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Original Paper

Necroptosis Contributes to Urban Particulate Matter-Induced Airway Epithelial Injury

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Key Words
Particulate matter • Necroptosis • Airway inflammation • Mucus hyperproduction

Abstract
Background/Aims: Necroptosis, a form of programmed necrosis, is involved in the pathologic process of several kinds of pulmonary diseases. However, the role of necroptosis in particulate matter (PM)–induced pulmonary injury remains unclear. The objective of this study is to investigate the involvement of necroptosis in the pathogenesis of PM-induced toxic effects in pulmonary inflammation and mucus hyperproduction, both in vitro and *in vivo*. ***Methods:*** PM was administered into human bronchial epithelial (HBE) cells or mouse airways, and the inflammatory response and mucus production were assessed. The mRNA expressions of IL6, IL8 and MUC5AC in HBE cells and Cxcl1, Cxcl2, and Gm-csf in the lung tissues were detected by quantitative real-time RT-PCR. The secreted protein levels of IL6 and IL8 in culture supernatants and Cxcl1, Cxcl2, and Gm-csf in bronchoalveolar lavage fluid (BALF) were detected by enzyme-linked immunosorbent assay (ELISA). We used Western blot to measure the protein expressions of necroptosis-related proteins (RIPK1, RIPK3, and Phospho-MLKL), NF-κB (P65 and PP65), AP-1 (P-c-Jun and P-c-Fos) and MUC5AC. Cell necrosis and mitochondrial ROS were detected using flow cytometry. In addition, pathological changes and scoring of lung tissue samples were monitored using hemoxylin and eosin (H&E), periodic acid-schiff (PAS) and immunohistochemistry staining. ***Results:*** Our study showed that PM exposure induced RIP and MLKL-dependent necroptosis in HBE cells and in mouse lungs. Managing the necroptosis inhibitor Necrostatin-1 (Nec-1) and GSK'872, specific molecule inhibitors of necroptosis, markedly reduced PM-induced inflammatory cytokines, e.g., IL6 and IL8, and MUC5AC in HBE cells. Similarly, administering Nec-1 significantly reduced airway inflammation and mucus hyperproduction in PM-exposed mice. Mechanistically, we found PM–induced necroptosis was mediated by mitochondrial reactive oxygen species-dependent early growth response gene 1, which ultimately promoted inflammation and mucin expression

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through nuclear factor κB and activator protein-1 pathways, respectively. ***Conclusions:*** Our results demonstrate that necroptosis is involved in the pathogenesis of PM–induced pulmonary inflammation and mucus hyperproduction, and suggests that it may be a novel target for treatment of airway disorders or disease exacerbations with airborne particulate pollution.

Introduction

Airborne particulate matter (PM) poses strong influences on the climate, environment and public health [1-3]. PM exposure has been shown to be a major risk factor for acute and chronic diseases including cardiovascular disease, liver fibrosis, various gastrointestinal diseases, and chronic respiratory disease, such as asthma and chronic obstructive pulmonary disease (COPD), along with lung cancer [4-8]. The sources of PM are complex and include transportation (e.g., vehicle exhaust), factory emissions (e.g., industries and coal-fired power plants), combustion (e.g., biomass and cigarette smoke) and agriculture (e.g., fertilizer and animal waste), and natural sources (e.g., volcanoes, forest fires and dust storms) [5]. Regardless of the origin, PM is a widespread air pollutant containing various toxins such as carbonaceous cores, polycyclic aromatic hydrocarbons (PAHs), quinones, metals, endotoxins, and many others [9]. Thus, elevated levels of short- and long-term PM exposure can induce many diseases associated with increased morbidity and mortality. It is important to find the molecular mechanisms and therapeutic targets in PM–induced pulmonary injury.

Apoptosis and necrosis are two major types of cell death. However, apoptosis is a programmed cell death modality, generally triggered by physiological processes, whereas necrosis is an uncontrolled and accidental cell death modality triggered by pathological processes. Necroptosis, a novel cell death modality, involves the loss of membrane integrity and occurs by a programmable mechanism with a characteristic necrotic cell death phenotype [10-13]. Necroptosis is initiated by the activation of receptor interacting protein (RIP) kinases and mixed-lineage kinase domain-like protein (MLKL). This leads to a loss of cellular integrity via the release of cytoplasmic content and the exposure of damage-associated molecular patterns (DAMPs) (e.g., high-mobility group box 1 [HMGB1], double-stranded DNA, heat shock protein 70, mitochondrial DNA, and ATP) [14-16]. Accumulating evidence suggested that necroptosis played an important role in the pathogenesis of pulmonary diseases [17]. One recent study described how the level of RIPK3, the hallmark of the activation of necroptosis, was increased in COPD lung tissue, and thus contributed to COPD pathogenesis [18]. Duprez also revealed that the inhibitor of RIPK1 (Nec-1) or deletion of RIPK3 (RIPK3 deficiency) could protect against systemic inflammatory response syndrome (SIRS) and sepsis [19]. Moreover, in lung cancer treatment, necroptosis exerted important roles in anti-tumor effect [20]. However, the functions and detailed mechanisms of necroptosis in PM–related pulmonary disorders still remain largely unknown.

The objective of this study is to investigate whether PM induces necroptosis in airway epithelium, and to understand its function in PM-induced epithelial injury. The upstream and downstream signals of PM-induced necroptosis are also explored.

Materials and Methods

Cell Culture

HBE human bronchial epithelial cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C in a water-saturated atmosphere with 5% CO_2. The cells were incubated with the following reagents, Nec-1 (25μ M, Sigma, USA), GSK'872 (5μ M, Selleck, USA), standard reference airborne PM (standard reference material 1649b, obtained from National Institute of Standards and Technology, Gaithersburg, MD, USA), and mito-TEMPO (10μ M, Alexis-Biochemicals, San Diego, CA, USA).

