RIPK1 is a key factor in black carbon-induced cell death

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(Received 18 December 2021; and accepted 23 December 2021)

ABSTRACT

Air pollution is associated with increased morbidity and mortality and with cell death at a cellular level. However, the exact mechanism of particulate matter-induced cell death remains to be elucidated. The aim of the present *in vitro* study using human alveolar epithelial cells (A549) was to determine the cell death pathway(s) induced by black carbon (BC) and ozone oxidized-black carbon (O-BC). BC and O-BC induced A549 cell death and the cytotoxic effect was dose-dependent. Cell death was significantly abrogated by inhibitor of receptor protein interacting kinase 1 (RIPK1) but only mildly inhibited by apoptosis inhibitor and RIPK3. BC- and O-BC-treated cells showed RIPK1 and RIPK3 protein overexpression and high phosphorylated levels of these proteins, as well as detectable levels of caspase-8 active form. BC- and O-BC-triggered cell death was also fully rescued in A549 cells that under-expressed RIPK1 with RIPK1 siRNA. Our results indicated that BC and O-BC could induce cell death through a multitude of pathways including apoptotic and necroptotic pathways and that RIPK1 is the upstream signal protein of these cell death pathways, with an important role in the regulation of BC-induced cell death.

INTRODUCTION

Air pollution is a serious problem on public health. Ambient air pollutants include particles and gases, and PM_{2.5} (particle mass with aerodynamic diameter of <2.5 µm) and tropospheric ozone have been selected as representative pollutants to quantify the health impact of ambient air pollution. Black carbon (BC) is major component of PM_{2.5} and is a byprod-

uct from the incomplete burning of solid fuels, residential cooking and heating, and vehicle exhausts. The adverse health effects of BC exposure have been investigated in epidemiological and experimental studies; exposure to BC increases the risk of cancer, chronic obstructive pulmonary disease (COPD), and cardiovascular dysfunction (Luben *et al.* 2017; Niranjana and Thakur 2017).

The mechanisms of the ill-effects of BC include oxidative stress, breakdown of double stranded DNA and formation of DNA adducts due to reactive oxygen species (ROS)-induced enhancement of DNA damage. Exposure to BC is also associated with changes in DNA methylation (Rider and Carlsten 2019). As a fine particulate, BC settles deep into the bronchoalveolar tree, then enters the circulation. Therefore, exposure to BC further induces inflammation that can lead to cell death and ultimately result in ventilatory dysfunction. BC-induced cell death is probably mediated through the apoptotic (An *et al.* 2019a) and autophagic pathways (An *et al.* 2017)

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and is even described sometimes to involve more complex molecular and cellular processes (Peixoto *et al.* 2017; An *et al.* 2019b). On the other hand, ozone, as a highly reactive irritant gas, causes oxidative stress, inflammation and lung tissue damage. Thus, the combination of ozone and PM_{2.5} aggravates lung damage and increases lung disease and mortality (Sunil *et al.* 2012).

In addition to the above mechanisms, ozone gases can cause oxidation of BC particles, which modifies their physicochemical characteristics, resulting in enhanced redox activity after emission into the atmosphere (Rattanavaraha *et al.* 2011). BC has a relatively large surface. After emission into the atmosphere, various pollutants such as heavy metals, sulfate, nitrate, polycyclic aromatic hydrocarbons (PHAs) and pathogen components adhere to the surface, synergistically increasing its toxicity. Experimentally, treatment of BC with ozone results in increased hydrophilicity, formulation of abundant oxygen-containing function groups and enhanced oxidative potential (Li *et al.* 2013; Xu *et al.* 2015). Several studies have assessed the differences in biological activities of BC and oxidized BC (O-BC) (Gao *et al.* 2017). O-BC generates more ROS and sufficient DNA damage repair responses than BC (An *et al.* 2019a). O-BC exposure also induces more pro-inflammatory cellular responses than BC (Chu *et al.* 2018). These results suggest that changes in particle surface properties through oxidation results in more severe genotoxicity and cytotoxicity responses than BC (Li *et al.* 2013; Gao *et al.* 2017).

