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The 3-methyl-4-nitrophenol (PNMC) compromises airway epithelial barrier function

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ABSTRACT

Background and aims: It is recognized that the air pollution is associated with the pathogenesis of airway diseases. This study aims to elucidate the role of the 3-methyl-4-nitrophenol (PNMC), one of the components of diesel-exhaust particles, in compromising the airway epithelial barrier integrity.
Methods: A549 cells, an airway epithelial cell line, were cultured to monolayers to be used as an in vitro epithelial barrier model. BALB/c mice were treated with nasal drops containing PNMC to test the effects of PNMC on alternating the airway epithelial barrier functions.
Results: Exposure of mice to PNMC induced nasal epithelial cell apoptosis and increased the permeability of the nasal epithelial barrier. PNMC increased casp8 and casp3 activities in nasal epithelial cells. Exposure to PNMC up regulated Fas and FasL expression in airway epithelial cells. Inhibition of caspase abolished the PNMC-induced airway epithelial barrier dysfunction.
Conclusion: Exposure of airway mucosa to PNMC induces epithelial cell apoptosis and compromises the epithelial barrier function, which can be prevented by the inhibition of caspases.

1. Introduction

A layer of epithelial cells covers on the surface of the airway mucosa. The cells connect each other via the tight junction complexes. The cell bodies and the tight junction complexes form the epithelial barrier. This barrier allows water and substances with small molecular weight to pass through. Once the epithelial cell barrier damaged, substances with macromolecular weight, such as protein antigens, may pass through the epithelial barrier to reach the deep regions of the mucosal tissue to contact immune cells (Georas and Rezaee, 2014; Heijink et al., 2014). The aberrant immune responses, such as allergic reactions and chronic inflammatory responses, may be initiated (Georas and Rezaee, 2014; Heijink et al., 2014). Published data indicate that the epithelial barrier dysfunction is associated with a large number of inflammatory diseases (Brune et al., 2015); the underlying mechanisms are not fully understood yet.

It is recognized that the air pollution is associated with the pathogenesis of a number of diseases in the body especially in the airway, such as allergic rhinitis and asthma (Fuertes et al., 2013; Kelly and Fussell, 2011). The automobile-exhaust particles, such as gasoline- and diesel-exhaust particles, are one of the major sources of the air pollution (Alexis and Carlsten, 2014). The particles include multiple substances, such as carbons, sulfate, nitrate, a number of metals, and more than sixty polynuclear aromatic hydrocarbons (Cadle et al., 1999). Cumulative studies demonstrate that the automobile-exhaust particles are closely associated with the airway inflammation (Fuertes et al., 2013; Ghio et al., 2012) via releasing pro-inflammatory cytokines, including cytokines involved in T helper (Th)17 differentiation, production of interleukin (IL)-1β, IL-6 and neutrophil chemokines (such as IL-8). The p38 mitogen activated protein kinase and nuclear factor-κB signaling pathway are involved in the air pollution-associated inflammatory reactions (Totlandsdal et al., 2010). Yet, remedies to prevent the air pollution-related inflammatory response are still limited currently.

One of the phenomena in the airway mucosa caused by air pollution is the airway epithelial barrier dysfunction (Nasreen et al., 2014). The abnormality of tight junction and loss of epithelial cells are the main

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causative factors involving in the epithelial barrier dysfunction (Chen and Yan, 2015; Fischer et al., 2013). The aberrant apoptosis may be one of the causes inducing the airway epithelial cell loss to cause the airway epithelial barrier dysfunction. Apoptosis is also called programmed cell death with the purpose to remove ageing cells or damaged cells under physiological condition. However, some unusual events, such as exposure to tumor necrosis factor, can abnormally initiate apoptotic process to induce cell apoptosis (Guo et al., 2014). Thus, in this study, we carried out an animal study and a cell culture study to observe the effects of the 3-methyl-4-nitrophenol (PNMC), one of the components of automobile-exhaust particles on compromising airway epithelial barrier functions. The results showed that exposure to PNMC induced the nasal epithelial barrier dysfunction via inducing epithelial cell apoptosis by activating caspase-8 (casp8), casp3 and increasing Fas/FasL expression in airway epithelial cells.

2. Materials and methods

2.1. Reagents

The antibodies of Fas, FasL, procaspase 8 and procaspase 3 were purchased from Santa Cruz Biotech (Santa Cruz, CA). Fluorometric assay kits for casp8 and casp3, 3-methyl-4-nitrophenol (PNMC), Z-VAD-FMK (caspase inhibitor) fluorescein isothiocyanate (FITC)-labeled dextran (MW = 40 kDa), annexin V kit and propidium iodide were purchased from Sigma Aldrich (St Louis, MO).

