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Mitochondrial dysfunction and inflammatory response in the cytotoxicity of NR8383 macrophages induced by fine particulate matter

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ABSTRACT

Exposure to fine particulate matter (fine PM) is known to cause severe public health problems. However, the potential mechanisms of alveolar macrophages cytotoxicity induced by fine PM exposure are still unclear. The aim of this study was to determine if both the mitochondrial dysfunction and the inflammatory response of NR8383 macrophages were triggered by Standard Reference Material 2786 (SRM 2786) for fine PM. NR8383 cells were exposed to different concentrations of SRM 2786, mitochondrial membrane potential, ultrastructure of mitochondria, activities of caspase 3/9, release of IL-6/TNF-α, expression of NF-κB and IκBα were evaluated. The results indicated that SRM 2786 induced mitochondrial dysfunction by increasing activities of caspase-3 and caspase-9, and structural damages of mitochondria with dissipation of mitochondrial membrane potential. The inflammatory response was triggered as evidenced by increased release of IL-6/TNF-α, and increased protein expression of NF-κB with decreased protein expression of IκBα. Consequently, these data indicate that both mitochondrial dysfunction and inflammatory responses might be responsible for SRM 2786 induced macrophage cytotoxicity.

1. Introduction

The respiratory tract is unique due to its directly linkage to the external environment. There is extensive evidence that long term exposure to particulate matter (PM), including fine PM ($PM_{2.5}$) is associated with an increase in the morbidity and mortality from respiratory diseases for the general population (Huang, 2014; Kan et al., 2009). Accumulated studies suggested that different types of PM might induce pulmonary diseases via oxidative damage and cell apoptosis (Pope et al., 1999; Ghio et al., 2012). However, the underlying mechanisms for fine PM induced cytotoxicity are largely unknown.

Mitochondria dysfunction is important in the development of pathology of many diseases, including lung disease (Kappos et al., 2004). One possible mechanism for mitochondria dysfunction induced PM-mediated lung injury is that mitochondria are major targets for PM as well as the main source of reactive oxygen species (ROS), since ROS production plays a key role in PM-induced oxidative stress process (Rodriguez-Cotto et al., 2015). Meanwhile, different cytokines released by lung macrophage are considered to contribute to the pathogenesis of lung injury by inducing inflammation and fibrosis (Fujiwara and Kobayashi, 2005; Kanj et al., 2005), but the underlying mechanisms remain poorly understood.

Both mitochondrial function and proinflammatory mediators are involved in cell cytotoxicity induced by nanoparticles stimulation (Ma et al., 2011), while they are also proven to have important influence on each other (Tsukahara and Haniu, 2011). Nakahira et al. (2011) found that mitochondrial dysfunction could increase the responsiveness of different types of cells to cytokine-induced inflammatory responses as demonstrated by accelerating the inflammatory responses through ROS generation and nuclear-factor-kappa B (NF-κB) activation. In addition, several studies reported that increased sensitivity to NF-κB binding sites might be essential for inflammatory gene expression (Ungvari et al., 2007; Vaamonde-García et al., 2012). On the contrary, a great number of cytokines, such as interleukin (IL)-6, IL-1β or tumor necrosis factor alpha (TNF-α) might also induce mitochondrial damage (Zell et al., 1997), as evidenced by decreasing the ATP production and mitochondrial membrane potential (ΔYm). Consequently, understanding the relationship between mitochondrial function and inflammatory response might be essential for preventing cell cytotoxicity induced by fine PM exposure.

Alveolar macrophage (AM) can react with ambient particulate directly (Hogg and Eden, 2009; Yuan et al., 2013), therefore it is recognized as the first line of defense against these foreign PM attack,

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which might be beneficial for PM-induced respiratory disorders treatment (Alexis et al., 2014). Our previous study (Xiong et al., 2015) showed that the standard reference material 2786 (SRM 2786) with relatively constant components for fine PM processed by the National Institute of Standards and Technology (NIST, Gaithersburg, MD) induced AM cytotoxicity mainly through apoptotic and oxidative stress response. The aim of the current study is to continue to investigate the potential underlying mechanisms of AM cytotoxicity induced by fine PM. Consequently, SRM 2786 with different concentrations based on previous studies (Mitkus et al., 2013; Xiong et al., 2015) is still applied to this research to assess whether the fine PM induced mitochondrial dysfunction in AM. In addition, we also want to examine if SRM 2786 exposure triggered inflammatory response in AM by evaluating the expressions of different inflammatory mediators.

2. Materials and methods

2.1. Preparation of fine particulate matter

Standard reference material (SRM 2786) was obtained from the NIST. In brief, the fine particulate matter was collected in 2005 from Prague, Czech Republic. The reported mean particle diameter is around 2.8 μm. The constitution and certificate of analysis for SRM 2786 are also available online (Mitkus et al., 2013; NIST, 2013; Xiong et al., 2015).

To prepare the stock suspension of SRM 2786, it was firstly made fresh in Kaighn's Modification F-12K culture media (Gibco, USA) at a final concentration of 2 mg/ml, then sonicated for 5 min on ice (Ningbo Scientz Biotechnology, China) and vigorously vortexed for another 2 min prior to each experiment, three different concentrations of SRM 2786 were prepared as discussed below.