The cell death processes of apoptosis and necrosis are also known as programmed and non-programmed cell death, respectively. The necrosis-like programmed cell death is currently termed necroptosis (Molnar *et al.* 2019). Receptor-interacting protein kinase 1 (RIPK1) is an upstream molecule on both the apoptosis and necroptosis pathways and is a key factor that regulates cell viability and inflammation (Wallach *et al.* 2016). It is known that TNF α binding to TNFR1 activates RIPK1, which in turn induces caspase-8-dependent apoptosis, or RIPK3 to trigger necroptosis. RIPK1 can also interact with NF- κ B pathway to induce inflammatory responses and cell death. Thus, RIPK1 is associated with a wide range of cellular events spanning from inflammation to cell death. Furthermore, exposure to BC or O-BC can result in the induction of apoptosis, autophagy and necroptosis (Peixoto *et al.* 2017; An *et al.* 2019b). Although the extent of oxidative stress is considered to play an important role in determining the cell death pathway (Peixoto *et al.* 2017), the

exact mechanism of BC- and OBC-induced cell death remains obscure.

The aim of this study was to determine the cell death pathway(s) induced by BC and O-BC using pharmacological approaches that included the use of specific inhibitors. The results showed that BC- and O-BC-dose-dependent induction of cell death was abrogated by RIPK1 inhibitors and partially by caspase inhibitors and RIPK3 inhibitors. The RIPK1 and RIPK3 protein and phosphorylated levels were increased in cells treated with BC and O-BC, and inhibition of RIPK1 expression using RIPK1 siRNA suppressed BC- and O-BC-induced cell death. Our results indicate that BC and O-BC induce multiple cell death pathways.

MATERIALS AND METHODS

Reagents. I κ B kinase (IKK) inhibitor BMS-345541 was purchased from Sigma Chemical Co. (St. Louis, MO); RIPK1 inhibitor Nec-1 and pan-caspase inhibitor zVAD-fmk were purchased from Selleck Chemicals (Houston, TX); and RIPK3 inhibitor GSK'872 was purchased from Calbiochem (Merck, Germany). The standard concentrations of inhibitors used in this study were selected based on previous studies: 20 μ M BMS-345541; 50 μ M Nec-1; 2 μ M GSK'872 and 25 μ M zVAD-fmk (Menon *et al.* 2017).

BC and O-BC were provided by Jing Shang (College of Environmental Sciences and Engineering, Peking University). As reported previously, Printex U carbon black (BC) was purchased from Degussa, O-BC was produced by exposure of BC to ozone, which was generated by photolysis of oxygen under an ultraviolet lamp (Li *et al.* 2013; Chu *et al.* 2018), and its physicochemical characteristics were similar to those described previously (Li *et al.* 2013). Briefly, physicochemical analysis showed a particle diameter of approximately 30 nm for both BC and O-BC with formation of larger size particles in water due to agglomeration. Treatment of the BC with ozone changes its surface charge from positive to negative, forms more oxygen species and free radicals on the BC surface and thus increases its oxidative capacity and cytotoxicity (Li *et al.* 2013).

Cell culture. Human alveolar epithelial cells (A549) were obtained from the American Type Tissue Collection (Manassas, VA) (Ouyang *et al.* 2018). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (10,000 U/mL) at 37°C and

maintained in an atmosphere of 5% CO₂.

Cell viability and inhibition assay. A549 cells were cultured in 96-well plates at a density of 5×10^4 cells. After 24-h culture, the cells were treated for 24 h with or without BC or ozone-BC (O-BC) at the concentration indicated in the figures. For the inhibition assay, the cells were cultured in 96-well plates at a density of 5×10^4 cells for 24 h, treated with each of the inhibitors at the concentrations indicated above for 30 min and then stimulated with BC or O-BC for 24 h. Cell viability was assessed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratory, Japan), which detects the absorbance value at 450 nm (OD 450) with SpectraMax 340PC384 96-well plate reader (Molecular Devices).

