Airborne Fine Particulate Matter Induces Oxidative Stress and Inflammation in Human Nasal Epithelial Cells

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Airborne fine particulate matter with an aerodynamic diameter equal to or smaller than 2.5 μm is abbreviated as PM2.5, which is one of the main components in air pollution. Exposure to PM2.5 is associated with increased risk of many human diseases, including chronic and allergic rhinitis, but the underlying molecular mechanism for its toxicity has not been fully elucidated. We have hypothesized that PM2.5 may cause oxidative stress and enhance inflammatory responses in nasal epithelial cells. Accordingly, we used human RPMI 2650 cells, derived from squamous cell carcinoma of the nasal septum, as a model of nasal epithelial cells, and exposed them to PM2.5 that was collected at Fudan University (31.3°N, 121.5°E) in Shanghai, China. PM2.5 exposure decreased the viability of RPMI 2650 cells, suggesting that PM2.5 may impair the barrier function of nasal epithelial cells. Moreover, PM2.5 increased the levels of intracellular reactive oxygen species (ROS) and the nuclear translocation of NF-E2-related factor-2 (Nrf2). Importantly, PM2.5 also decreased the activities of superoxide dismutase, catalase and glutathione peroxidase. Pretreatment with N-Acetyl-L-cysteine (an anti-oxidant) reduced the degree of the PM2.5-induced oxidative stress in RPMI 2650 cells. In addition, PM2.5 increased the production of granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-α, interleukin-13 and eotaxin (C-C motif chemokine ligand 11), each of which initiates and/or augments local inflammation. These results suggest that PM2.5 may induce oxidative stress and inflammatory responses in human nasal epithelial cells, thereby leading to nasal inflammatory diseases. The present study provides insights into cellular injury induced by PM2.5.

Keywords: inflammatory response; nasal epithelial cells; Nrf2; oxidative stress; PM2.5

Introduction

Nowadays, exposure to airborne particulate matter (PM) is closely related to public health hazards in many developing countries. PM with an aerodynamic diameter equal to or smaller than 2.5 μm is abbreviated as PM2.5. Epidemiological studies have found that PM2.5 exposure increases the risk of cardiac (Delfino et al. 2005) and respiratory (Kloog et al. 2012) diseases. The size, morphology and component of PM2.5 play key roles in the adverse effects on human health. Generally, smaller PM has higher health risks. PM2.5 is a small particulate, which is inhalable and able to penetrate to tissues (Harrison and Yin 2000). Due to numerous sources, the chemical constituents of PM2.5 can contain nitrate, sulfate, ammonium, metals and poly aromatic hydrocarbons (PAHs) (Sun et al. 2004). Although specific constituents in charge of their toxicity have not been fully investigated, metals, PAHs, biological agents and ultrafine particles that adhere to particles are considered to be the constituents that have adverse effects on the respiratory system (Li et al. 2002). Inhaled PM2.5 makes contact with human nasal epithelial cells and may impair the function of these cells, thereby causing nasal inflammatory diseases such as chronic and allergic rhinitis. However, its underlying molecular mechanism is largely unknown.

Oxidative stress is a state of imbalance between oxidation and anti-oxidation, and plays an important role in the pathology of many human diseases (Limón-Pacheco and Gonsebatt 2009). One source of oxidative stresses results from the generation of reactive oxygen species (ROS). PM2.5 can induce ROS that directly interact with superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), and alter their enzymatic activities (Pamplona and Costantini 2011). Nuclear factor NF-E2-
related factor-2 (Nrf2) plays a key role in keeping cellular redox balance by protecting cells from oxidative stress (Li et al. 2004). The activation of Nrf2 increases the expression of antioxidant enzymes, which in turn removes ROS and decreases cell damage (Satoh et al. 2010). However, whether PM2.5 alters the activity of Nrf2 remains elusive.

