Glucocorticoid Enhances Viability of Human Respiratory Epithelial Cells Inflicted by Ambient Particulate Matter

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Particulate matter (PM) is one of the most common ambient air pollutants, which is derived from diesel engines, wildfires, yellow dust, and other combustion sources. Especially, PM with an aerodynamic diameter of <2.5 μm (PM2.5) enhances risks of damaging respiratory organs. Previous studies have reported that PM-induced autophagy and apoptosis can be considered as a phenomenal molecular mechanism of PM-mediated cytotoxicity in lung cancer epithelial cells. In this study, we investigate the effects of PM2.5 on cellular responses in human nasal epithelial primary cells and lung cancer cells. The respiratory epithelial cells showed both time- and concentration-dependent decrease of cell viability when the cells were exposed to PM, which can be attributed to increased levels of reactive oxygen species (ROS) in the cells. In addition to inducing autophagy via increase of microtubule-associated protein light chain-3 (LC3) puncta, PM exposure also induced the expression of inflammatory cytokines such as IL-6 and 8 in human respiratory epithelial cells. Thus, inflammation response, as well as autophagosome formation due to increased ROS, is responsible for apoptotic death of respiratory epithelial cells under challenge of PM. The glucocorticoid ciclesonide used to treat asthma and allergic rhinitis was shown to be effective in reducing cellular mortality, probably by reducing inflammatory cytokines. These results suggest that PM2.5-induced inflammation plays a key role in apoptotic cell death in respiratory epithelial cells, which may be treated by an anti-inflammatory glucocorticoid.

Keywords: Particulate matter, Respiratory epithelial cells, Reactive oxygen Species, Glucocorticoid, Inflammatory cytokines

Introduction

Particulate matter (PM) is one of the most common urban ambient air pollutants, which can be produced by industrialization and various other combustions such as fossil fuels, vehicle smoke, and so on. In particular, PM with an aerodynamic diameter of <2.5 μm (PM_{2.5}) has been reported to cause a variety of respiratory and cardiovascular diseases. Recent studies have reported various PM_{2.5}-induced cellular responses such as autophagy and apoptosis response in lung cancer cells. Autophagy is known to be the catabolic mechanism for cell survival that helps to maintain cellular homeostasis, which involves subcellular degradation of unnecessary or dysfunctional cellular components. Through autophagy, decomposed proteins and organelles can be used as the energy source. Expression of various autophagy-related proteins involving microtubule-associated proteins light chain 3 (LC3), and degradation of cytosolic components can lead to the formation of autophagosome, which is a double membrane structure and which subsequently fuses with lysosomes to form autolysosomes. Thus, autophagy has been recognized as a cellular pathway to maintain homeostasis and adaptation to environmental stress.

Recently, it has been reported that PM can induce oxidative stress as an important molecular mechanism of cell toxicity. Oxidative stress is caused by reactive oxygen species (ROS), which are signaling molecules in both cell survival and cell death. Several studies have suggested that inorganic and organic components from PM exert toxic effects on cells by increasing oxidative stress in lung epithelial cells. Deng et al. recently observed that autophagy is triggered by PM_{2.5}-induced oxidative stress in human lung epithelial A549 cells. To date, however, the presence of the PM_{2.5}-induced autophagy has not been investigated in nasal epithelial cells, which is important to assess front-line effects incurred by PMs in the human respiratory system. Inflammatory response is a nonspecific host defense mechanism, which is generated by external pathogens such as viruses, bacteria, and parasites. Proinflammatory cytokines are soluble signaling peptides molecules, which include interleukin-6 (IL-6), IL-8, and tumor necrosis factor-α (TNF-α). These molecules assist immune response, which could induce...
or reduce inflammatory responses. Very recently, one study reported that exposure of PM$_{2.5}$ to mouse-bone-derived macrophage cells enhanced inflammatory potential by increased secretion of cytokines such as TNF-α and IL-1β in vitro. This study, however, did not use human primary cells derived from respiratory tissues, which are more suitable to investigate combined cellular responses such as inflammatory cytokine release and the accompanying morbidity of neighboring cells. In pharmacology, steroids or glucocorticoids are used to suppress the adverse effects of inflammatory response by inhibiting the induction of inflammatory cytokines. Inhalation of a glucocorticoid such as ciclesonide to treat asthma and allergic rhinitis can inhibit the induction of cytokines implicated by inflammatory response in the nasal mucous membrane and bronchial mucosa cells.

