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Paper

$PM_{2.5}$ induces inflammatory responses via oxidative stress-mediated mitophagy in human bronchial epithelial cells

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Background: Fine particulate matter ($PM_{2.5}$) is a ubiquitous air pollutant, and it has been reported to be closely associated with lung inflammatory injury. In this study, the potential molecular mechanisms underlying $PM_{2.5}$-induced cellular inflammation in human bronchial epithelial (BEAS-2B) cells were investigated.

Materials and methods: Ambient $PM_{2.5}$ particulates from Suzhou, China, were collected and re-suspended in ultrapure water. Cellular damages, characterized by oxidative stress, mitochondrial injury, and inflammatory cytokine production, were determined in 24 h $PM_{2.5}$-treated BEAS-2B cells with or without 3-methyladenine (3-MA; autophagy inhibitor) pretreatment. Biomarkers related to oxidative damage, inflammatory injury and autophagy signaling pathways were also measured.

Results: Uptake of $PM_{2.5}$ in BEAS-2B cells induced cellular oxidative damage, mitochondrial injury, and inflammatory responses as indicated by a significant decrease in GSH/GSSG ratio, increased MDA content, dilated mitochondria with loss and rupture of crista, and production of inflammatory cytokines. Activation of Nrf-2/TXNIP-mediated NF-κB and Bnip3L/NIX-dependent mitophagy signaling pathways, as well as accumulation of autophagosomes and autolysosomes, were also observed. A 6 h pretreatment of 3-MA increased $PM_{2.5}$-induced oxidative damage and cellular inflammation as indicated by increasing protein levels of HO-1, TXNIP, Bnip3L/NIX and *IL*-8 gene expression.

Conclusions: $PM_{2.5}$ induced cellular inflammatory injury by oxidative stress, mitochondrial dysfunction, and mitophagy initiation. Although induction of Bnip3L/NIX-mediated mitophagy in BEAS-2B cells appeared to confer protection in response to $PM_{2.5}$, dysfunction of autophagic flux may be a critical contributor to defective mitophagy and cellular inflammatory response.

Key words: $PM_{2.5}$; oxidative stress; mitochondrial dysfunction; mitophagy; inflammation; autophagic flux.

Introduction

Ambient fine particulate matter ($PM_{2.5}$; aerodynamic diameter $\leq$ 2.5 μm), the primary hazardous pollutant present within haze, is of great concern because of its potential detrimental effects on human health. As the initial deposition site, the respiratory tract is the major target of $PM_{2.5}$. With their extended sedimentation time, $PM_{2.5}$ particles can deposit in distal small airways and alveoli, accumulate in lung parenchyma, and consequently cause serious adverse effects, such as inflammatory injury and oxidative damage, to respiratory systems [1, 2]. Additionally, growing epidemiological investigations have demonstrated associations between $PM_{2.5}$ exposure and airway inflammation, decreased lung function, and the development of respiratory diseases including asthma, chronic obstructive pulmonary disease, and lung cancer [3–5]. Despite its public health importance, the molecular mechanisms underlying the clinical consequences of $PM_{2.5}$ on the human pulmonary system remain largely unknown.

In vitro and in vivo studies have demonstrated that $PM_{2.5}$ increased the production of reactive oxygen species (ROS) in a variety of ways. Sustained ROS stimulated by environmental contaminants adsorbed onto $PM_{2.5}$ particles, such as heavy metals and polycyclic aromatic hydrocarbons, resulted in oxidative injury characterized by lipid peroxidation, DNA breakdown, and protein damage [6, 7]. Recent in vitro studies also documented that $PM_{2.5}$ exposure elicited oxidative stress, followed by a series of impairments to airway epithelial cells and alveolar macrophages, as indicated by structural damage and the release of inflammatory mediators [5, 6]. Upon the initiation of inflammatory responses, the cytokine production disordered by $PM_{2.5}$ may further increase injury to lung tissue and disruption of pulmonary function [6].

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While oxidative stress and the accompanying inflammation are proposed to be important causal events leading to $PM_{2.5}$-induced pulmonary injury or exacerbation of respiratory diseases [8, 9], the current research on $PM_{2.5}$ exposure and lung injury via ROS-mediated inflammation is still limited, and further investigation is needed to understand the underlying mechanisms.

Mitochondria are the main sources of intracellular ROS during energy generation. Without sufficient oxygen during electron transport, uncoupled free electrons will promote ROS production [10]. With ROS accumulation, permeability of the mitochondrial membrane increases. As a result, excessive ROS are released into the cytoplasm leading to imbalances in the intracellular redox system, and ultimately resulting in severe damage both to the function and morphology of mitochondria [11]. Mitochondrial quality is sustained through complex molecular mechanisms including biogenesis of new mitochondria, dynamics of fusion and fission, and elimination of damaged mitochondria via a selective form autophagy–lysosomal degradation, also known as mitophagy [12–14]. Disruption of these regulatory systems has been shown to be associated with pulmonary disorders [15]. Additionally, accumulating evidence suggests that excessive damage to lung mitochondria may be one of the critical mechanisms underlying $PM_{2.5}$-induced lung injury [16].