Table 1. Primers used for quantitative real time PCR analysis

Species	Genes	Primer sequence (5'–3')	
Human	IL8	Forward: ACTGAGAGTGATTGAGAGTGGAC	Reverse: AACCCTCTGCACCCAGTTTTC
Human	IL6	Forward: ACTCACCTCTTCAGAACGAATTG	Reverse: CCATCTTTGGAAGGTTCAGGTTG
Mouse	Cxcl1	Forward: CTGGGATTCACCTCAAGAACATC	Reverse: CAGGGTCAAGGCAAGCCTC
Mouse	Cxcl2	Forward: TGTCCCTCAACGGAAGAACC	Reverse: CTCAGACAGCGAGGCACATC
Mouse	Gm-csf	Forward: CACAAGTTACCACCTATGC	Reverse: CAAGTTCCTGGCTCATTAC
Mouse	Muc5ac	Forward: CTGTGACATTATCCCATAAGCCC	Reverse: AAGGGGTATAGCTGGCCTGA

Preparation of particle matter samples

We used standard reference airborne PM (average diameter: 10.5 μm), which primarily contains polycyclic aromatic hydrocarbons. PM was dispersed in phosphate buffered saline (PBS) or saline at a concentration of 2000 μ g/ml (mass/volume). *In vitro*, PM was dispersed in sterile PBS, and pulmonary cells were treated with PM at 100 μ g/ml. *In vivo*, PM was dispersed in sterile saline at 100 μ g PM (in 50μl saline) per day by intratracheal instillation.

Transfection with siRNAs

Control siRNA, Egr-1 siRNA, P65 siRNA, and Jun siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection of siRNA was carried out using the transfection reagent according to the manufacturer's instructions.

RNA isolation and quantitative real-time PCR analysis

Total RNA from lung tissues and treated cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The reverse transcriptase reaction was performed using Reverse Transcription Reagents (Takara Biotechnology, Shiga, Japan). The resulting cDNA was used for realtime RT-PCR using SYBR Green Master Mix (Takara Biotechnology, Shiga, Japan) on a StepOne real-time PCR system (Applied Biosystems, Foster City, CA, USA). The sequences of primer pairs are presented in Table 1.

Flow cytometry

After exposure to PM, cells were collected and stained with MitoSOX (Invitrogen, 5 μ M for 15 min at 37 °C). Cell death was assessed with double staining with FITC-labeled Annexin-V in combination with PI (Multi Sciences, Hangzhou, China). Flow cytometry assays were performed according to the manufacturer's instructions.

Western blot

After exposure to PM, cells lysates and lung tissue homogenates were lysed in RIPA buffer containing protease and phosphatase inhibitors. For Western blot, equal amounts of protein for each group were subjected to sodium dodecyl sulfate polyacrylaminde gel electrophoresis (SDS–PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were then blocked at room temperature for 1 h, and incubated for overnight in 4 °C with the following antibodies against the following proteins: β-actin (Sigma-Aldrich), Egr-1 (Cell Signaling Technology, Danvers, MA, USA), NF-κB P65 (Cell Signaling Technology, MA, USA), Phospho-NF-κB P65 (Cell Signaling Technology, MA, USA), Phospho-c-Fos (Cell Signaling Technology, MA, USA), Phospho-c-Jun (Cell Signaling Technology, MA, USA), MUC5AC (Abcam, MA, USA), RIPK1 (Cell Signaling Technology, MA, USA), RIPK3(Cell Signaling Technology, MA, USA), and Phospho-MLKL(Cell Signaling Technology, MA, USA). After washing in Tris-Buffered Saline and Tween 20 (TBST) three times (5 min each), membranes were incubated with the secondary antibody (1:1000 dilutions) at room temperature for 1 h.

ELISA

Cell culture supernatants of IL8 and IL6 and BALF supernatants of CXCL1, CXCL2, and GM-CSF collected were used to determine protein levels. ELISA assays were performed using ELISA kits from R&D Systems following the manufacturer's instructions.

Animal Experiments

Experiments were conducted in accordance with the Ethical Committee for Animal Studies at Zhejiang University. The mice were maintained in individual ventilated cages under specific pathogen-free conditions. Male C57BL/6 mice (aged 6-8 weeks) were randomly divided into the different exposure groups (n = 5–8 mice/group). Mice were exposed to PM treated with 100 μg PM (in 50μl saline) per day by intratracheal instillation for 7 d using a method as previously described [4]. Nec-1 (5 mg/kg) was injected intraperitoneallly before PM challenge. Control mice received the same volume of saline and DMSO instead of PM and Nec-1, respectively.

Histological analysis

The samples were stained by hemoxylin and eosin (H&E) or periodic acid-schiff (PAS) and imaged under an Olympus BX53 inverted microscope (Olympus, Melville, NY, USA). We assessed inflammation according to published guidelines [21], and scored PAS-stained goblet cells in airway epithelium as described previously [22].

Statistical analysis

Statistical analysis of differences between the groups was performed using GraphPad Prism Program (GraphPad, San Diego, CA, USA). Data are expressed as mean ± standard error of the mean (SEM). Comparisons between the two groups were calculated using the Mann-Whitney U test. For multiple groups, one-way analysis of variance followed by the Newman-Keuls test was used. P values <0.05 were considered to indicate statistical significance.

Results

PM exposure induces necroptosis in pulmonary epithelial cells as well as in mouse lungs

To address the possible role of necroptosis in PM-induced airway injury, we first examined the expression of necroptosis-related proteins in pulmonary epithelial cells and mouse lungs after being exposed to PM. As shown in Fig. 1A, western blot analysis revealed that the expression of necroptosis-related proteins (RIPK1, RIPK3, and Phospho-MLKL) were significantly increased in time-dependent induction after stimulation with PM. Consistently, these biomarkers were significantly enhanced in dose-dependently (Fig. 1B). Necroptosis markers were also expanded in mouse lungs after treatment with PM (Fig. 1C). Such findings corroborate that necroptosis represents a response to PM exposure *in vivo* and *in vitro*.

