hydrogen free radicals (H•). The benzene ring could be hydrogenated under the catalysis of some enzymes in vivo. △ means "under the catalysis of some enzymes".

the inhaled hydrogen in vivo (hydrogenation reaction model was shown in Fig. 8), which would change the structures of PAHs and reduce their affinities with AhR. Thus, the consumption of AhR was reduced after hydrogen inhalation as shown in Fig. 7 (PM + H group compared with PM group). In other words, hydrogen inhalation could affect the affinity of PAHs with AhR, and reduce the consumption of AhR induced by PAHs from PM2.5 inhalation, thus decrease the AhR-related toxicity of PAHs such as oxidative or inflammatory damages.

In addition, it has been proved by previous research that the inhibition of AhR degradation would result in a three- to fourfold increase in AhR-triggered target genes following ligand binding in cells compared to controls in which the decline of AhR was not blocked [36,37]. AhR could regulate many signaling pathways containing oxidant and antioxidant responses, such as the AhR-Nrf2 and AhR-CYP1A1 pathways [11]. Since the PM2.5-induced decline of AhR was inhibited by hydrogen treatment, hydrogen may selectively strengthen protective AhR-dependent pathway so that the antioxidant response of AhR predominated. But AhR-dependent protective effects of hydrogen inhalation need to be proved by further study, such as the negative effect of hydrogen in AhR-/- mice.

The water electrolysis-derived hydrogen applied in our study has a 66.7% concentration of hydrogen, which is much higher than 1–4% in previous researches [42]. Recently, a dose-response effect of hydrogen gas inhalation was observed and higher concentrations of hydrogen (22% and 41.6%) showed a better outcome in preventing cigarette smoke-induced lung injury [8]. And the inhaled 66.7% hydrogen and 33.3% oxygen produced by the hydrogen/oxygen nebulizer are aerosol mixtures, which may avoid the risk of explosion for hydrogen at this high concentration [20,21]. Our result showed that water electrolysis-derived hydrogen, the high concentration of hydrogen, is a potential new resource for prevention and treatment of PM2.5-induced lung injury. But, hydrogen inhalation was performed with a single time and concentration in our study. So the most effective concentration and time for hydrogen inhalation still need to be clarified in the future.

5. Conclusions

Our study showed that a subacute term exposure (4 weeks) to high concentration of concentrated ambient PM2.5 could cause severe respiratory damages in rats, including pulmonary dysfunction, airway mucus hypersecretion, oxidation damage, and inflammation response. While, hydrogen with high concentration could reduce the damages. Importantly, hydrogen alleviates lung injury induced by PM2.5, possibly through AhR-dependent mechanisms, which requires further researches.

Declaration of Competing Interest

There is no conflict of interest in this paper.

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The authors declare no competing interests.

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