In this study, we investigated the cellular effects by exposure to PM$_{2.5}$ regarding apoptotic cell death, autophagy, and inflammatory responses by employing human lung epithelial A549 cells and human nasal epithelial primary (HNEp) cells. Compared to the previous studies using the lung cancer cell line such as A549 cells, our study used human respiratory primary cells such as HNEp cells cultured from human tissues, which provide a better in vitro system to assess the physiological responses against PMs than immortalized cancer cell lines. Furthermore, we demonstrated that ciclesonide is an effective reagent to attenuate inflammation-related death of respiratory epithelial cells inflicted by PM.

**Experimental**

**Human Respiratory Epithelial Cells and Glucocorticoid.** The human lung cancer epithelial cell line, A549, was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). HNEp cell line HNEpC was provided by the Kosin University College of Medicine (Busan, Korea). Cell line A549 and HNEpC were cultured in Dulbecco’s modified minimal essential medium (DMEM) and RPMI-1640 media supplemented with 100 U of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum (FBS) and fetal calf serum (FCS). The cells were maintained at 37 °C in 5% CO₂ incubator. The anti-inflammatory glucocorticoid, ciclesonide (Omnaris®), micronized as nasal spray type, was obtained from Takeda Pharmaceutical Co. (Osaka, Japan).

**PM$_{2.5}$ Sampling and Preparation.** PM$_{2.5}$ samples were collected in the Dong-A University (Busan, Korea) in April 2014. PM$_{2.5}$ samples were collected on Whatman® Nuclepore™ filters (diameter 47 mm, pore size 0.2 μm, purchased from GE healthcare life sciences, Piscataway, NJ, USA) using a cyclone sampler (about 16.7 L/min, URG Corporation, Chapel Hill, NC, USA). The filters were equilibrated in 40% relative humidity and 25 °C for 48 h, and then weighed on a high-precision microbalance. PM$_{2.5}$ particles were extracted from nitrocellulose filters by immersing them in PBS buffer and sonicating for 30 min. The extracted samples were then stored at −80 °C until cell exposure. Blank control filters were also prepared using the same method as above except for the sampling. The amount of PM samples was estimated by the difference in weight between the sampled filter and the blank filter.

**Cell Viability Assay.** Cell viability was measured by the methylene blue colorimetric microassay. A549 and HNEp cells (3 × 10⁴ cells/well) were treated with 50, 100, and 200 μg/mL of PM$_{2.5}$ for 24 and 48 h. Cells were fixed with 4% paraformaldehyde for 1 h and stained with methylene blue (1% w/v in borate buffer, Sigma-Aldrich, St. Louis, MO, USA) for 10 min. After dying at room temperature, the dye was solubilized using 0.1 M HCl. The absorbance was read at 595 nm using a VICTOR X3 multilabel plate reader (PerkinElmer, Waltham, MA, USA).

**ROS Assay.** A549 and HNEp cells were seeded into 96-well black plates at a density 3 × 10⁴ cells/well and then incubated at 37 °C. After 24 h incubation, cells were treated with 50, 100, and 200 μg/mL of PM$_{2.5}$ or parquat (Sigma-Aldrich) for 6 h and then washed with PBS and added to 10 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA; Sigma-Aldrich). The fluorescence of DCF was measured by the VICTOR X3 multilabel plate reader (PerkinElmer, Waltham, MA, USA) with excitation and emission wavelengths of 485 and 535 nm, respectively.

**Confocal Microscopy.** A549 and HNEp cells were cultured on cover slips in a 12-well plate for 24 h. The GFP-LC3 plasmid was transfected with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) for 4 h. Transfected cells were treated with 100 and 200 μg/mL of PM$_{2.5}$ or parquat (50 μM) for 24 h. Cells were fixed with 4% paraformaldehyde for further 10 min and washed three times with PBS. After washing, the treated cells were visualized using a confocal laser scanning microscope (FV-1000, Olympus, Tokyo, Japan).