Necroptosis is required for the release of PM-induced inflammatory cytokines and mucin MUC5AC in HBE cells

We next explore whether necroptosis is required for the release of PM-induced inflammatory cytokines. Interestingly, we found that Nec-1 treatment significantly attenuated the mRNA levels of IL6 and IL8 induced by PM exposure (Fig. 2A and C). The secreted protein levels of IL6 and IL8 were also decreased in culture supernatants upon PM exposure (Fig. 2B and D). Similar to cytokines, Nec-1 treatment significantly reduces the expression of MUC5AC (Fig. 2E). We use the PI positive percentage to detect necrotic cells and determine whether Nec-1 can reduce HBE cell death after PM exposure. As shown in Fig. 2F-G, flow cytometry results showed that Nec-1 reduced the number of PI positive cells after PM exposure, and indicates that inhibiting necroptosis with NEC-1 in pulmonary cells may increase cell survival and decrease PM–induced inflammation and mucus production. To further emphasize the impact of necroptosis on PM-induced airway inflammation and mucus production, RIPK3 inhibitor GSK'872 was used in HBE before PM exposure. Similar to the effects of Nec-1, GSK'872 treatment significantly reduced the PM-induced expression of IL6 and IL8 at both mRNA and protein levels in HBE cells (Fig. 2H-K). As such, GSK'872 treatment markedly attenuated the PM-induced production of MUC5AC (Fig. 2L). Our results further suggest that necroptosis may contribute to a pathogenic process leading to PM–induced inflammation and mucus production in airway epithelial cells.

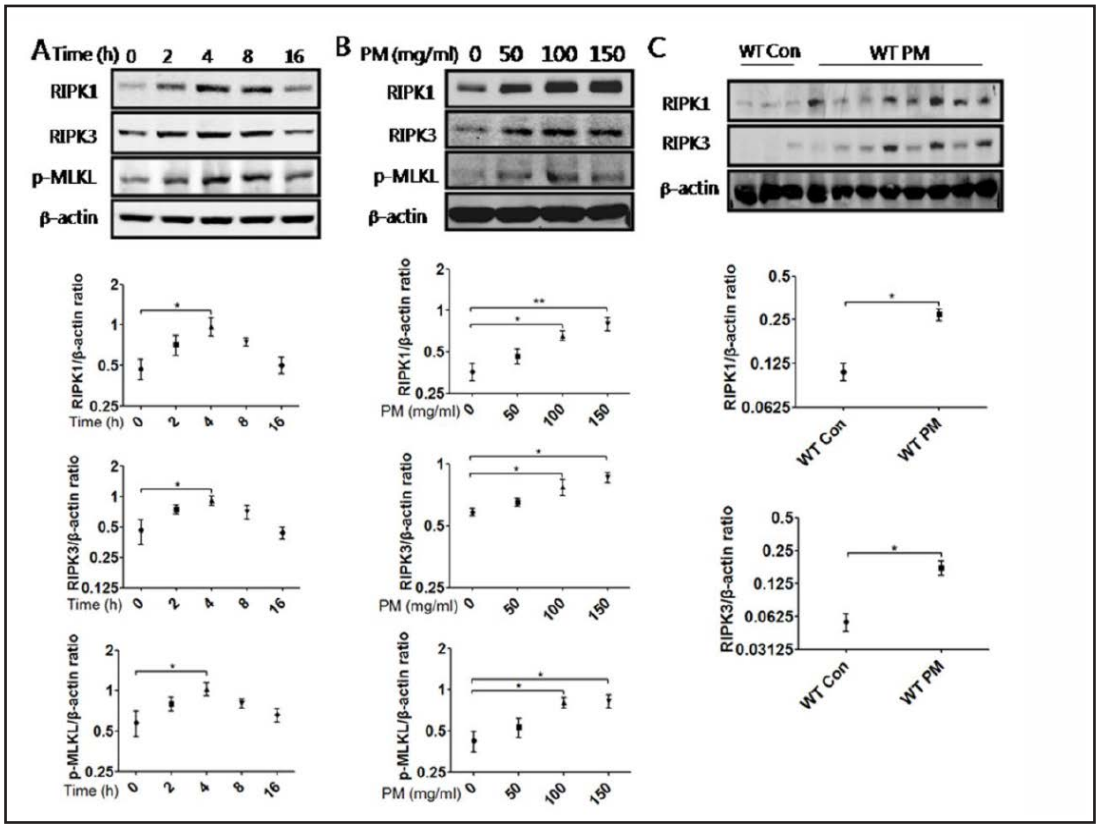


Fig. 1. PM exposure induces necroptosis in pulmonary epithelial cells as well as in mouse lungs. (A-B) Cells were exposed to PM at indicated times or concentrations. (A-B) Western blot analysis for a time course (PM at 100 μg/ml for various times) or dose response (various concentrations of PM for 4h) of RIPK1, RIPK3, and p-MLKL protein expression in HBE cells treated with PM. (C) Western blot analysis of RIPK1, RIPK3, and p-MLKL protein expression in lung tissues of WT mice treated with 100 μg PM (in 50μl saline) by intratracheal instillation for 7 d. The data are presented as the mean ± SEM. *P < 0.05, ** P < 0.01, and *** P < 0.001.

Necroptosis inhibitor Nec-1 attenuates PM-induced pulmonary inflammation in vivo

To elucidate the critical role of necroptosis in PM-induced pulmonary inflammation, we carried out *in vivo* experiments. In agreement with *in vitro* findings, treatment with Nec-1 strongly decreased the PM-induced total number of inflammatory cells and neutrophils in bronchoalveolar lavage fluid (BALF) (Figs. 3A-B). Also, the PM-induced mRNA levels of inflammatory cytokines, such as Cxcl1, Cxcl2, and Gm-csf in the lung tissues (Figs. 3C, E, and G), and the protein levels of these inflammatory cytokines in the BALF (Figs. 3D, 3F, and H) were very much affected by Nec-1 treatment. Moreover, lung histopathology analysis also confirmed that the PM-induced airway inflammation was significantly affected in Nec-1 treated mice (Figs. 3I-J).