Recently, the relationship between inflammation and mitophagy has raised increasing interest that requires further exploration [17]. Although the specific mechanisms of mitophagy are not fully understood, numerous studies have highlighted the pivotal role of mitophagy in the regulation of the immune system [18]. Mitochondrial danger signals can lead to an exaggerated inflammatory response, potentially initiating chronic and systemic inflammatory injury [17, 19]. In most cases, the mitophagy process is initiated by mitochondrial membrane depolarization and mediated by several receptors including phosphatase and tensin homolog-induced putative protein kinase 1 (PINK1), Parkin, BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), and its homolog BNIP3 like (Bnip3L/NIX) [20]. These receptors can directly bind microtubule-associated protein 1 light chain 3 (LC3) proteins that allow the recruitment of phagophores to damaged mitochondria. After encapsulated by autophagosomes and fused with lysosomes, dysfunctional mitochondria are eliminated, thereby preventing the accumulation of potential harmful mitochondria that could cause an exaggerated inflammatory response [19]. Impaired or defective mitophagy and malfunctioning mitochondria provide a feedback on the inflammatory response and have been implicated in inflammatory cytokine production and dysregulation of immune cell homeostasis, and thus, contributing to the development and pathogenesis of both acute and chronic lung diseases [19, 21, 22]. Emerging lines of evidence have also suggested that mitophagy plays a crucial role in airborne $PM_{2.5}$-induced lung injury [23]. However, whether mitophagy is a deleterious process or is playing a protective role in $PM_{2.5}$-induced toxicity remains to be elucidated.

Suzhou, a growing city located in the Yangtze River Delta Region of China, currently faces a problem with ambient $PM_{2.5}$ pollution, especially in winter [24]. The present study aimed to investigate the possible roles of mitochondria, oxidative stress, and mitophagy in Suzhou winter $PM_{2.5}$-induced inflammatory responses in human bronchial epithelial (BEAS-2B) cells. After a 24 h treatment of $PM_{2.5}$ fractions, levels of cellular damage including oxidative stress, mitochondrial injury, inflammatory cytokine production, as well as the indicated biomarkers related with oxidative stress, inflammation, and mitophagy signaling pathway, were determined. To further elucidate the role of mitophagy in $PM_{2.5}$-induced lung injury, 3-methyladenine (3-MA), a classic autophagy inhibitor, was used to pretreat the BEAS-2B cells before $PM_{2.5}$ exposure.

Materials and methods

Chemicals and materials

Dulbecco's modified Eagle's minimal (DMEM) essential medium, trypsin, and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY, USA). Cell counting kit-8 (CCK-8), total superoxide dismutase (SOD) assay kits, and cellular glutathione peroxidase (Gpx) assay kits were purchased from Biyuntian Biotechnology Co., Ltd (Shanghai, China). Cell malonaldehyde (MDA) assay kits were obtained from Jiancheng Bio Company (Nanjing, China). CellTiter-Glo luminescent cell viability assay and glutathione (GSH)/oxidized GSH (GSSG)-Glo assay kits were purchased from Promega (Madison, WI, USA). 5,5′,6,6′-tetrachloro1,1′,3,3′-tetramethylbenzimidazolylcarbocyanine iodide (JC-1) apoptosis detection kits were obtained from Jiangsu KeyGen Biotech Co.Ltd (NanJing, China). Human interleukin-6 (IL-6) and interleukin-8 (IL-8) enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D systems (Minneapolis, MN, USA). Rabbit anti-nuclear factor erythroid 2-related factor 2 (Nrf-2), Heme oxygenase-1 (HO-1), Thioredoxin 1 (Trx-1), Trx-interacting protein (TXNIP), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phospho-c-JUN, phospho-nuclear factor kappa-B (NF-κB) p65, phospho-inhibitor of NF-κB (IκBα), Beclin-1, autophagy-related genes (Atg) 12, Atg5, p62, Atg7, Bnip3L/NIX, LC3 primary antibodies, and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-Gpx-4 antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). ECL luminescence reagent was obtained from Absin Co., Ltd (Shanghai, China).

$PM_{2.5}$ collection and preparation

Ambient $PM_{2.5}$ particles were collected on a university campus in Suzhou, China, from September 2018 to January 2019. Teflon filters were extracted with ultrapure

water for 4 h in a sonication bath on ice, and then $PM_{2.5}$ solution was vacuum freeze-dried in a refrigerated Centrivap concentrator and weighed. The concentrated $PM_{2.5}$ particulates were combined and UV-irradiated overnight to sterilize them and inactivate any contaminating endotoxin, as reported by Peeters et al. [25]. Then, $PM_{2.5}$ particles were resuspended in ultrapure water, and a stock solution was prepared at a concentration of 10 mg/mL. After digestion in a HNO_3 and HCl mixture (1:3, v/v), concentrations of 22 metal elements in the $PM_{2.5}$ suspensions were determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo Fisher, Waltham, MA, USA). Before cell treatment, $PM_{2.5}$ suspension was diluted with cell culture medium, sonicated, and mixed completely.