2.2. Cell culture preparation

Rat alveolar NR8383 cells were applied to all experiments (Shanghai Institute of cell library, China). NR8383 cells were grown in 75 cm^2 tissue culture flasks (Corning Incorporated, USA) in 80% Kaighn's Modification F-12K culture media supplemented with 20% heat-inactivated fetal bovine serum (Gibco, USA), the antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml) were also included. In brief, cells were incubated at 37 °C/5% CO_2 incubator for 2–3 days to grow to confluence, then the cells were spun at 1000 rpm for 6 min at room temperature and finally these cells were harvested and seeded in either 96-cell-well culture plates, 24-cell-well culture plates, 6-cell-well culture plates or culture flasks (Becton, Dickinson and Company, USA) at a density of 5×10^3, 3×10^5, 1×10^6 and 5×10^6 for diverse experimental procedures. All procedures were conducted in triplicate and each experiment was performed in triplicate.

2.3. Exposure of cells to SRM 2786

Stock suspension of SRM 2786 was further diluted in fresh Kaighn's Modification F-12K culture media at concentrations of 125, 250 or 500 μg/ml based on our previously published study and other related studies (Li et al., 2003; Mitkus et al., 2013; Xiong et al., 2015) NR8383 cells were then incubated with these different concentrations of SRM 2786 for 24 h incubation period at 37 °C/5% CO_2 incubator.

2.4. Transmission electron microscopy

The ultrastructural changes in NR8383 cells mitochondria post different concentrations of SRM 2786 treatment were observed by electron microscopy. Similar procedures were followed by our previous published study and other related study (Xiong et al., 2014; Gualtieri et al., 2011). Briefly, cells were firstly harvested, spun at 1000 rpm for 6 min, and then removed the supernatants. The remaining cells were fixed with 2.5% glutaraldehyde in cacodilate buffer, pH 7.6 for 1 h and post-fixed with 1% OsO_4 in the same buffer for 2 h. After PBS washing, cells were dehydrated in a series graded ethanol and embedded in EPON® 812. Ultrathin sections of the cell layers were prepared and put onto 300 mesh copper grids, and then these sections were stained with uranyl acetate and lead citrate. The mesh copper grids were examined by Tecnai G2 transmission electron microscope (FEI Corporation, USA) operating at 160 kV with a CCD camera.

2.5. Evaluation of mitochondrial membrane potential

Changes in ΔYm during the early stages of apoptosis were analyzed by Muse MitoPotential assay (Merck Millipore, Germany) in NR8383 cells with different concentrations of SRM 2786 treatment in 6-well culture plates for 24 h. Firstly, cells were harvested and the cell pellet was suspended in Muse™ MitoPotential dye working solution (diluting the dye 1:1000 with 1 × Assay Buffer at the concentration of 10^5 cells/100 μl Muse™ MitoPotential dye working solution) and the cell samples were incubated at 37° C for 20 min, and finally 5 μl of Muse MitoPotential 7-AAD dye was also added in each group and incubated for another 5 min at room temperature. Changes in mitochondrial membrane potential were determined by flow cytometry (Muse™ Cell Analyzer, Germany).

2.6. Measurement of Caspase-3 and Caspase-9 activities

Both caspase-3 (Cat No C1115, Beyotime Company, China) and caspase-9 (Cat No C1157, Beyotime Company, China) activities were determined by commercially available Caspase Activity Assay kits in triplicate. Briefly, different groups of NR8383 cells treated by SRM 2786 were initially cultured on culture flasks for 24 h and then collected these cell samples according to the instruction book. Then all the samples incubated with either test buffer or substrate (Ac-DEVD-pNA for casepase-3 and Ac-LEHD-pNA for caspase-9) at 37° C . The detailed experimental procedures were performed following manufacturer's instructions. The results were measured by microplate reader (Thermo Scientific, USA) at 405 nm. The activities of caspase-3 and caspase-9 in different groups were calculated based on corresponding standard curves.

2.7. Cytokine secretion of TNF-α and IL-6

NR8383 cells with different treatments by SRM 2786 were cultured on 24-well culture plate for 24 h and then the supernatants were collected. The secretion of TNF-α (Cat No EK0526, Boster Company, China) and IL-6 (Cat No EK0412, Boster Company, China) from NR8383 cells were examined by commercially available Elisa kits according to the manufacturer's instructions. Cytokine secretions were determined using the microplate reader at 450 nm. Standard curves for each cytokine were plotted. Three independent cytokine secretion experiments were performed.

2.8. Western blot analysis

NR8383 cell proteins were extracted, homogenized and protein concentrations from different groups were detected by BCA assay kit (Boster Company, China). SDS-PAGE loading buffer was added to each group, and boiled for 5 min. Then 20 μg proteins of samples were examined by 12% SDS-PAGE gel and transferred to PVDF membrane (Merck Millipore, USA). For immunoblotting, membranes were blocked with 5% (w/v) milk in TBST for 1 h at RT. Primary antibodies (Cell Signaling, USA) including rabbit anti-NF-κB (1:1000), mouse anti-IκBα (1:800) or rabbit anti-β-actin (1:1000) incubated with PVDF membranes in 5% (w/v) milk in TBST at 4 °C overnight. Then the membranes were washed and incubated with a 1:3000 dilution of HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Boster