Environmental Toxicology and Pharmacology 59 (2018) 190–198

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

High molecular weight hyaluronan attenuates fine particulate matter-induced acute lung injury through inhibition of ROS-ASK1-p38/JNK-mediated epithelial apoptosis

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ARTICLE INFO

Keywords:
Hyaluronan
Fine particulate matter
Lung injury
Reactive oxygen species

ABSTRACT

Inhalation of fine particulate matter ($PM_{2.5}$) is asscoiated with lung injury. High molecular weight hyaluronan (HMW-HA) is an essential constituent of extracellular matrix (ECM), exhibiting anti-oxidative and anti-inflammatory properties when administered by injection, inhalation, nebulization or gene delivery of HA synthases. The aim of the present study is to determine whether HMW-HA alleviates $PM_{2.5}$-induced acute lung injury (ALI) and investigate the underlying mechanisms. We observed that HMW-HA suppressed pathological injury, inflammation, oxidative stress, edema and epithelial damage caused by $PM_{2.5}$ in the lungs of the rats. The protective mechanism of HMW-HA was further explored *in vitro*. The results elucidated that reactive oxygen species (ROS) was involved in $PM_{2.5}$-induced cell apoptosis, and HMW-HA mitigated the oxidative potential of $PM_{2.5}$, subsequently inhibiting phosphorylation of ASK1 at Thr845, downstream phosphorylation of p38 and JNK, and eventual apoptosis. Our study indicates that HMW-HA is a promising strategy in the prevention of $PM_{2.5}$-induced pulmonary damage.

1. Introduction

Fine particulate matter ($PM_{2.5}$; aerodynamic diameter < 2.5 μm) is a well-known air pollutant threatening public health. Long-term exposure to $PM_{2.5}$ has been associated with reduction in pulmonary function, exacerbation of chronic respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD), and increased incidence and mortality of lung cancer (Wu et al., 2014; Karakatsani et al., 2012; Raaschou-Nielsen et al., 2013). Epidemiological studies have revealed an inverse correlation between $PM_{2.5}$ inhalation and average life span (Xing et al., 2016). Experimental evidence has indicated that administration of $PM_{2.5}$ to animals by intratracheal instillation induces lung inflammation, hyperemia, pulmonary oxidative stress, lung vascular hyperpermeability, alveolar epithelial dysfunction and lung injury (Li et al., 2015a,b; Wang et al., 2015). The toxicity of $PM_{2.5}$ is mainly due to the small size of the particle that allows $PM_{2.5}$ to bypass human innate defense mechanism and go deeply into the bronchial to deposit in the alveolar, and the adsorbed toxic substances including endotoxin, polycyclic aromatic hydrocarbons (PAHs), sulfate and heavy metals (Falcon-Rodriguez et al., 2016). The research regarding the distribution of chemical species in $PM_{2.5}$ and PM_{10} demonstrated that SO_4^{2-}, NH_4^+, organic carbon (OC) and elemental carbon (EC) mainly existed in $PM_{2.5}$, while crustal species were the most abundant components in PM_{10}, indicating a crucial role of $PM_{2.5}$ in poor air quality (Zhou et al., 2016). Since the environmental problem cannot be solved immediately, it is urgent to identify novel preventive and therapeutic strategies to protect human respiratory system.

Hyaluronan (HA) is a major component of extracellular matrix (ECM) with important roles in physiological and pathological processes in almost all tissues. High molecular weight HA (HMW-HA; > 500 kDa) exists predominantly in healthy tissue, suppressing inflammatory cytokine production, neutrophil-endothelial cell adhesion, tumor progression and macrophage phagocytosis by interaction with HA binding proteins such as CD44, RHAMM, SHAP and LYVE1 (Hussain et al., 2016; Tian et al., 2013; He et al., 2013; Alam et al., 2005; Ruppert et al., 2014). Besides, the anti-apoptotic and anti-oxidative role of HMW-HA has been revealed in multiple cellular models, including UV-induced epithelial corneal cell apoptosis and IL-17A-mediated nasal epithelial cell inflammation (Albano et al., 2016; Pauloin et al., 2009). For patients with osteoarthritis (OA), intra-articular injection of HMW-HA is an effective and safe treatment to relieve pain and prevent cartilage degradation (Hashizume and Mihara, 2010). Animal studies

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https://doi.org/10.1016/j.etap.2018.03.020
Received 3 February 2018; Received in revised form 27 March 2018; Accepted 28 March 2018
Available online 30 March 2018

demonstrated that HMW-HA improved measures of surface activity, lung compliance, gas exchange and pulmonary mechanics in meconium-treated rats, and ameliorated pulmonary inflammation, lung edema, airway epithelial cell apoptosis and airway mucous plugging in rats exposed to smoke (Lu et al., 2005; Huang et al., 2010).

The protective role of HMW-HA in inflammatory diseases and pulmonary damage led us to investigate whether $PM_{2.5}$-induced ALI could be attenuated by HMW-HA and the underlying mechanisms. In this study, we used a rat model to evaluate the effect of HMW-HA on pulmonary histology, lung inflammation, oxidative stress, lung edema and epithelial apoptosis after $PM_{2.5}$ exposure, and an *in vitro* model to study whether HMW-HA would repress $PM_{2.5}$-induced apoptosis through its anti-oxidative property. The study demonstrated that $PM_{2.5}$ stimulated reactive oxygen species (ROS) production and subsequent activation of ASK1 and p38/JNK MAPK pathway, resulting in airway epithelial cell apoptosis and consequent ALI, and HMW-HA could act as an antioxidant to mitigate $PM_{2.5}$-associated airway epithelial apoptosis, lung inflammation and ALI.