Cell culture and treatment

The BEAS-2B cell line was purchased from Shanghai Institutes for Biological Sciences (SIBS, Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

For $PM_{2.5}$ exposure, cells were seeded in 96-well plates (for CCK-8, ATP, and GSH/GSSG Glo assays) or 6-well plates (for western blot, PCR, and cell MDA, mitochondrial membrane potential (MMP), SOD, Gpx, and ELISA assays) for 24 h. Then, cells were exposed to increasing concentrations of $PM_{2.5}$ (50, 100, and 150 μg/mL) for 24 h. To verify the relationship between $PM_{2.5}$-induced autophagy and inflammation, cells were pretreated with 2 mM 3-MA for 6 h before $PM_{2.5}$ exposure.

Cell-viability assay

Cell viability of BEAS-2B cells were determined using CCK-8 assay kits. After the 24 h $PM_{2.5}$ treatment, cells were incubated with CCK-8 solution at 37°C for another 4 h. The absorbance at 450 nm was then detected by a SYNERGY 2 microplate reader (Bio-Tek, USA). Cell viability of the untreated control was set to be 100%.

ATP detection assay

After $PM_{2.5}$ treatment, CellTiter-Glo reagent was added to each well and mixed for 2 min on an orbital shaker, according to the manufacturer's protocol. After equilibrated for 10 min at room temperature, the luminescent signals were then recorded using the SYNERGY 2 microplate reader. The ATP levels of each group were calculated based on an ATP standard curve, and the results were expressed as the percentage of ATP production relative to the untreated control.

MMP assay

The MMP of BEAS-2B cells was measured using the JC-1 apoptosis detection kit, according to the manufacturer's instruction. After treated with 5 μM JC-1 working solution at 37°C for15 min, cells were washed three times with incubation buffer. Then, the fluorescence intensity of the JC-1 dye was immediately analyzed using flow cytometry. Levels of MMP were expressed as the percentage of fluorescence intensity relative to the untreated control.

Table 1. Primer sequences for quantitative real-time PCR.

Gene	Primer sequence (5′–3′)
GAPDH	Sense-CGACCACTTTGTCAAGCTCA
	Antisense-AGAGTTGTCAGGGCCCTTTT
IL-8	Sense-ACATGACTTCCAAGCTGGCC
	Antisense-CAGAAATCAGGAAGGCTGCC

Transmission electron microscopy analysis

After 24 h exposure to 150 μg/mL $PM_{2.5}$, BEAS-2B cells were harvested and fixed at 4°C in freshly prepared 2.5% glutaraldehyde. Then, the pellets were fixed for another 2 h at room temperature in 1% osmium tetroxide dissolved in .01 M phosphate buffer (pH 7.4). After dehydration in ethanol followed by acetone, samples were embedded in epoxy resin (Epon 812), cut into ultrathin sections (approximately 60–80 nm), and were then stained with 2% uranyl acetate and lead citrate. Finally, samples were examined with transmission electron microscopy (TEM, HITACHI HT7700, Japan).

Determination of oxidative stress indices

GSH/GSSG ratio, the enzyme activities of Gpx and total SOD, and MDA contents were measured using the GSH/GSSG-Glo, Gpx, SOD, and cell MDA assay kits, respectively. All the assays were conducted following the supplier's instructions. The luminescent signals and light absorptions were measured using the SYNERGY 2 microplate reader. The enzyme activities of Gpx and total SOD, and the MDA concentration, were expressed as fold-change of the untreated control.

ELISA assay

After $PM_{2.5}$ treatment, levels of IL-6 and IL-8 in cell culture supernatants were determined by ELISA kits according to the manufacturer's instructions. The concentrations of IL-6 and IL-8 were calculated as fold-change of the untreated control.

Quantitative real-time polymerase chain reaction

After $PM_{2.5}$ treatment, RNA was extracted by using RNeasy Mini Kit (QIAGEN, Germantown, MD, USA). Then, 1 μg of total RNA was reverse-transcribed to cDNA using PrimeScript RT reagent kit (Takara Biotechnology, RR037A). The expression of the human *IL-8* gene was measured by quantitative real-time PCR using TB Green Premix EX Taq II (Takara Biotechnology, RR820A) on a QuantStudio 6 Flex system (Thermo Fisher, Waltham, MA, USA). The primer sequences used in this study are listed in Table 1.