**Flow Cytometric Analysis of Apoptosis.** Apoptosis was monitored by flow cytometric analysis. A549 and HNEp cells were seeded into the 24-well plates at a density 6 × 10⁴ cells/well and then incubated at 37 °C. After 24 h incubation, cells were treated with 50, 100, and 200 μg/mL of PM$_{2.5}$ or staurosponine (2 μM as positive control, Sigma-Aldrich) for 48 h. Cells were then harvested and washed twice with cold PBS and resuspended in a staining buffer containing fluorescein-5-isothiocyanate, annexin V, and propidium iodide, provided in the apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer’s instructions. Flow cytometric data were obtained using a FACSCalibur flow cytometer equipped with BD CellQuest Pro software (BD Biosciences, San Jose, CA, USA).

**Enzyme-linked Immunosorbent Assay (ELISA).** A549 and HNEp cells were seeded into 24-well plates at a density 6 × 10⁴ cells/well and then incubated at 37 °C. After 24 h incubation, cells were treated with 50, 100, and 200 μg/mL of PM$_{2.5}$ or lipopolysaccharides at 50 ng/mL (LPS, Sigma-Aldrich) for 24 h. The amounts of proinflammatory cytokines IL-6 and IL-8 were measured with ELISA kits (Quansys Biosciences, Logan, UT, USA) according to the manufacturer’s instructions.
Results and Discussion

Cytotoxicity of PM$_{2.5}$ in Human Respiratory Epithelial Cells. First, cell viability assay was performed to assess cellular toxicity after exposure of respiratory epithelial cells to PM$_{2.5}$. All previous studies have used lung cancer epithelial cells for monitoring PM-incurred effects. In addition to lung epithelial cells, we used the HNEp cells which were isolated from human nasal mucosa tissues. The use of primary cells is better to assess physiological effects of PM because the lung epithelial cells such as A549 cells are immortalized as cancer cells. A549 and HNEp cells were treated with 50, 100, and 200 $\mu$g/mL of PM$_{2.5}$ for 24 and 48 h. The cell viability measured by methylene blue colorimetric microassay showed that PM$_{2.5}$ exposure had significant cytotoxicity in respiratory epithelial cells in a dose- and time-dependent manner (Figure 1). These results indicated that exposure of PM$_{2.5}$ causes cytotoxicity, leading to cell death in respiratory epithelial cells.

PM$_{2.5}$ Generates Oxidative Stress in Respiratory Epithelial Cells. ROS as signaling molecules are involved in autophagy and/or cell death. Previous studies have indicated that PM$_{2.5}$ can increase the ROS levels in cells. To monitor the ROS level in the respiratory epithelial cells upon exposure to PM$_{2.5}$, intracellular fluorescence level of ROS was determined using an ROS-sensing reagent (DCF-DA). After treating with various concentrations of PM$_{2.5}$ for 6 h, the respiratory epithelial cells showed increased DCF fluorescence level in a dose-dependent manner, indicating that a significantly higher amount of ROS was generated compared to unexposed control cells (Figure 2). Exposure of PM$_{2.5}$ at 100 $\mu$g/mL induced ROS accumulation to a similar extent as with the treatment of 50 $\mu$M ROS-generating reagent (paraquat) in the cells.