2.2. Mice

Male BALB/c mice, 8–10 week old, were purchased from the Beijing Experimental Animal Center (Beijing, China). The mice were maintained in a specific pathogen facility with accessing food and water freely. The using animals in the present study was approved by the Animal Ethics Committee at Shanxi Medical University.

2.3. Assessment of the effects of PNMC on alternating the epithelial barrier functions of the mouse airway mucosa

Mice were treated with nasal drops (20–30 μl/drop) containing PNMC (10 μg/ml, or 20 μg/ml, or 30 μg/ml) daily for 1 week, or 2 weeks, or 3 weeks. Each mouse was treated with 3 drops per nostril each time. Before sacrificing, the mice were treated with nasal drops (Each mouse was treated with 3 drops per nostril each time) containing FITC-labeled dextran (MW = 40 kDa; 1 mg/ml). The mice were sacrificed 1 h after the last nasal drop. The blood samples were collected from each mouse. The serum levels of dextran were determined with a fluorometer and used as the indicators of the airway epithelial barrier functions.

2.4. Assessment of apoptotic nasal epithelial cells

Mice were treated with nasal drops for 3 weeks. After sacrifice, the nasal mucosal samples were collected and processed for cryosections. The sections were stained with the terminal deoxynucleotidyl transferase (TUNEL) staining with a reagent kit following the manufacturer's instructions. The positively stained cells were observed under a microscope.

2.5. Assessment of apoptotic A549 cells

After treating with PNMC for 48 h, the A549 cells were harvested and stained with propidium iodide (PI) and annexin V kit following the manufacturer's instructions. The cells were analyzed by flow cytometry. The annexin V^+ or PI^+ annexin V^+ cells were regarded as apoptotic cells.

2.6. Cell culture

A549 cells and RPMI2650 cells (human airway epithelial cell lines, ATCC (Manassas, VA)) were cultured in DMEM supplemented with fetal bovine serum (10%), penicillin (100 U/ml), streptomycin (0.1 mg/ml) and glutamine (2 mM). The medium was changed daily. The cell viability was greater than 99%as assessed by the Trypan blue exclusion assay. The cells were harvested from culture dishes by incubating with 0.25% trypsin and EDTA no-serum solution for 5–10 min and used in further experiments.

2.7. Assessment of activity of casp8 and casp3

Casp8 and casp3 activity was measured in A549 cells (1×10^6/ml) and nasal epithelial scraping samples. The assay was performed by monitoring cleavage of the casp8- or casp3-specific fluorogenic substrate according to the manufacturer's instructions.

2.8. Real-time quantitative RT-PCR (RT-qPCR)

The total RNA was extracted from cells collected from relevant experiments with the TRIzol reagents. The cDNA was synthesized with the RNA and a reverse transcription kit following the manufacturer instructions. The samples were amplified in a qPCR device with the SYBR Green Master Mix and the presence of relevant primers (Table 1). The results were presented as fold change against the housekeeping gene β-actin.

2.9. Preparation of protein extracts from cells

Cells were collected from relevant experiments and lysed with a lysing buffer. The lysates were centrifuged for 10 min at 13,000 rpm. The supernatant was collected and used as the cytosolic extracts. All the procedures were performed at 4 °C.

2.10. Western blotting

The proteins were fractioned by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. The membrane was blocked by incubating with 5% skim milk for 30 min, followed by incubating with antibodies of interest overnight at 4 °C, washed with Tris-buffered saline Tween (TBST) 20 for 3 times, incubated with peroxidase-labeled second antibodies at room temperature for 2 h. After washing with TBST, the immunoblots on the membrane were developed with the enhanced chemiluminescence and photographed with an imaging device.

2.11. RNA interference (RNAi)

The A549 cells were treated with RNAi of casp8, or RNAi of casp3, or control RNAi with purchased shRNA reagent kits with the aid of electroporation using a Bio-Rad Gene Pulse set at 950 μF and 280 V. The effects of RNAi were assessed by Western blotting 48 h after the transfection.

Table 1
Primers used in the experiments.

Molecules	Forward	Reverse
ZO-1	acgatctcctgaccaacgtt	gctttgggtggatgatcgtc
Occludin	ctacggaggtggctatggag	agcgctgactatgatcacga
Claudin 5	cgttgtccgcgagttctatg	gagtacttgaccgggaagct
Fas (mouse)	acctccagtcgtgaaaccat	ctcagctgtgtcttggatgc
Fas (human)	gtgctttgcttagggttccc	aacttgcacttctggccatg
FasL (mouse)	atagccaaccccagtacacc	gctggttgttgcaagactga
FasL (human)	cagaaggcagggaagtgaga	ccatcctcgctactcccatt