Western blot assay

Whole cell protein extracts were separated using 10% or 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat

Table 2. Concentration of metal elements detected in $PM_{2.5}$ collected in Suzhou, China.

Metal elements	Concentration (μg/mg $PM_{2.5}$)
Mg	5.432 ± 0.137
Al	10.553 ± 0.960
Fe	17.727 ± 0.515
Ti	0.024 ± 0.001
Cr	0.961 ± 0.086
Mn	0.671 ± 0.067
Co	0.020 ± 0.001
Ni	0.269 ± 0.018
Cu	0.949 ± 0.022
Zn	4.481 ± 0.071
As	0.075 ± 0.007
Cd	0.030 ± 0.002
Pb	0.597 ± 0.058
Ag	0.018 ± 0.0008
Ba	0.453 ± 0.003
Be	0.010 ± 0.001
Se	0.057 ± 0.013
Sb	0.070 ± 0.006
Sn	0.118 ± 0.001
Tl	0.005 ± 0.0001
B	3.386 ± 0.016
V	0.054 ± 0.0004
Total metals	45.958 ± 1.989

milk, the membranes were then incubated overnight at 4°C with the primary antibodies. GAPDH was used as the loading control for total protein. Specific antibody–antigen complexes were detected by ECL luminescence reagents after 1 h incubation of HRP-conjugated secondary antibodies at 37°C. All proteins were analyzed by at least three independent experiments.

Statistical analysis

Statistical analysis was performed using the SPSS 17.0 software program. All data are presented as the mean ± standard deviation (SD) and were analyzed by one-way analysis of variance followed by Turkey's post hoc test. $P < 0.05$ was considered statistically significant.

Results

Elemental composition of $PM_{2.5}$

As shown in Table 2, the total mass concentration of all the elemental compositions was 45.958 μg/mg $PM_{2.5}$. In this $PM_{2.5}$ sample, elements considered to be from soil-related (i.e. Al, Fe, and Mn) and anthropogenic sources (i.e. Cr, Cu, Pb, Ni, Zn, and Cd) were detected [26–28]. Additionally, Fe, Al, Mg, Zn, and B were the most abundant elements, of which Fe was at the highest concentration (17.727 μg/mg $PM_{2.5}$), and then Al (10.553 μg/mg $PM_{2.5}$), while, Mg, Zn, and B followed in order.

$PM_{2.5}$ decreases cell viability, ATP production, and MMP of BEAS-2B cells

The CCK-8 assay showed that the ambient $PM_{2.5}$ particles could significantly decrease BEAS-2B cell viability compared with the control ($P < 0.01$, Fig. 1a).

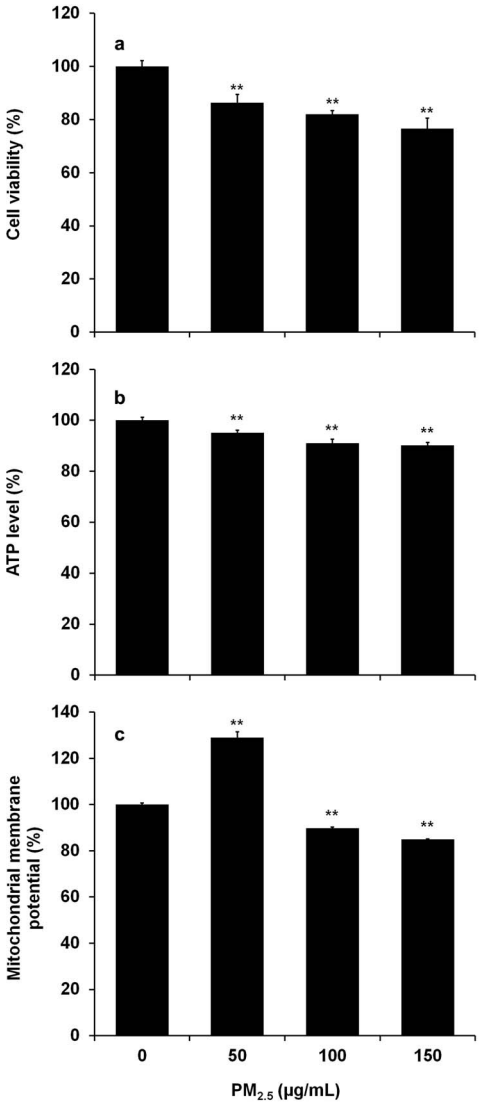

Figure 1. Effects of $PM_{2.5}$ on BEAS-2B cell viability, ATP production, and mitochondrial membrane potential. Cells were treated with increasing concentrations of $PM_{2.5}$ solution (50, 100, and 150 μg/mL) for 24 h. Cell viability (a), levels of ATP production (b), and MMP (c) were determined by CCK8 assay kits, CellTiter-Glo luminescent cell-viability assay and JC-1 apoptosis detection kits, respectively. **$P < 0.01$, versus the control.