Necroptosis inhibitor Nec-1 lowers airway mucus production induced by PM exposure in vivo

We next sought to confirm the effect of necroptosis in the regulation of PM-induced mucus production *in vivo.* PAS staining and immunohistochemistry analysis of MUC5AC demonstrated less mucus production in Nec-1 treated mice (Figs. 4A-D). The mRNA levels of Muc5ac in the lungs were also reduced in response to PM exposure in Nec-1 treated mice (Fig. 4E). These findings suggest that inhibition of necroptosis lowers pulmonary mucus production as a result of PM exposure.

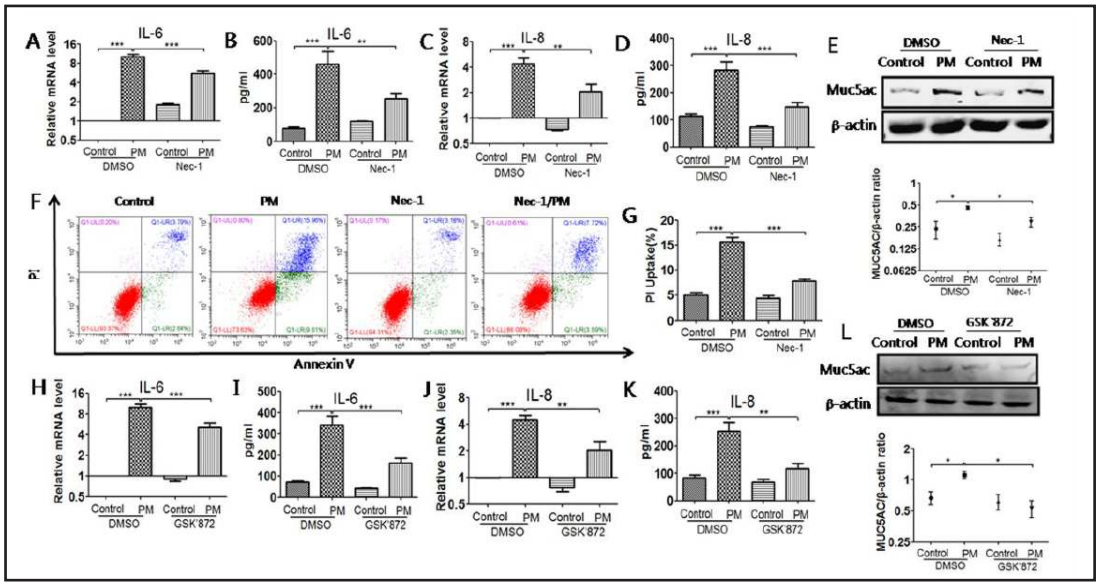


Fig. 2. Necroptosis is required for the release of PM-induced inflammatory cytokines and mucin MUC5AC in HBE cells. (A-D and F-G) Cells were incubated in the absence or presence of NEC-1, and then were treated with PM (100 μg/ml) for 24 h. The relative levels of IL6 (A), and IL8 (C) mRNA transcripts were measured by quantitative RT-PCR, and the protein levels of IL6 (B) and IL8 (D) in the culture supernatants were measured by ELISA. (E) Cells were incubated in the absence or presence of NEC-1, and treated with PM (100 μg/ml) without FBS for an additional 48 h to measure the protein of MUC5AC via the Western blot. (F) Representative graphs of cell death through flow cytometry analysis after Annexin-V/PI dual staining. (G) Statistical analysis of PI positive cell ratio. (H-K) Cells incubated in the absence or presence of GSK'872, and treated with PM (100 μg/ml) for 24 h. The relative levels of IL6 (H), and IL8 (J) mRNA transcripts were measured by quantitative RT-PCR, and the protein levels of IL6 (I) and IL8 (K) in the culture supernatants, which are in turn were measured by ELISA. (L) Cells were incubated in the absence or presence of GSK'872, and then were treated with PM (100 μg/ml) without FBS for an additional 48 h to measure the protein of MUC5AC by Western blot. The data are presented as the mean ± SEM. *P < 0.05, ** P < 0.01, and *** P < 0.001.

MitoROS-dependent Egr-1 activation is involved in PM-induced necroptosis

We then examined the involvement of mitoROS and the early growth response gene 1 (Egr-1) in PM-induced necroptosis expression in HBE cells. As illustrated in Figs. 5A–B, flow cytometric analysis of mitoSOX demonstrated that higher levels of mitochondrial ROS were generated after PM exposure. Furthermore, PM exposure resulted in a time-dependent increase in Egr-1 (Fig. 5C). Specific inhibition of mitoROS by mitochondria-targeting antioxidant mito-TEMPO effectively downregulated PM-induced Egr-1 (Fig. 5D), and also attenuated PM-induced activation of RIPK1, RIPK3, and Phospho-MLKL (Fig. 5E) in HBE cells. Genetic inhibition of Egr-1 by siRNA significantly decreased PM-induced RIPK1, RIPK3, and Phospho-MLKL (Fig. 5F). These results suggest that PM-induced necroptosis in HBE cells can be mediated, at least in part, by mitoROS-dependent Egr-1 activation.