Cell death assay by Hoechst/PI double staining. A549 cells were cultured on 8-chamber slides (Nunc International, IL) for 24 h and treated subsequently with BC or O-BC for another 24 h. Apoptosis and necroptosis were assessed by Hoechst and propidium iodide (PI) double staining, according to the instructions provided by the manufacturer (Solarbio, China). The cells were then examined by confocal laser scanning microscopy (Nikon A1, Tokyo).

RNA interference. Silencer Pre-designed siRNAs for knockdown of RIPK1 proteins (siRNA ID #112846, 112847, 112848) and negative control (N.C) siRNA (siRNA ID #462001) were purchased from Invitrogen (Carlsbad, CA). Treatment of cells with siRNA RIPK1 was performed using Lipofectamine 2000, according to the instructions provided by the manufacturer. After 24-h transfection, the cells were divided into three groups and treated with or without BC or O-BC for 24 h. Cell viability was assessed using the CCK8 assay.

Western blot. A549 cells were cultured in 6-well plates at a density of 1.5×10^5 cells, and after 1 day of culture, the cells were treated with BC or O-BC for 24 h. The cells were lysed in a buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1% ProteinSafe Protease Inhibitor Cocktail (TransGen Biotech, Beijing, China) and 5 mM EDTA. The protein concentrations of the lysates were detected by Pierce BCA Protein Assay (Thermo Fisher Scientific). The proteins were subjected to SDS-PAGE, and the separated protein bands were transferred to PVDF membrane (Merck Millipore, Billerica, MA) for immunoblotting using the indicated antibodies. The primary antibodies

were purchased from Cell Signaling Technology (Danvers, MA). The immunoreactive bands were detected using the enhanced chemiluminescence (ECL) system (Amersham, UK) and visualized using Amersham Imager 600 (GE Healthcare, Freiburg, Germany).

RESULTS

Effects of BC and O-BC on cell viability

Physicochemical characterization of BC and O-BC was described in Materials and Methods section. First, human alveolar epithelial cells (A549) were incubated in the presence or absence of BC and O-BC, and the cell viability was assessed using the CCK-8 assay. As shown in Fig. 1, both BC and O-BC significantly and dose-dependently reduced cell viability. Treatment with 50 µg/mL of BC and O-BC decreased cell viability to 23.04% and 17.26%, respectively. These results suggest that both forms of BC are potentially cytotoxic and can induce cell death.

Cell death mode

Next, the A549 cells were incubated in the presence or absence of various cell death inhibitors to determine the cell death mode triggered by BC and O-BC. The IKK inhibitor BMS-345541 (BMS), a broad pan-caspase inhibitor zVAD-fmk (zVAD), RIPK1 inhibitor Nec-1 and RIPK3 inhibitor GSK'872 were added to the cell culture medium before the addition of BC or O-BC treatment, followed by assessment with CCK-8 analysis. Inhibition of IKK with BMS, inhibition of apoptosis with zVAD-fmk and inhibition of necroptosis with GSK'872 resulted in only mild inhibition of cell death (Fig. 2). On the other hand, RIP1 kinase inhibitor, Nec-1, significantly reduced BC and O-BC-induced cytotoxicity (Fig. 2). The inhibitory effects of these inhibitors on BC and O-BC-induced cell death seemed comparable. These results suggest that BC and O-BC-induced cell death involves more than one cell death pathway. RIPK1 plays a key role in regulating the death of cells exposed to BC and O-BC.

Complexity of BC and O-BC-induced cell death

We also used western blot analysis to investigate changes in the expression levels of various cell death-related proteins induced by BC and O-BC. Treatment of A549 cells with BC and O-BC upregulated the protein expression levels of RIPK1, RIPK3 and IκBα proteins and their phosphorylated levels. Furthermore, BC and O-BC also upregulated

Fig. 1 Cytotoxic effect of BC and O-BC on A549 cells. The A549 cells were pre-cultured in 96 well plate, after 24 h, cells were treated with BC and O-BC in concentration as indicated. Twenty-four hours later, cell viability was subjected to CCK-8 assay. Data are mean $\pm$ SD of at least 3 independent experiments.