In the 150 μg/mL $PM_{2.5}$-treated group, the percentage of viable cells decreased to 76.67% of the untreated control (Fig. 1a). As shown in Figure 1b, the 24 h-$PM_{2.5}$ treatment resulted in a decrease in ATP production with the lowest level detected after 150 μg/mL of $PM_{2.5}$ exposure ($P < 0.01$, compared with the control). The MMP assay using JC-1 loading showed different responses depending on incubation concentration of $PM_{2.5}$ (Fig. 1c), and a significant reduction in membrane potential was observed in BEAS-2B cells treated with higher concentrations (i.e. 100 and 150 μg/mL) of $PM_{2.5}$ ($P < 0.01$).

Uptake of $PM_{2.5}$ induced oxidative stress and inflammatory responses

TEM analysis showed particle uptake and morphology changes in $PM_{2.5}$-exposed cells. As shown in Figure 2,

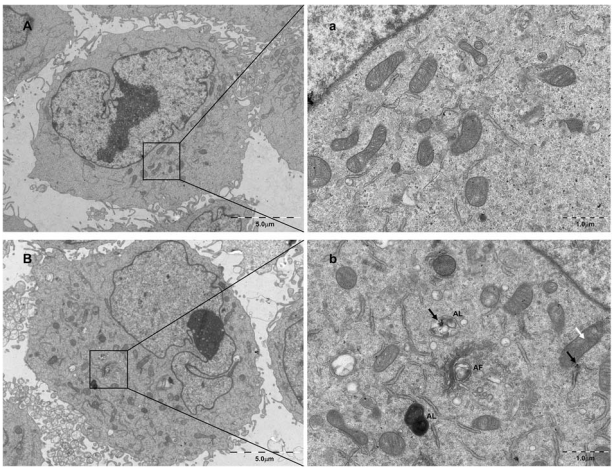

Figure 2. TEM images of BEAS-2B cells after a 24 h exposure to 150 μg/mL of $PM_{2.5}$. Morphological change of cells and intracellular localization of $PM_{2.5}$ were investigated by TEM (A-B with corresponding inserts a-b). Untreated BEAS-2B cells showed no morphological changes (A, a). After $PM_{2.5}$ exposure, particles were taken up and some were contained within membrane-bound structures (b). Autophagic vesicles and mitochondrial injury were observed in $PM_{2.5}$-treated cells (b). $PM_{2.5}$ particle (black arrows); AF (autophagosome); AL (autolysosome); mitochondria (white arrow). The magnifications for A-B and a-b were 2, 500, and 8,000, respectively.

compared with the well-ordered organelle morphology in untreated control, $PM_{2.5}$ treatment (150 μg/mL) resulted in observable cell structure changes including dilated mitochondria and endoplasmic reticulum, and accumulation of autophagosomes and autolysosomes. Loss and rupture of cristae were also found in some mitochondria after $PM_{2.5}$ exposure. Some particles were observed within membrane-bound structures of the exposed cells, which may have resulted in mitochondrial injury and excessive formation of autophagosomes.

Based on the findings obtained from TEM observations, we next investigated mitochondria-related oxidative stress and inflammatory responses after $PM_{2.5}$ exposure. As shown in Figure 3, $PM_{2.5}$ activated antioxidant enzymes (i.e. SOD and Gpx) accompanied by loss of GSH, an important antioxidant for removal of excessive ROS. Lipid peroxidation indicated by MDA production (Fig. 3d) and cellular inflammation indicated by increasing production of inflammatory cytokines (i.e. IL-6 and IL-8, Fig. 3e and f) were also detected after the 24 h of $PM_{2.5}$ treatment.

To further explore whether $PM_{2.5}$ could facilitate oxidative damage and inflammation within BEAS-2B cells, western blot analysis of Nrf-2/TXNIP-mediated NF-κB signaling pathway-related proteins was conducted. In accordance with the changes in antioxidant activities, lipid peroxidation, and production of inflammatory cytokines, several important proteins regulating oxidative stress and inflammation were significantly up-regulated in BEAS-2B cells ($P < 0.05$, Fig. 4). With increased $PM_{2.5}$ concentration, Nrf-2 and its downstream targets HO-1 and Gpx4 were significantly increased along with the up-regulation of corresponding phosphorylation of NF-κB signaling pathway-related proteins (i.e. c-JUN, IκBα, and NF-κB p65, $P < 0.05$, Fig. 4). TXNIP and Trx-1 associated with cellular inflammatory response were also activated by $PM_{2.5}$. Taken together, our results showed that $PM_{2.5}$ particles could be engulfed by BEAS-2B cells and trigger oxidative damage and inflammatory injury.