In addition, airway epithelial cells play a key role in nasal inflammatory diseases by producing and releasing numerous inflammatory cytokines, leading to inflammatory response. Previous studies have suggested that PM2.5 has a potential to stimulate cells to produce and release inflammatory cytokines, leading to inflammatory nasal inflammatory diseases by producing and releasing numerous inflammatory cytokines, leading to inflammatory response. Previous studies have suggested that PM2.5 has a potential to stimulate cells to produce and release inflammatory cytokines (Ogino et al. 2014). In vitro studies have found that diesel exhaust particles (DEP) and PM increase the expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), interleukin 8 (IL-8), IL-6, IL-1β and eotaxin (C-C motif chemokine ligand 11) in airway epithelial cells (Boland et al. 1999; Gioda et al. 2011; Patel et al. 2011; Tang et al. 2012). Since nasal epithelial cells function as the first epithelial barrier in the nasal cavity to protect from inhaled particles, PM2.5 may induce inflammatory responses in nasal epithelial cells.

We hypothesize that inhaled PM2.5 is involved in the pathogenesis of nasal inflammatory disorders by inducing oxidative stress and inflammatory response in nasal epithelial cells. In this study, we used well-characterized PM2.5 collected from the atmosphere and investigated its effects on cell viability, intracellular ROS generation, enzymatic antioxidant activity, nuclear translocation of Nrf2, and expression of inflammatory cytokines in RPMI 2650 human nasal epithelial cells.

Materials and Methods

**PM2.5 sampling and preparation**

Sampling of PM2.5 was performed as previously described (Huang et al. 2012). In brief, PM2.5 was collected on Whatman 41 filters (Whatman, Maidstone, UK) using TSP/PM0/PM2.5-2 samplers (Dinkel, Beijing, China) with the flow rate of 77.59 Liter/min. The samplers were on the roof (approximately 20 meters high to the ground) of a building at Fudan University (31.3’N, 121.5’E) in Shanghai, China. This site could be regarded as representative of the megacity Shanghai, standing for the mixture of residential, traffic, construction and industrial sources. Before and after sampling, the filters were stabilized in constant temperature (20 ± 1°C) and humidity (40 ± 1%), and weighed using a Sartorius 2004MP analytical balance (Sartorius, Germany) with a reading precision of 10 µg.

PM2.5 was prepared according to the method as described (Imrich et al. 2000). Briefly, the sample filter containing PM2.5 was immersed in deionized water and sonicated for 45 min using a KQ-50B water-bath sonicator (Kunshan Ultrasonic Instruments, Jiangsu, China). PM2.5 was extracted and stored at −20°C. Aliquots containing 1 mL of PM2.5 were placed on filters and air-dried, and the samples and filters were weighed on a Sartorius 2004MP analytical balance (Sartorius) after stabilization at constant temperature (20 ± 1°C) and humidity (40 ± 1%) to determine the concentration of PM2.5.

**PM2.5 physical and chemical characterization**

In order to determine the size and morphology of PM2.5, scanning electron microscopy (S4800; Hitachi, Japan) was used to perform a particle analysis. One-fourth of the sample filter was extracted ultrasonically by 20 mL of deionized water. Inductively coupled plasma atomic emission spectroscopy (ICP-OES; SPECTRO, Germany) was used to measure 18 metals (Al, As, Ba, Be, Co, Cr, Cu, Fe, Ge, K, Mn, Na, Ni, Sb, Se, Sn, V and Zn). Ten inorganic ions (F−, CH3COO−, HCOO−, Cl−, NO3−, SO42−, PO43−, Na+, NH4+ and K+) were analyzed by ICS 3000 ion chromatography (Dionex, USA). Detailed procedures of the element and ion analysis were based on the procedures of as procedures previously described (Huang et al. 2013). The PAH analytical procedure was carried out as previously described (Guo et al. 2009). PAHs in PM2.5 were measured using a gas chromatography-mass spectrometer (GC-MS; Agilent, USA). The sixteen targeted PAHs analyzed in PM2.5 samples were as follows: acenaphthylene, acenaphthene, anthracene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[g,h,i]perylene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorene, indeno[123-cd]pyrene, naphthalene, phenanthrene and pyrene.