PM$_{2.5}$ Induces Autophagy and Apoptosis. Previous studies have indicated that PM$_{2.5}$ as well as nanoparticles induced autophagy by oxidative stress. As we observed that exposure to PM$_{2.5}$ of respiratory epithelial cells increased the ROS levels, we investigated whether PM$_{2.5}$ induces autophagy and/or apoptosis via oxidative stress. To assess autophagosome formation in cells, expression and puncta of the GFP-LC3 protein was monitored under confocal fluorescence microscopy (Figure 3(a)). Respiratory epithelial cells increased autophagosome protein GFP-LC3 vacuoles (i.e., LC3 puncta) in the cytoplasm after treatment with PM$_{2.5}$. This result indicates that increased levels of ROS in the cells (shown in Figure 2) triggered autophagy in the cells inflicted by PM$_{2.5}$. Next, the progress of apoptotic cell death was investigated using flow cytometric analysis of cells exposed to PM$_{2.5}$. As the cells were treated with PM$_{2.5}$, the cells underwent apoptosis in a dose-dependent manner (Figure 3(b)). Thus, human respiratory epithelial cells exposed to PM$_{2.5}$ undergo cellular autophagy and subsequent apoptotic cell death due to enhanced ROS in cells.

Induction of Inflammatory Cytokines by PM$_{2.5}$ in Respiratory Epithelial Cells. Previous studies have reported that PM$_{2.5}$ induced inflammatory cytokines such as IL-8 and TNF-$\alpha$ in lung cancer cells. To observe the inflammatory response by the PM$_{2.5}$, we measured the expression of cytokines.
including IL-6 and IL-8 by using ELISA assay in respiratory epithelial cells. A549 and HNEp cells were treated with various concentrations (50, 100, and 200 μg/mL) of PM2.5 for 24 h.

In both cell lines, inflammatory cytokines released after the exposure were significantly higher than those in the control (Figure 4). Both cell lines showed similar sensitivity to PM2.5 in producing IL-6 and IL-8 upon exposure to PM2.5, which was comparable to that when treated with the cytokine-inducing reagent (i.e., LPS). It has been previously reported that PM2.5 exposure synergistically increased the levels of TNF-α and IL-1β in mouse macrophages in vitro. Unlike that in the previous report, our result indicated that IL-6 and IL-8 were mainly secreted in the respiratory HNEp cells.

**Figure 3.** Induction of autophagy and apoptotic cell death by PM2.5 exposure in respiratory epithelial cells. (a) Confocal fluorescence microscopy images of A549 and HNEp cells treated with PM2.5 or paraquat (50 μM) for 24 h. (b) Flow cytometric estimation of apoptotic cell numbers of A549 and HNEp cells. Cells were treated with PM2.5 or staurosporine (2 μM) for 48 h.

Anti-Inflammatory Glucocorticoid Attenuates Cell Toxicity Incurred by PM2.5. Corticosteroids are known to play a
role in reducing the inflammatory response in cells. We investigated the cell viability of respiratory epithelial cells exposed to PM$_{2.5}$ with or without treatment with ciclenoside (Figure 5(a)), which is an FDA-approved glucocorticoid drug to treat asthma and allergic rhinitis. A549 and HNEp cells were treated with various concentrations of PM$_{2.5}$ in the presence or absence of ciclenoside for 24 h.

For both cell lines, cell viabilities decreased significantly less compared to the ciclenoside treatment than without the drug treatment (Figure 5(b)). Thus, glucocorticoid ciclesonide at optimal concentration (i.e., 5 μg/mL) was shown to be effective in reducing cellular toxicity incurred by PM$_{2.5}$, probably by reducing inflammatory cytokines.

**Conclusion**

We have observed that PM$_{2.5}$ can induce cellular toxicity by generating oxidative stress, which can cause the onset of autophagy and eventual apoptotic cell death in human respiratory epithelial lung cells and nasal epithelial primary cell lines. Moreover, PM$_{2.5}$ induced secretion of inflammatory cytokines such as IL-6 and IL-8 in both cell lines. The results suggest that PM$_{2.5}$-induced oxidative stress and inflammatory cytokines probably play a key role in causing cell toxicity in human respiratory epithelial cells. The adverse effect of PM$_{2.5}$ on the cell viability was ameliorated by treatment with the anti-inflammatory glucocorticoid, which might be contributing to the treatment of PM$_{2.5}$-induced impairment of
respiratory function. Our study is the first in which the glucocorticoid ciclesonide is shown to be effective in treating asthma and allergic rhinitis by reducing cellular mortality in human respiratory epithelial cells inflicted by ambient PM by probably reducing inflammatory cytokines.

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