$PM_{2.5}$ induced autophagy but block autophagic flux in BEAS-2B cells

With the observations of oxidative stress and changes to mitochondria structure, we examined if mitophagy was activated for the elimination of damaged mitochondria. As shown in Figure 5, increased expression levels of Beclin-1, Atg5, Atg7, and Atg12 provided evidence of autophagy induction by $PM_{2.5}$. Although PINK1 and Parkin are two important proteins commonly involved in mitophagy, no obvious changes in these two proteins or genes were observed after $PM_{2.5}$ exposure (data not shown). However, western blot analysis showed a pronounced increase in the level of the Bnip3L/NIX protein that peaked after 150 μg/mL of $PM_{2.5}$ treatment (Fig. 5a and g). To better understand the impact of $PM_{2.5}$ on mitophagy, we analyzed several markers of autophagic flux. As shown in Figure 5a, e, and h, $PM_{2.5}$ induced accumulation of p62 and LC3-II at the highest incubation concentration. These findings suggested that apart from stimulating autophagosomes and autolysosomes (Fig. 2b), $PM_{2.5}$ also blocked the autophagic flux, especially at the higher exposure concentration (i.e. 150 μg/mL), which thereby lead to accumulation of single-membrane autolysosomes with nondegraded cargo as shown in Figure 2b.

Mitophagy confers protection against oxidative stress and inflammation in response to $PM_{2.5}$

Finally, we investigated the relationship between mitophagy, oxidative stress and inflammation in response to $PM_{2.5}$ by using 3-MA, an autophagy antagonist. As shown in Figure 6, a 6-h 3-MA pretreatment aggravated $PM_{2.5}$-induced oxidative stress and inflammatory responses as indicated by increasing protein levels of HO-1 and TXNIP and gene expression of *IL-8* (Fig. 6 b, c, e, and f). Although 3-MA pretreatment resulted in no obvious change in the ratio of LC3-II/LC3-I (Fig. 6c and d) in the 150-μg/mL $PM_{2.5}$-treated group, Bnip3L/NIX protein was significantly upregulated ($P < 0.01$, Fig. 6 c and g). Together these data suggested that induction of mitophagy in BEAS-2B cells may confer protection in response to $PM_{2.5}$.

Discussion

Epidemiological and experimental data have suggested that $PM_{2.5}$ can cause a variety of pulmonary diseases, and the possible mechanisms involve metabolic activation, oxidative stress, genotoxicity, and inflammation [4]. Nevertheless, the specific modes of action from the perspectives of subcellular structure damage have not