**Cell culture and PM2.5 exposure**

RPMI 2650 cell line derived from squamous cell carcinoma of nasal septum was used as a model of human nasal epithelial cells. The cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Eagle’s minimal essential medium (ATCC) supplemented with 10% fetal bovine serum (FBS; HyClone, GA, USA), 2 mM of L-glutamine (Gibco, Paisley, UK), 100 U/mL of penicillin (Gibco) and 100 µg/mL of streptomycin (Gibco). Cells were maintained at 37°C with 5% CO2 and 95% air. All cell exposure experiments were performed at 80-90% of cell confluence. Then, cells were harvested and sub-cultured into six-well plates, 24-well plates or 96-well plates, according to the selection of experiments. Subsequently, cells were exposed to serum-free medium with different concentrations of PM2.5 with or without N-Acetyl-L-cysteine (NAC; Sigma, USA) pretreatment. Cell growth status was observed with a phase contrast microscope (Olympus, Japan).

**Cell morphology characterization**

Cell morphology after PM2.5 exposure was evaluated with SEM SU8010 (Hitachi). RPMI 2650 cells were seeded in six-well plates and cultured for 24 hours. After attachment, the cells were exposed to 800 µg/mL of PM2.5 for 24 hours and were examined by SEM.

**Acute cytotoxicity assay**

Acute cytotoxicity of PM2.5 was evaluated in RPMI 2650 cells with Cell Counting Kit-8 (CCK-8; Ohio Technology, Shanghai, China). RPMI 2650 cells were seeded in 96-well plates (2,000 cells/well) for 24 hours, and treated with PM2.5 at final concentrations of 50, 100, 200, 400, 600 or 800 µg/mL for 12, 24 or 48 hours, respectively. The doses and exposure time of PM2.5 were based on preliminary experiments, and appropriate doses and exposure time were selected according to the growth curves. Ten µL of the CCK-8 solution was added into each well and incubated at 37°C for two hours. Absorbance at 490 nm was measured with a microplate reader (Tecan US, Inc., Charlotte, NC, USA).

**Reactive oxygen species assay**

A Reactive Oxygen Species Assay Kit (Beyotime, Hainan,
PM2.5 Induces Stress and Inflammation

Antioxidant enzyme assays

The intracellular superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities were measured with SOD, CAT and glutathione peroxidase assay kits, according to manufacturer’s instructions (Beyotime). Briefly, RPMI 2650 cells were seeded at a density of 1×10^5 cells/ml in six-well plates and were allowed to attach for 24 hours, followed by exposure to 100, 400 or 800 μg/ml of PM2.5 for 24 hours at 37°C with or without one hour treatment of NAC (0.5 mM). Cell extracts were used to measure the activities of SOD, CAT and GSH-Px. Total protein concentration was determined with Bicinchoninic Acid protein assay kit (Beyotime). The intracellular superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities were measured with SOD, CAT and glutathione peroxidase assay kits, according to manufacturer’s instructions (Beyotime). Briefly, RPMI 2650 cells were seeded at a density of 1×10^5 cells/ml in six-well plates and were allowed to attach for 24 hours, followed by exposure to 100, 400 or 800 μg/ml of PM2.5 for 24 hours at 37°C with or without one hour treatment of NAC (0.5 mM). Cell extracts were used to measure the activities of SOD, CAT and GSH-Px. Total protein concentration was determined with Bicinchoninic Acid protein assay kit (Beyotime), and enzyme activity was standardized to milligram protein.

Real-time quantitative reverse transcription PCR (qRT-PCR)

After exposure of cells to zero, 100, 400 or 800 μg/ml of PM2.5 for 24 hours, the mRNA levels of GM-CSF, TNF-α, IL-13, etoxin, IL-6 and IL-8 were measured by real-time qRT-PCR as previously described (Teng et al. 2015). Briefly, total RNA of RPMI 2650 cells was extracted using Trizol reagent (Invitrogen, Shanghai, China), and reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen). The synthesized cDNA was then quantified by SYBR Green assay on a CFX96 Touch™ Real-Time Detection System (Bio-Rad, Hercules, CA, USA). β-actin was used as an endogenous reference for mRNAs. All primers were obtained from Invitrogen, and their sequences are presented in Table 1. Relative expression was calculated using the comparative cycle threshold method (Livak and Schmittgen 2001).