NF-κB is critical for PM-induced inflammatory responses in HBE cells

These intriguing results encouraged further exploration of the possible mechanisms in PM-induced expression of IL6 and IL8. Interestingly, we noted that inhibition of the NF-κB pathway by P65 siRNA reduced the PM-induced expression of IL6 and IL8 at mRNA, as well as protein levels in HBE cells (Figs. 6A-D). As seen in Fig. 6E, PM exposure resulted in time-dependent increases in NF-κB-related proteins (P65 and PP65). Interestingly, mito-TEMPO effectively lowered the PM-induced NF-κB activation (Fig. 6F). Furthermore, genetic inhibition of Egr-1 by siRNA or Nec-1 treatment dramatically decreased the PM-

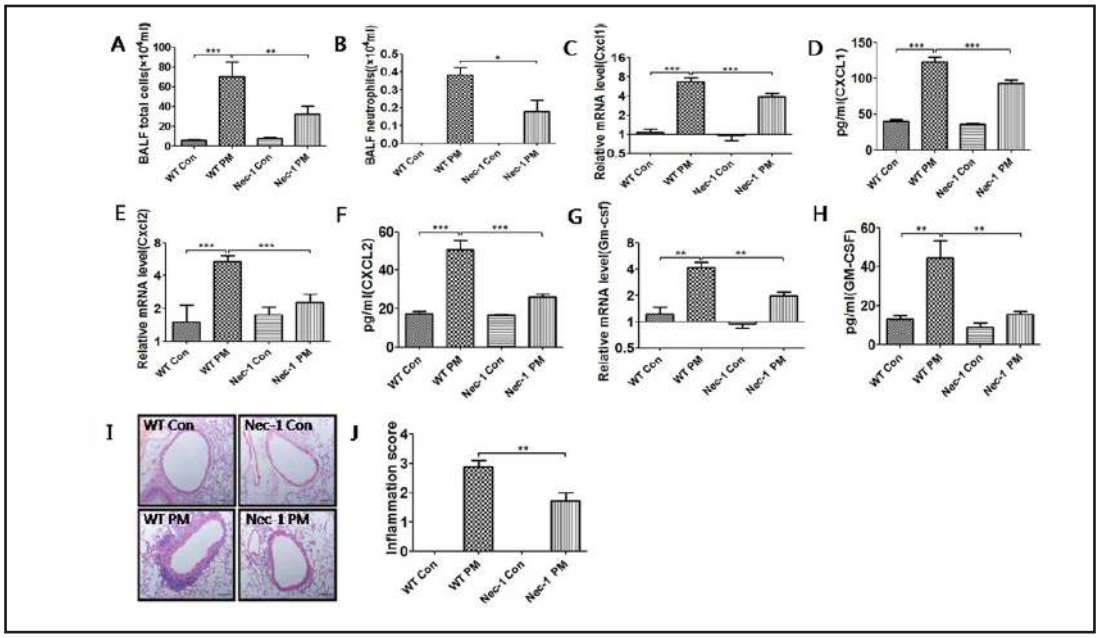
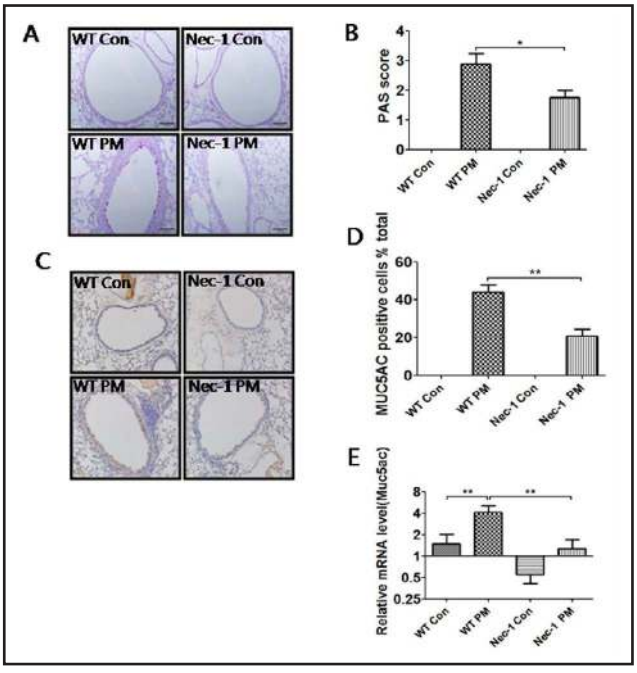


Fig. 3. Necroptosis inhibitor Nec-1 attenuates PM-induced pulmonary inflammation *in vivo.* Mice were anesthetized and instilled intratracheally with PM at 100 μg/d for 7 d. After 24 h, mice were sacrificed and the total inflammatory cells (A) and the number of neutrophils (B) in the BALF were measured. Expression of the mRNA levels of Cxcl1 (C), Cxcl2 (E), and Gm-csf (G) in lung tissues were assessed by quantitative PCR. Expression of the protein levels of CXCL1 (D), CXCL2 (F), and GM-CSF (H) in the BALF were measured by ELISA. (I) Lung sections stained with H&E were performed to assess lung inflammation. (J) Semiquantified inflammation by H&E staining. The data are presented as the mean ± SEM. *P < 0.05, ** P < 0.01, and *** P < 0.001.

Fig. 4. Necroptosis inhibitor Nec-1 lowers airway mucus production induced by PM exposure *in vivo.* (A) Representative images of lung sections with PAS staining. (B) Semiquantified PAS score. (C) Immunohistochemical staining was assessed with MUC5AC. (D) Quantification percentage of MUC5AC positive cells in the epithelium. (E) The mRNA expression of Muc5ac in lung tissue. The data are presented as the mean ± SEM. *P < 0.05, ** P < 0.01, and *** P < 0.001.



induced activation of NF-κB pathway (Fig. 6G-6I). This strongly suggests that PM-induced inflammation may be mediated via the mitoROS/Egr-1/necroptosis cascade, which then activates the NF-κB pathway.