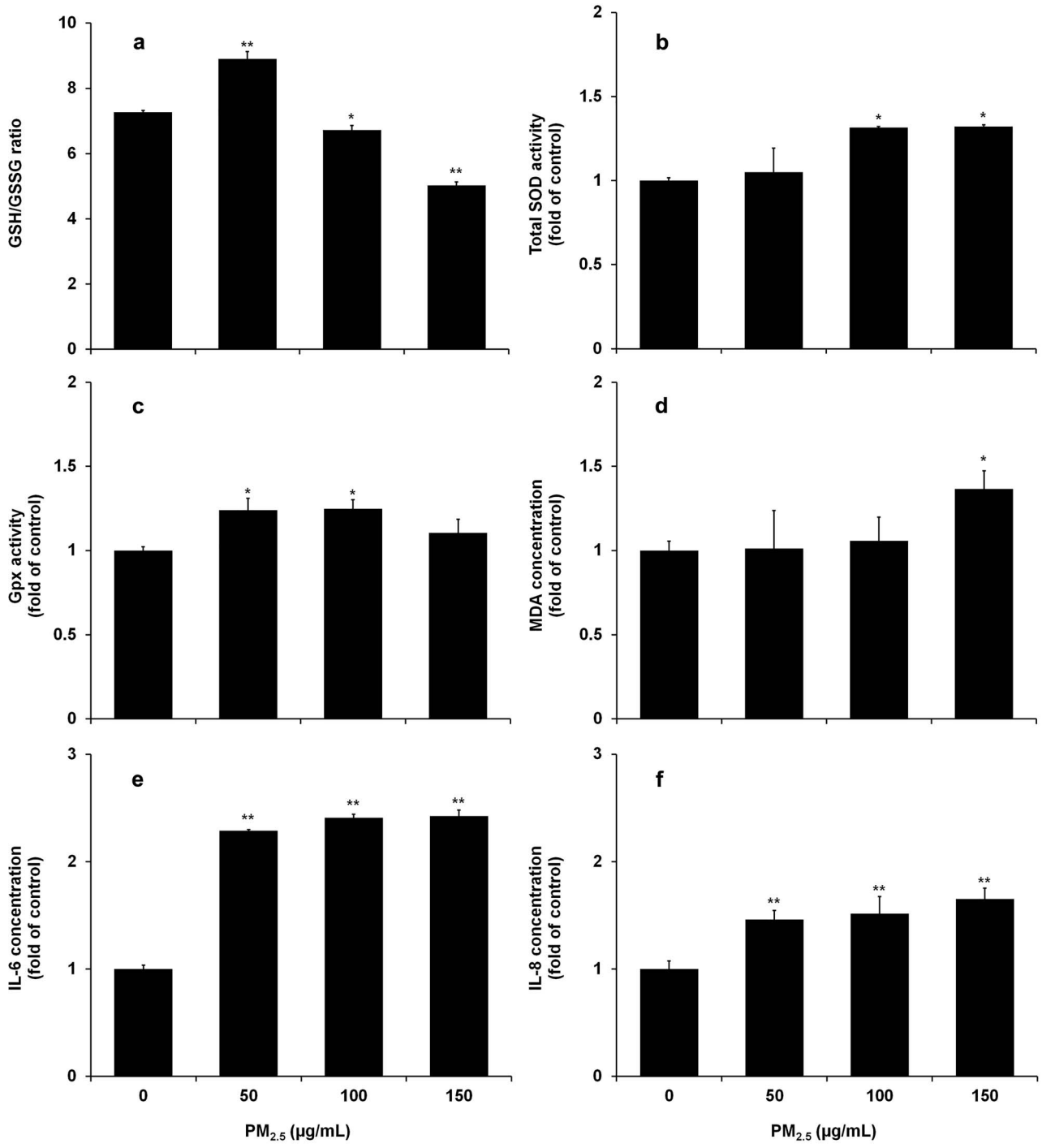

Figure 3. Effects of $PM_{2.5}$ on redox balance and production of inflammatory cytokines in BEAS-2B cells. Cells were treated with increasing concentrations of $PM_{2.5}$ solution (50, 100, and 150 μg/mL) for 24 h. The contents or enzyme activities of antioxidants including GSH (a), SOD (b), Gpx (c), and levels of lipid peroxidation (i.e. MDA, d) were determined by assay kits. Concentrations of IL-6 (e) and IL-8 (f) in cell culture supernatants were measured by ELISA kits, and the results were calculated as fold of the untreated control. *$P < 0.05$, **$P < 0.01$, versus the control.

been fully elucidated. As the primary respiratory organ and the major target of ambient airborne pollutants, in vivo and in vitro models of the pulmonary system are commonly used to assess $PM_{2.5}$ toxicity. Therefore, in this study, we investigated the potential toxicity of $PM_{2.5}$ and its underlying mechanisms in bronchial epithelial cells. After 24 h of $PM_{2.5}$ exposure, we correlated particle uptake with subcellular structure dysfunction and oxidative stress in BEAS-2B cells, which in turn, facilitated mitophagy and cellular inflammation. Pretreatment of cells with 3-MA, an autophagy antagonist, indicated a protective role of mitophagy in BEAS-2B cells in response to $PM_{2.5}$.

Although the mechanisms underlying the toxicological effects of $PM_{2.5}$ exposure are unclear, oxidative stress, the imbalance between oxidation and the antioxidant system, induced by particles and/or the coated organic chemicals and transition metals has been the main