Fig. 2 Forms of cell death induced by BC and O-BC. A549 cells were pre-cultured in 96-well plate for 24 h, and then the indicated inhibitors were added 30 min before the addition of 50 μg/mL of BC and O-BC. Twenty-four hours later, cell viability was assessed by CCK-8 assay. Data are mean $\pm$ SD of at least three independent experiments.

caspase-8 expression and enhanced the detection of the cleaved form of caspase-8 (Fig. 3A).

Exposure of A549 cells to BC and O-BC also induced apoptosis and necroptosis (Fig. 3B), which were confirmed with Hoechst/PI double staining. Staining with Hoechst/PI demonstrated cell nuclear staining with high Hoechst condensation, which is indicative of apoptosis. These results suggest that

OB and O-BC-induced cell death is mediated through apoptosis and necroptosis pathways as well as the NF-κB pathway.

RIPK1 regulates black carbon-induced cell death

In addition to the above pharmacological approaches, we also examined the role of RIPK1 in the regulation of BC and O-BC particle-induced cell death

A

Fig. 3 Western blot analysis of apoptosis- and necroptosis-related proteins in A549 cells treated with BC and O-BC. Proteins were extracted from cells treated for 24 h with 50 μ g/mL of BC or O-BC. (A) The proteins were detected with the listed antibodies. Actin was used as a loading control.

by gene silencing technique. In these experiments, we used RIPK1 siRNA to knockdown RIPK1. We used three pre-designed independent siRNA sequences directed against human RIPK1. Western blot analysis was used to determine the knockdown efficiency of this technique. The relative RIPK1 protein level was markedly decreased in A549 cells after transfection with #1 and #2 si-RIPK1 (Fig. 4A). Based on these results, we used #2 RIPK1 siRNA in the following experiment. Compared to the control siRNA, cells transfected with RIPK1-specific siRNA were completely protected against cell death induced by BC and O-BC. CCK-8 assay showed recovery of cell viability from 36.2% to 82.4% and 31.8% to 83.0% under RIPK1 silence conditions in A549 cells treated with BC and O-BC, respectively (Fig. 4B). These results are consistent with those obtained by the inhibitor assays and confirm the key role of RIPK1 in BC and O-BC-induced cell death.

DISCUSSION

Air pollution has adverse effects on human health. It is known that inhalation of PM_{2.5} leads to increased generation of reactive oxygen species (ROS), which can ultimately cause DNA damage, resulting inflammation and cell death. Although it has long been reported that cell death induced by BC and O-BC is either apoptosis (An *et al.* 2019b), necrosis (An *et al.* 2017) and a combination of the two (Peixoto *et al.* 2017; An *et al.* 2019b), this does not reflect well the complexity of the pathology.

In the present study, first, the cell toxicity of BC and O-BC was examined using the cell viability test by CCK-8. We found that BC and O-BC resulted in cell death in a dose-dependent manner. These results are in agreement with those reported in other previous studies (Li *et al.* 2013; An *et al.* 2017). Second, we used a variety of specific inhibitors to explore the mechanism of cell death caused by BC and O-BC. The results showed that IKK inhibitor, apoptosis-specific pan-caspase inhibitor zVAD-fmk and necroptosis RIPK3 inhibitor partially suppressed cell death, while RIPK1 specific inhibitor Nec-1 abrogated BC- and O-BC-induced cell death. We also detected a cleaved caspase-8 protein in the apoptotic active form, and up-regulation of protein expression and phosphorylation of RIPK1 and RIPK3 in BC- and O-BC-treated cells. Finally, the use of RIPK1 siRNA to suppress gene expression demonstrated a limited cell death. An *et al.* (2019a) proposed the involvement of other forms of cell death, in addition to apoptosis, underlying BC cytotoxicity. Our results indicate that the cell death caused by BC and O-BC is complex and exists in various forms, including apoptosis and necroptosis and are in agreement with those reported previously by other investigators, including necrosis and apoptosis in ROS-induced cell damage (Arakawa *et al.* 2015; Sun *et al.* 2018).