Enzyme-linked immunosorbent assay (ELISA)

After exposure of cells to zero, 100, 400 or 800 μg/ml of PM2.5 for 24 hours, GM-CSF, TNF-α, IL-13, etoxin, IL-6 and IL-8 levels in the cell culture supernatant were measured using GM-CSF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA), TNF-α Quantikine ELISA Kit (R&D Systems), IL-13 Quantikine ELISA Kit (R&D Systems), CCL11/etoxin Quantikine ELISA Kit (R&D Systems), IL-6 Quantikine ELISA Kit (R&D Systems) and IL-8 Quantikine ELISA Kit (R&D Systems), respectively, in accordance with manufacturers’ instructions.

Western blotting analysis

After exposure of cells to zero, 100, 400 or 800 μg/ml of PM2.5 for 24 hours, a nuclear and cytoplasmic protein extraction kit (Beyotime) was used to purify the nuclear proteins and cytoplasmic proteins of RPMI 2650 cells, according to the manufacturer’s instruction. Nrf2 and Histone H1 in nuclear lysates, as well as Nrf2 and β-actin in cytosolic lysates, were evaluated by western blot analysis, as described (Yu et al. 2013). Anti-Nrf2 primary antibody was purchased from Abcam (Cambridge, UK). Protein bands were visualized using ECL detection reagent (GE Healthcare Life Sciences, Piscataway, NJ, USA). Scanned images were quantified using Quantity One software.

Statistics

Statistical analysis was performed using SPSS software for Windows (version 16.0; SPSS, Inc., Chicago, IL, USA). Results are expressed as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA). A P value < 0.05 was considered statistically significant.

Results

Physical and chemical characteristics of PM2.5 used in this study

Physical characteristics of PM2.5 such as size and morphology were analyzed with SEM. PM with a diameter equal or smaller than 2.5 μm were the most abundant on the porous membrane. Some morphological differences between PM2.5 particulates were observed according to their respective origins. These particles possessed diverse forms including spheres, clusters, plate and reticular forms (Fig. 1A). ICP-OES, ICS and GC-MS were used to analyze PM2.5 chemical characteristics by analyzing metals, ions and PAHs constituents of PM2.5 (Table 2). Analytical results of the 18 elements revealed that Na, K, Al, Zn, Cu and Fe were the most abundant elements in PM2.5. Among the 10 ions analyzed, NO_3^−, SO_4^{2−} and NH_4^+ were the domi-
nant ions in PM2.5. The analysis of PAHs revealed that benzo[b]fluoranthene, phenanthrene, fluoranthene, pyrene and chrysene were the dominant PAHs in PM2.5.

PM2.5 alters morphology and decrease the viability of RPMI 2650 cells

In order to elucidate whether PM2.5 is able to affect the morphology of cells, RPMI 2650 cells were challenged with 800 µg/ml of PM2.5 for 24 hours and scanned by SEM. PM2.5 firmly adhered to the ciliated cell membrane of RPMI 2650 cells, and PM2.5 provoked the rearrangement of microvilli (Fig. 1B). In order to explore the cytotoxic effects of PM2.5, a phase contrast microscope was used to measure the morphology of RPMI 2650 cells following treatment with different concentrations of PM2.5 for 24 hours. Results revealed that with the increase of PM2.5 concentration, the number of particles on cells obviously increased, and cell growth was inhibited (Fig. 1C-F). Next, cells were exposed to different concentrations of PM2.5 for 12, 24, or 48 hours, and cell proliferation was assessed by CCK-8 assay. Compared with unexposed control cells, PM2.5 decreased the cell viability of RPMI 2650 cells in a time- and concentration-dependent manner (Fig. 1G). These results demonstrate that PM2.5 was toxic to RPMI 2650 cells.