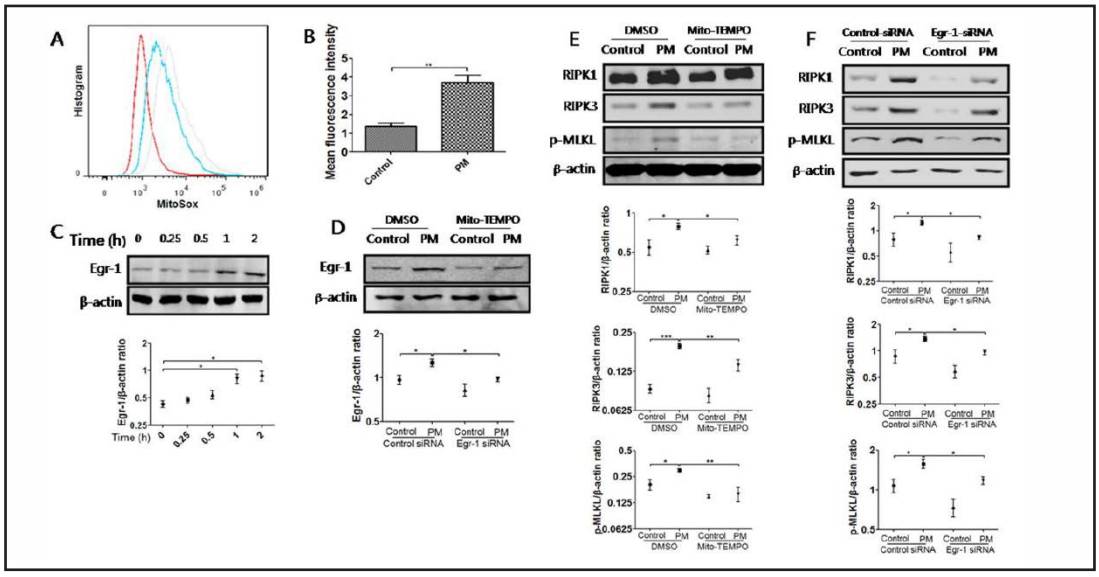


Fig. 5. MitoROS-dependent Egr-1 activation is involved in PM-induced necroptosis. (A-B) Cells were treated with PM (100 μg/ml) for 6 h, and the level of mitoROS was determined. (A) Flow cytometry analysis of HBE cells were stained with mitoSOX (red, unstained; blue, control; gray, PM); (B) Quantification data of A. (C) Western blot analysis for time course (PM at 100 μg/ml for various times) of Egr-1 protein expression in HBE cells treated with PM. (D-E) Cells treated with PM (100 μg/ml) in the absence or presence of 10μM mito-TEMPO. (D) Western blot for PM-induced Egr-1 expression. (E) The levels of RIPK1, RIPK3, and Phospho-MLKL were determined by Western blots. (F) HBE cells were transfected with control-siRNA or Egr-1-siRNA for 24 h, and were treated with PM (100 μg/ml) for an additional 4 h. (F) RIPK1, RIPK3, and p-MLKL protein expression were assessed by Western blots. The data are presented as the mean ± SEM. *P < 0.05, ** P < 0.01, and *** P < 0.001.

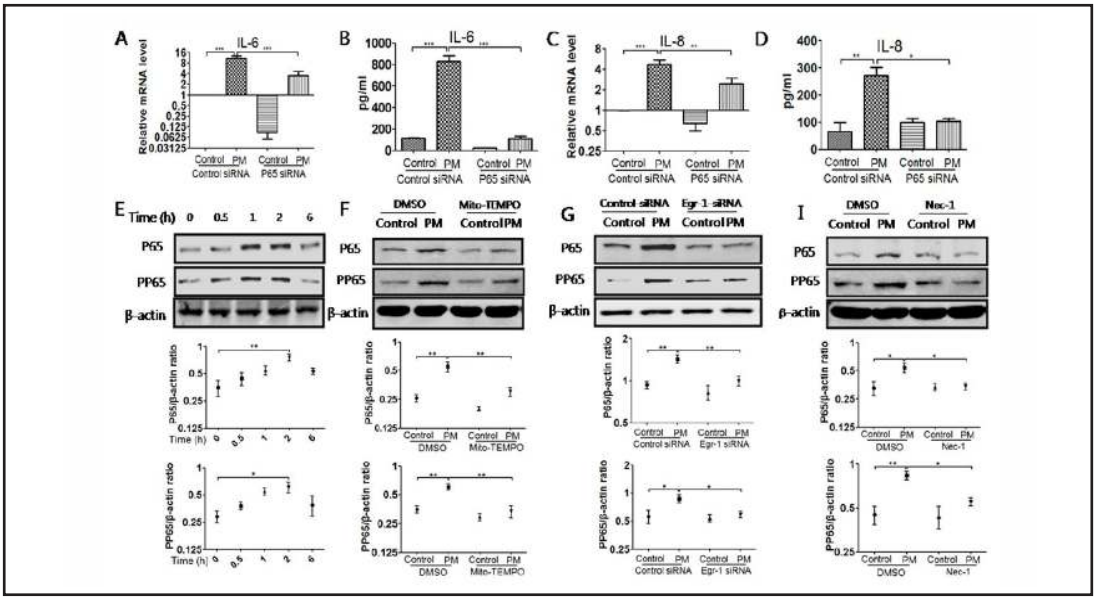


Fig. 6. NF-κB is critical for PM-induced inflammatory responses in HBE cells. (A-D) Cells were transfected with control-siRNA, Jun-siRNA or P65-siRNA for 24 h, and then were treated with PM (100 μg/ml) for an additional 24 h. The relative levels of IL6 (A) and IL8 (C) mRNA transcripts were measured by quantitative RT-PCR, and the protein levels of IL6 (B) and IL8 (D) in the culture supernatants were measured by ELISA. (E) Western blot analysis for time course (PM at 100 μg/ml for various times) of NF-κB (detected with p65 or phospho-p65 antibodies) protein expression in HBE cells treated with PM. (F) Cells were treated with PM

(100 μg/ml) for 2h in the absence or presence of 10μM mito-TEMPO to measure the level of NF-κB. (G) Cells were transfected with control-siRNA or Egr-1-siRNA for 24 h, and then were treated with PM (100 μg/ml) for 2h to measure NF-κB. (I) Cells were incubated in the absence or presence of NEC-1, and then treated with PM (100 μg/ml) for with 2h to measure the level of NF-κB. (F, G, I) The level of NF-κB was determined by Western blots. The data are presented as the mean ± SEM. *P < 0.05, ** P < 0.01, and *** P < 0.001.