2. Materials and methods

2.1. Particle collection, preparation and analysis

$PM_{2.5}$ samples were collected on quartz fiber filters (General Electric, USA) with a pump flow rate of 1.13 m^3/min using $PM_{2.5}$ high volume air sampler (Thermo Anderson, USA) 24 h per day from June to December 2016 in Hangzhou, China. The filters containing $PM_{2.5}$ were cut and immersed in sterile water with sonication (four cycles of 20 min each) to extract $PM_{2.5}$. $PM_{2.5}$ suspensions were vacuum-freeze dried, weighed and stored at −20 ℃. The resulting pellets were resuspended in sterile saline at a concentration of 10 mg/ml for *in vivo* and *in vitro* studies, and $PM_{2.5}$ suspension was always sonicated and vortexed before administration.

The chemical characterization of particles was determined as previously reported (Wang et al., 2013). Briefly, the concentrations of metal element were measured by inductively coupled plasma mass spectrometry (Elan DRC-e, PerkinElmer, USA), and the amounts of PAHs and phthalic acid esters (PAEs) were detected by gas chromatograph-mass spectrometer (7890 A, Agilent Technologies, USA).

2.2. Animals and administration

Male Sprague-Dwaley (SD) rats (200 ± 20 g) were supplied by Zhejiang Academy of Medical Sciences. The animals were housed in a specific pathogen-free (SPF) environment at a temperature of 24 ± 1 ℃ and a 12 h light: 12 h dark cycle and maintained on standard diet with water *ad libitum*. All the animal experiments were handled in accordance with the "Guide for the Care and Use of Laboratory Animals" and the "Principles for the Utilization and Care of Vertebrate Animals". After acclimatization to the laboratory, the rats were weighted and randomly divided into three groups (n = 6): (1) intratracheal instillation of normal saline for 3 days (NS group); (2) exposure to $PM_{2.5}$ (8 mg/kg b.w.) and 200 μl of saline by intratracheal instillation for 3 days ($PM_{2.5}$ group); (3) intratracheal instillation of $PM_{2.5}$ suspension (8 mg/kg b.w.) and 200 μl of 0.2% HMW-HA (1500 kDa-1700 kDa; Sigma-Aldrich, St. Louis, MO, USA) for 3 days ($PM_{2.5}$ + HA group). Rats were sacrificed 24 h after last intratracheal instillation to collect bronchoalveolar lavage fluid (BALF) and lung tissues for further analysis.

2.3. Measurement of cell counts, SOD activity, MDA, TNF-α and IL-1β levels in BALF

The cells present in the lavage fluid were counted using a hemocytometer. To perform cell differentials, cells were fixed on glass slides by cytospin and stained with geimsa. Superoxide dismutase (SOD) activity was determined by the xanthine oxidase test, and malondialdehyde (MDA) level was measured by thiobarbituric acid (TBA) reaction method (Beyotime Biotechnology, Nantong, China). TNF-α and IL-1β levels were analyzed using ELISA kits (Neobioscience Technology Co., Ltd, China)

2.4. Histological analysis

The lung tissues were perfused with 4% paraformaldehyde and embedded with paraffin. The tissues were cut into 4-μm-thick sections to make slices. The histological sections were stained with hematoxylin & eosin (H&E) by standard procedure, and the histopathological changes were observed under a light microscope.

2.5. Immunohistochemistry (IHC)

Cleaved caspase-3 protein in lung tissues was examined by IHC. Paraformaldehyde-fixed, paraffin-embedded lung samples were sliced into 4 μm, and then the slides were deparaffinized, rehydrated and incubated with cleaved caspase-3 antibodies (Cell Signaling Technology, MA, USA) at 4 ℃ overnight followed by incubation with a HRP-conjugated secondary antibody at 37 ℃ for 30 min. The sections were visualized using diaminobenzidine and the results were observed under a light microscope.

2.6. DNA damage and apoptosis in lung tissue

Terminal deoxynucleotidyl transferase-mediated dUTP nick end label (TUNEL) staining was performed using a DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WI, USA) according to manufacturer's protocol.

2.7. Analysis of lung water

Lungs were removed, weighted and dried at 80 ℃ for 48 h. Lung wet: dry weight ratio was used as an index of pulmonary edema condition.

2.8. Detection of total NO level in lung tissue

Lung tissue was homogenized with Cell and Tissue Lysis Buffer for Nitric Oxide Assay (Beyotime Biotechnology, Nantong, China) according to manufacturer's instruction, and total Nitric Oxide (NO) level was determined by Total Nitric Oxide Assay Kit (Beyotime Biotechnology, Nantong, China).

2.9. Cell culture

BEAS-2B cells (human bronchial epithelial cells) were obtained from the Type Culture Collection (Chinese Academy of Sciences, Shanghai, China) and maintained in DMEM (Invitrogen, San Diego, CA, USA) supplemented with 10% FBS (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Thermo Scientific, Waltham, MA, USA) at 37 ℃ in an atmosphere of 5% CO_2.

2.10. Cell viability assay

100 μl of BEAS-2B cells at a concentration of 7×10^4 cells/ml were placed in a 96-well plate. After treatment with different concentrations of $PM_{2.5}$ and/or HMW-HA for 24 h, cells were stained with 20 μl of MTT (5 mg/ml). The medium was removed 4 h later and formazan crystals were dissolved by addition of DMSO. Absorbance was measured at 570 nm using a microplate reader.