RIPK1 is the major modulator of cell fate under a wide range of cellular stress conditions. It is known as a molecular switch that can regulate inflammation and cell survival as well as apoptosis and necroptosis in response to various pathologic settings (Wallach *et al.* 2016). RIPK1 can activate the NF- κ B pathway through the IKK complex to cause an inflammatory response to circumvent cell death. IKK phosphorylates the inhibitory I κ B α protein. As a result, NF- κ B migrates to the nucleus from the cytoplasm and activates transcription of genes involved in inflammation and survival. IKK also phosphorylates and inhibits RIPK1 kinase activity to avert TNF-induced cell death (Dondelinger *et al.* 2015). When the NF-

Fig. 3 (B) Cell death after 24-h treatment with 50 $\mu\text{g/mL}$ of BC or O-BC was assessed by Hoechst/PI double staining and confocal microscopy. Merge: merge of transmitted detector channel, blue and red channels. Representative results of three independent experiments.

κB signaling pathway is inhibited, RIPK1 can form a complex with Fas-associated protein with death domain (FADD) and caspase-8 (complex II) to cause apoptosis (Wegner *et al.* 2017). When apoptosis is inhibited through the caspase-8 pathway, RIPK1 is phosphorylated to RIPK3 with further phosphorylation of mixed lineage kinase domain-like (MLKL), leading to the formation of a necrosome complex, with resultant switch from cell death by apoptosis to necroptosis. Our results demonstrated the induction of RIPK1 overexpression and phosphorylation by BC and O-BC. Both RIPK1 inhibition and expression silencing effectively promoted cell survival

compared with inhibitors of apoptosis and RIPK3, indicating that RIPK1 seems to act upstream of the cell death pathway through apoptosis and necroptosis to regulate cell death. Since RIPK1 is an important regulatory factor, the signaling pathways of apoptosis and necrosis seem to be linked, though how RIPK1 precisely regulates necrosis and apoptosis is still incompletely understood (Wegner *et al.* 2017). We do not know which specific form of death plays a major role in BC-related cytotoxicity, and which form of cell death occurs first and occurs second, or whether they are both induced simultaneously. Further research is needed to provide answers

Fig. 4 Knockdown efficiency of RIPK1 siRNA and cell viability following silencing RIPK1 in A549 cells. **(A)** Proteins from lysates of cells transfected with RIPK1 siRNA (#1, #2 and #3) or control siRNA were analyzed by western blotting using an antibody against RIPK1 or actin. **(B)** A549 cells were transfected with RIPK1 siRNA (#2) for 24 h and treated with 50 μ g/mL of BC or O-BC. Cell viability was determined 24 h after transfection by the CCK-8 assay. Data are mean $\pm$ SD of at least three independent experiments.

to these issues.

Although the experimental conditions were limited to lung cell lines in this study, BC and O-BC-induced cytotoxicity, inflammation and oxidative stress at human nasal epithelial cells (hNECs) (Li *et al.* 2021; Ouyang *et al.* 2021) and human corneal epithelial cells were reported (Long *et al.* 2020). Further study will evaluate the possible pathological machineries with various cell-lines including macrophages and intestinal epithelial cell-lines and other immune cells.

We conclude that RIPK1 is a key molecule involved in the regulation of cell death induced by BC and O-BC. The results suggest that RIPK1 is a potentially useful target for the design of new treatments for diseases associated with BC exposure.

Acknowledgments

The project was funded by the Program for Tongjiang Scholars and Fujian Natural Science Foundation (#2017J01152); Quanzhou City Science & Technology Program of China (#2018Z171, #2018N108S), and Program for Fujian Collaborative Innovation Center (Quanzhou Medical College).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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