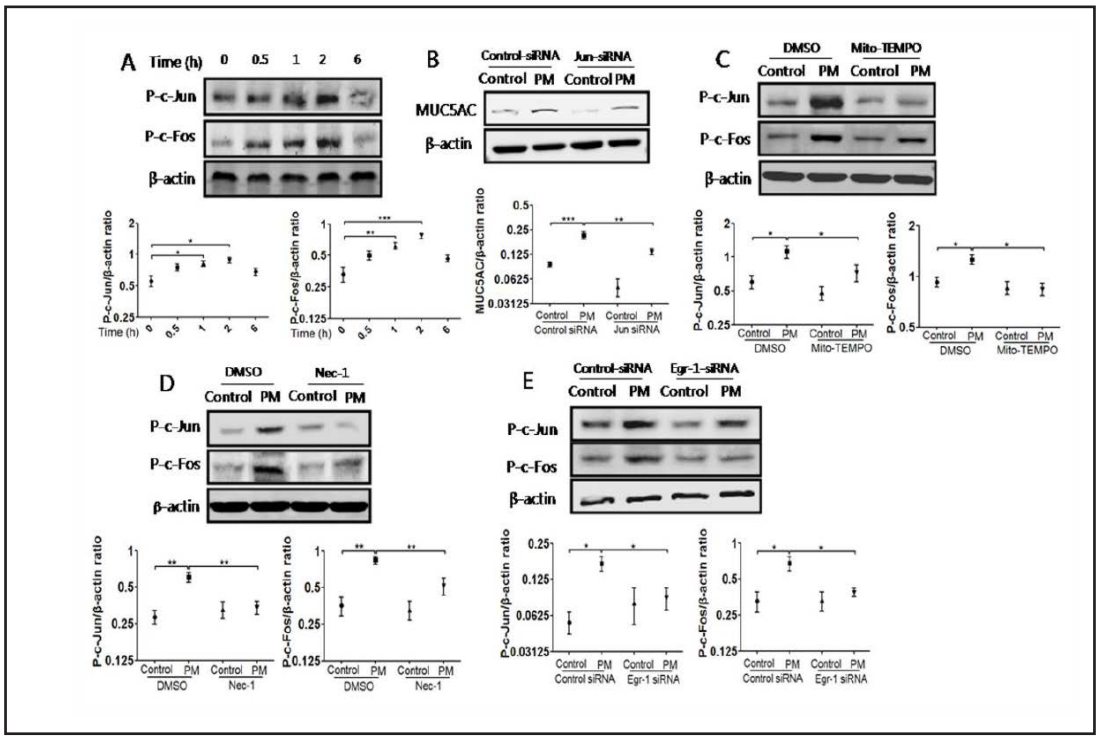


Fig. 7. AP-1 is critical for PM-induced expression of mucin MUC5AC in HBE cells. (A) Western blot analysis for time course (PM at 100 μg/ml for various times) of AP-1 detected with P-c-Jun and P-c-Fos protein expression in HBE cells treated with PM. (B) Cells were transfected with control-siRNA or Jun-siRNA for 24 h, and then were treated with PM (100 μg/ml) without FBS for an additional 48 h to measure the protein of MUC5AC. (B) Levels of MUC5AC were measured by Western blot. (C) Cells were treated with PM (100 μg/ml) for 2h in the absence or presence of 10μM mito-TEMPO to measure the level of AP-1. (D) Cells were transfected with control-siRNA or Egr-1-siRNA for 24 h, and then were treated with PM (100 μg/ml) for 2h to measure the level of AP-1. (E) Cells were incubated in the absence or presence of NEC-1, and then were treated with PM (100 μg/ml) for with 2h to measure the level of AP-1. (C, D, and E) The levels of AP-1 protein were detected with phospho-c-Jun and phospho-c-Fos antibodies, as determined by Western blots. The data are presented as the mean ± SEM. *P < 0.05, ** P < 0.01, and *** P < 0.001.

AP-1 is critical for PM-induced expression of mucin MUC5AC in HBE cells

To verify the role of AP1 in CSE-induced expression of MUC5AC, we detected AP-1 expression on PM exposure. In HBE cells, AP-1-related proteins (P-c-Jun and P-c-Fos) were increased in a time-dependent manner after PM exposure (Fig. 7A). Moreover, the inhibition of the AP-1 pathway by its specific siRNA markedly attenuated the PM-induced production of MUC5AC (Fig. 7B). We use mito-TEMPO to suppress mitoROS and further investigate the role of the mitoROS pathway. Interestingly, the expression of PM-induced AP-1 was significantly decreased (Fig. 7C). Again, genetic inhibition of Egr-1 by siRNA or Nec-1 treatment significantly decreased the PM-induced activation of the AP-1 pathway (Fig. 7D-E). These results suggest that PM exposure triggers the mitoROS/Egr-1/necroptosis pathway, which accordingly activates AP-1, as a signal to produce MUC5AC in airway epithelial cells.

Discussion

A growing body of evidence suggests that PM exposure leads to a greater incidence of respiratory diseases associated with increased morbidity and mortality. However, the cause of this condition is complex. Necroptosis is a regulated form of necrosis that features plasma membrane rupture, thereby releasing proinflammatory intracellular components. Classical necroptosis is characterized by RIP and its substrate MLKL activation, both of which are useful biomarkers for assessing necroptosis *in vitro* and *in vivo* [23]. When caspases are inhibited under certain physiological conditions, RIPK1 via the RIP-homotypic interaction motif (RHIM) interacts with downstream RIPK3 to form the necrosome to then recruit MLKL [24]. Activated MLKL oligomers eventually cause membrane rupture and release of DAMPs, which trigger inflammatory reactions and accelerate cell death [25, 26]. Thus, necroptosis is recognized as a cause of inflammation and is linked to pathological conditions with an overt inflammatory signature [25]. Necroptosis execution depends on RIPK1, RIPK3, and MLKL activation, and can be blocked by these inhibitors. It has been recently reported that necroptosis plays an important role in the pathogenesis of pulmonary diseases, including COPD, lung cancer, infection, and sepsis [17]. In our study, necroptosis inhibitor Nec-1 and GSK'872 could decrease inflammation and alleviate mucus hyperproduction in airway epithelial cells induced by PM exposure. Nec-1 also reduced these pathologic features in mouse airways. Therefore, our *in vitro* and *in vivo* data demonstrate that airway epithelium necroptosis may play a pathogenic role in PM-induced pulmonary injury.