PM2.5 induces oxidative stress and Nrf2 nuclear translocation in RPMI 2650 cells

In order to assess whether PM2.5 induces oxidative stress, RPMI 2650 cells were exposed to PM2.5 at zero, 100, 400 or 800 µg/ml for four hours in the presence or absence of anti-oxidant NAC, and intracellular levels of ROS were determined by 2′,7′-Dichlorofluorescin (DCF) fluorescence intensity assay. The exposure to PM2.5 for four hours significantly elevated intracellular ROS levels in a concentration-dependent manner (Fig. 2A). However, NAC significantly reduced ROS generation with 800 µg/ml of PM2.5. Moreover, exposure of RPMI 2650 cells to PM2.5 for 24 hours significantly and concentration-dependently decreased the activities of SOD (Fig. 2B), CAT (Fig. 2C) and GSH-Px (Fig. 2D); however, the degree of the decreases in these activities was significantly alleviated by NAC pretreatment (Fig. 2B-D). These results demonstrate that PM2.5 induces oxidative stress and decrease intracellular antioxidant enzymatic defenses in RPMI 2650 cells.
Nrf2 plays a key role in protecting cells from oxidative stress (Li et al. 2004). We further explored the nuclear translocation of Nrf2 after exposure of RPMI 2650 cells to different concentrations of PM2.5 for 24 hours with or without NAC. Treatment of RPMI 2650 cells with PM2.5 for 24 hours significantly increased the nuclear protein levels of Nrf2, while at the same time, decreased cytosolic Nrf2 levels in a concentration-dependent manner. In contrast, NAC significantly prevented the nuclear translocation of Nrf2 following 800 µg/ml of PM2.5 treatment. Thus, PM2.5 induced Nrf2 translocation from the cytoplasm into the nucleus, which was inhibited by NAC.

PM2.5 stimulates production of inflammatory cytokines in RPMI 2650 cells

In order to examine the impact of PM2.5 on inflammatory cytokines production, RPMI 2650 cells were treated with different concentrations of PM2.5 for 24 hours, and the mRNA levels of GM-CSF, TNF-α, IL-13, eotaxin, IL-6 and IL-8 were measured by real-time qRT-PCR. GM-CSF, TNF-α, IL-13, eotaxin, IL-6 and IL-8 mRNA levels significantly increased in RPMI 2650 cells treated with PM2.5 for 24 hours when compared with untreated cells (Fig. 3A-F). However, there was no significant difference in mRNA expression levels of IL-13 and eotaxin between cells exposed to 100 µg/ml of PM2.5 and control. Consistent with
the upregulation of mRNA levels, ELISA analysis revealed that treatment of RPMI 2650 cells with PM$_{2.5}$ for 24 hours significantly increased the protein levels of GM-CSF, TNF-$\alpha$, IL-13, eotaxin, IL-6, and IL-8 in culture supernatants (Fig. 4A-F). These results indicate that PM$_{2.5}$ induced the expression of inflammatory cytokines in RPMI 2650 cells.

**Discussion**

In this study, we demonstrated that PM$_{2.5}$ altered the morphology and decreased the cell viability of RPMI 2650 cells. Moreover, PM$_{2.5}$ increased ROS levels and reduced intracellular antioxidant enzymatic activity accompanied with Nrf2 translocation from the cytoplasm into the nucleus; all of which were inhibited by NAC. Importantly, PM$_{2.5}$ induced the expression of inflammatory cytokines GM-CSF, TNF-$\alpha$, IL-13, eotaxin, IL-6 and IL-8. These results revealed that PM$_{2.5}$ induced oxidative stress and inflammatory response in RPMI 2650 cells. Our findings suggest that oxidative stress and inflammatory response may play an important role in the cell growth of RPMI 2650 cells and contribute to the impairment of nasal epithelial barrier dysfunction following PM$_{2.5}$ exposure.