Oxidative stress is integral to the general inflammatory response, which occurs due to a metabolic imbalance due to excess production of ROS and reduced level of host antioxidant defences [27-29]. PM induced acute and chronic effects via an inflammatory mechanism of oxidative stress [30-32], and PM could play an important role in adverse respiratory effects through generating ROS in the respiratory tract [33]. Substantial evidence accumulated to show that reactive oxygen species (ROS) have long been considered to be a driving force for necroptosis [34-36]. A recent study showed that mitoROS promoted RIP1 autophosphorylation via modification of three crucial cysteine residues, and this phosphorylation event allowed efficient recruitment of RIP3 to RIP1 to form a functional necrosome for TNF-induced necroptosis [37]. In accordance with previous studies, we found that PM exposure induced mitoROS, and mitoROS mediated PM-induced necroptosis in HBE cells. Egr-1, also known as NGFI-A, krox-24, Zif-268, or TIS8, belongs to the immediate-early gene family and encodes a Cys2-His2-type zinc finger transcription factor [38]. In previous work, we found that Egr-1 played a negative role in PM-induced pulmonary injury in airway epithelium. We further found a positive interaction between mitoROS and Egr-1 activation, which in turn increase PM-induced necroptosis. Our data suggest that mitoROS and Egr-1 might be selected targets in PM-induced pulmonary diseases.

Several studies indicate that NF-κB plays a strong role in the initiation of inflammatory responses [39-41]. Recently, RIPK3, the critical biomarker for necroptosis, was shown to facilitate inflammation through NF-κB [26, 42]. Our data demonstrated that inhibiting necroptosis reduced the PM-induced pulmonary inflammation via the NF-κB pathway. Airway mucus hypersecretion may be considered to be an important pathological feature of chronic airway diseases, in that it clogs small airways, which impair respiration and contributes to recurrent infection and mortality, particularly in patients with more severe diseases. Mucins are the major component of mucus secretions, and MUC5AC is the primary mucin in human airways [43]. Previous studies demonstrated that activator protein-1 (AP-1), composed of homo- and heterodimers of c-Jun and c-Fos proteins, was the transcriptional activator of MUC5AC [44]. Thus, we first detected AP-1 expression in PM exposure. AP-1 was reported to mediate mucin transcription through two binding sites on -3700/-3337 in the MUC5AC 5'-flanking region [45]. Our results showed that inhibiting necroptosis was correlated with a decrease in mucus production through AP-1 pathway by PM exposure. As such, it is possible that RIPK1 interacts with its downstream RIPK3 to form the necrosome to recruit MLKL, in turn causing membrane rupture that releases DAMP, and facilitates NF-κB to induce

inflammatory cytokines and AP-1 to induce mucus production in response to PM exposure (Fig. 8).

We found that necroptosis was required for the release of PM-induced inflammatory cytokines and mucin MUC5AC. The present study had limitations, though, in terms of what we do about solid control groups. Numerous reports classify PM by particle size, but it may oversimplify or overlook the molecular makeup of the PM sample. PM is a complex mixture containing components such as carbonaceous core, PAHs, quinones, metals, endotoxins and others [9]. It is difficult to know what the main substance in the induction of the inflammatory response may be. We use PBS or saline as controls for PM treatment (PM is generally dispersed in sterile saline or PBS), similar to previous studies [4, 8, 46, 47]. It might be better to add an a-specific stimulus as a control which might enhance the robustness of our findings.

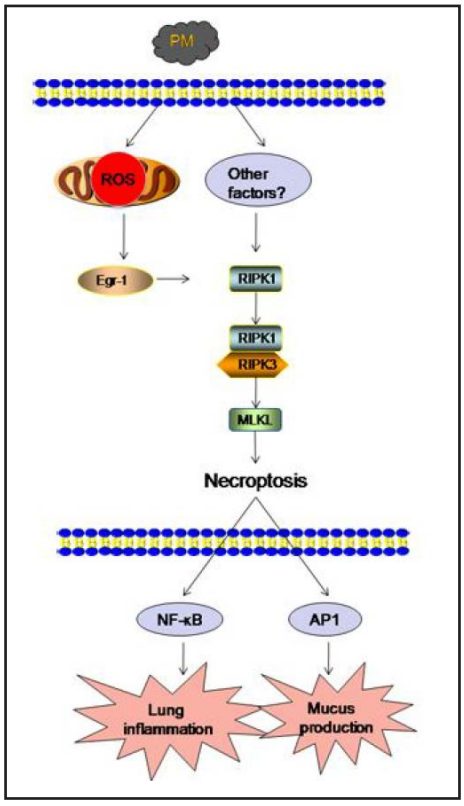


Fig. 8. Schematic representation of the mechanisms of PM-induced necroptosis in the differential regulation of subsequent inflammation and mucus hyperproduction in airway epithelium. PM exposure initially triggers production of mitoROS and elicits Egr-1, which induces RIPK1, RIPK3, and Phospho-MLKL; this then regulates inflammation and mucus hyperproduction, partially through modulation of two distinct signaling cascades, NF-κB and AP-1 pathways.

Conclusion

In summary, we demonstrate a novel mechanism of how PM induces mitoRos and Egr-1 signaling, which leads to necroptosis in pulmonary epithelial cells; ultimately, they activate two distinct signaling cascades, NF-κB and AP-1 pathways, to induce airway inflammation and mucus hyperproduction. Our results suggest that necroptosis contributes to the pathogenesis of PM-induced pulmonary injury, so that related strategies targeting this pathway may lead to novel therapies for airway disorders or disease exacerbations associated with airborne particulate pollution.

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Disclosure Statement

The authors declare that there is no conflict of interest regarding publication of this manuscript.

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