Organic and inorganic components of PM$_{2.5}$ are involved in toxicological effects (Deng et al. 2013). It was reported that metal content in particles is the main composition that induce inflammatory response, and trace metals were the most toxic substances in ambient PM (Ghio and Devlin 2001). In the present study, both natural sourced elements (Na, K, Al, etc.) and anthropogenic inorganic elements (Zn, Cu, Fe, Ni, Mn, etc.) were detected with high levels of Na, K, Al, Zn, Cu and Fe, indicating that PM$_{2.5}$ was from both natural and anthropogenic sources. The analysis of ions revealed that NO$_3^-$, SO$_4^{2-}$ and NH$_4^+$ were dominant ions in PM$_{2.5}$, which are the representative of secondary aerosols. Furthermore, PAHs analysis revealed that a high proportion of benzo[b]fluoranthene, chrysene, fluoranthene, phenanthrene and pyrene were found in PM$_{2.5}$ samples. These analytical results of PM$_{2.5}$ compositions indicate that PM$_{2.5}$ samples are a complex mixture of chemicals, including natural sourced elements, anthropogenic inorganic elements, secondary aerosols and PAHs. This study focused on the toxic effect of atmospheric PM$_{2.5}$ on human nasal epithelial cells. PM$_{2.5}$ was collected in the atmosphere and used in this study. PAHs and heavy metals are the main components of the fractions, although these components in PM$_{2.5}$ accounted for a small proportion.
Future studies are needed to assess the impact of these components on human health.

Cell viability assays have been used to explore the cellular effects of toxicants (Lee et al. 2011). We found that the cell viability of RPMI 2650 cells exposed to PM$_{2.5}$ decreased in a time- and concentration-dependent manner. Further studies found that PM$_{2.5}$ triggered an increase of intracellular ROS and decrease in activities of SOD, CAT and GSH-Px in RPMI2650 cells. Meanwhile, PM$_{2.5}$ induced the nuclear translocation of Nrf2 in RPMI2650 cells. Moreover, pretreatment with NAC significantly reduced the PM$_{2.5}$-induced generation of ROS, suppression of SOD, CAT and GSH-Px activities, and nuclear translocation of Nrf2 in RPMI2650 cells. Nrf2-mediated defense mechanisms protect cells from oxidative stress. These results suggest that PM$_{2.5}$-induced oxidative stress may play a crucial role in cell growth, which may contribute to the nasal epithelial barrier dysfunction induced by PM$_{2.5}$. Nevertheless, the underlying molecular mechanisms of PM$_{2.5}$ and NAC on ROS generation, enzyme activities and nuclear translocation of Nrf2 have not been fully elucidated, and need future studies.

Nasal epithelial cells make up the epithelial barrier in the nasal cavity, and secrete various inflammatory media-

Fig. 3. PM$_{2.5}$ increases inflammatory cytokine mRNA expression in RPMI 2650 cells.

The mRNA expression levels of GM-CSF (A), TNF-α (B), IL-13 (C), eotaxin (D), IL-6 (E), and IL-8 (F) were measured by qRT-PCR analysis. The expression levels of mRNA were normalized to β-actin and given as fold induction compared to the mRNA level in control cells. All data are presented as the mean ± SD ($n=5$).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control.
Inflammatory cytokines GM-CSF and eotaxin can be synthesized and released by airway epithelial cells in local inflammation, and infiltrating inflammatory cells to local tissues (Matsuwaki et al. 2012). IL-13 is considered to be a crucial mediator of the pathogenesis of inflammation (Ingram and Kraft 2012; Corren 2013). This study revealed that PM$_{2.5}$ increased the expression of GM-CSF, TNF-α, IL-13, eotaxin, IL-6 and IL-8. These findings suggest that PM$_{2.5}$ may initiate and augment local inflammation, and lead to disease exacerbation by stimulating the production of GM-CSF, TNF-α, IL-13, eotaxin, IL-6 and IL-8.

In summary, we have shown that exposure to PM$_{2.5}$ causes oxidative stress and inflammatory response, which in turn may inhibit the cell growth and impair the nasal epithelial barrier function. Our results provide insights into the PM$_{2.5}$-mediated cellular injury.

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**Conflict of Interest**

The authors declare no conflict of interest.

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