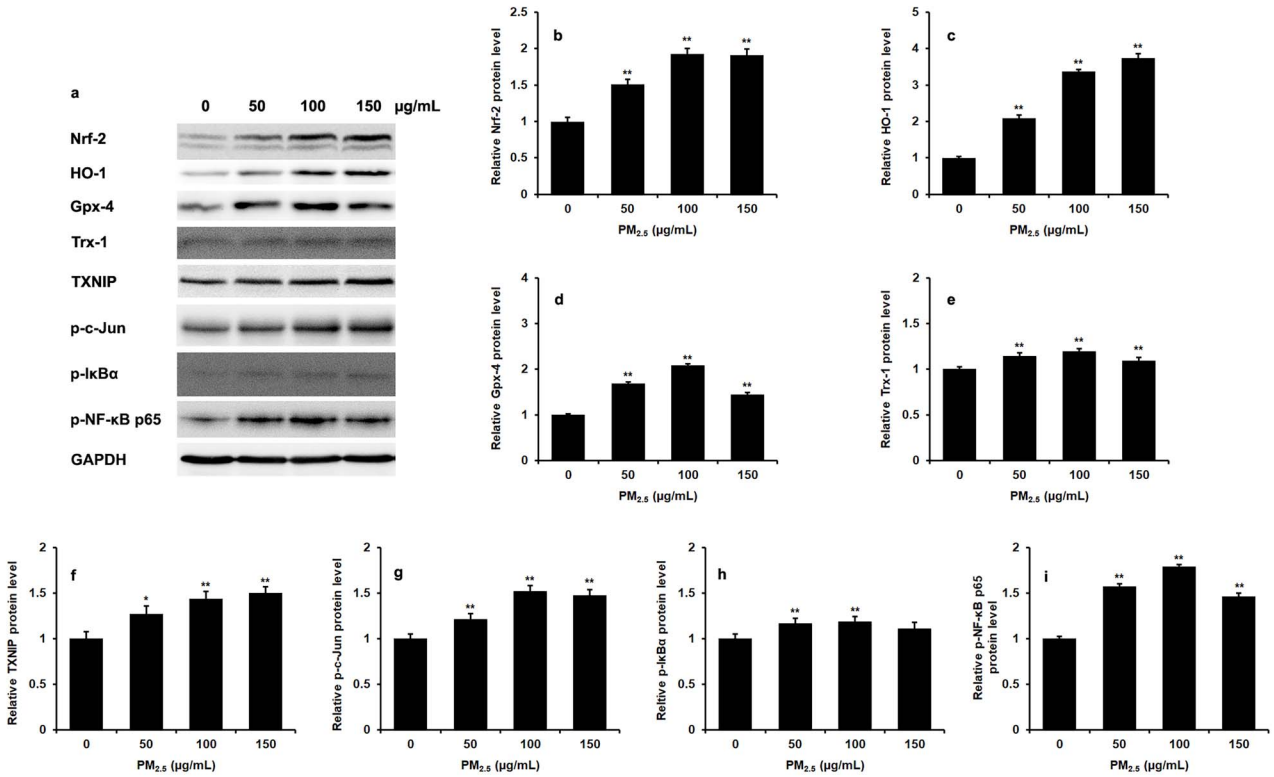

Figure 4. Effects of $PM_{2.5}$ on the Nrf-2/TXNIP-mediated NF-κB signaling pathway in BEAS-2B cells. (a) Representative western blot of Nrf-2, HO-1, Gpx-4, Trx-1, TXNIP, phospho-c-Jun, phospho-IκBα, and phospho-NF-κB p65 protein levels in cells treated with increasing concentrations of $PM_{2.5}$ solution (50, 100, and 150 μg/mL) for 24 h. (b-i) Quantification analysis of protein expression conducted by Image J software. *$P < 0.05$, **$P < 0.01$, versus the control.

cause of $PM_{2.5}$-mediated toxicity [4]. Previous studies have reported that $PM_{2.5}$ particles contributed to cellular oxidative stress and damage in several in vitro models, such as human bronchial epithelial, hepatic stellate, and neuroblastoma cell lines [7, 29, 30]. Our results showed that Suzhou-collected $PM_{2.5}$ induced redox imbalances as indicated by significant decreases in the GSH/GSSG ratio and elevation of MDA content, which are crucial steps for further occurrence of subcellular mitochondrial structure dysfunction, initiation of mitophagy and inflammatory response. Intriguingly, in this study, we observed increased activities of antioxidant enzymes (i.e. SOD and Gpx) and protein expression of Gpx-4, HO-1, and Nrf-2, which were reported to be the primary intracellular defense mechanisms against GSH exhaustion and oxidative stress [4]. Therefore, it can be explained that, due to the sharp consumption of GSH, the antioxidant system was activated as a negative feedback of cellular response to $PM_{2.5}$ [31]. In addition, cell proliferation requires continuous ATP production and transport, and thus intact mitochondrial structure and function are critical for normal physiological homeostasis of BEAS-2B cells. In accordance with a recent study [30], ambient $PM_{2.5}$ particles from Suzhou resulted in decreased cell viabilities of BEAS-2B cells accompanying a similar reduction of cellular ATP contents. We therefore considered that the imbalance of redox states and induction of oxidative stress were of great importance in BEAS-2B cell injury induced by $PM_{2.5}$.

As the main site of ROS production, mitochondria are much more susceptible to oxidative stress than other cellular components. Cellular oxidative stress induced by $PM_{2.5}$ produces continuous attacks on the mitochondrial membrane [32]. ROS can directly impair the mitochondrial respiratory chain, impeding the flow of electrons through the respiratory chain, and increasing the permeability of mitochondrial membrane [7, 10]. Moreover, previous studies have reported that exposure to $PM_{2.5}$ could induce ultrastructure alterations of mitochondria, such as mitochondrial swelling and cristae disorder [16]. In the present study, we found that $PM_{2.5}$ could be absorbed into BEAS-2B cells, which resulted in dilated mitochondria and endoplasmic reticulum and loss or rupture of mitochondrial cristae. Additionally, significant reduction in MMP, which is associated with decreased electron transport, were observed in BEAS-2B cells treated with 100 and 150 μg/mL of $PM_{2.5}$. Yet, an increase in MMP, which always indicates an increase in ATP production, was detected in 50 μg/mL $PM_{2.5}$ treated group. Whereas considering the slight reduction of ATP induced by 50 μg/mL of $PM_{2.5}$ in the present study, we suggested that a transient block of electron transfer or proton use by enzymes within the mitochondria and a change in the mitochondrial pH lead to this increase in MMP [33]. Together these results indicated that mitochondria were important cellular targets of $PM_{2.5}$, and $PM_{2.5}$ toxicity was closely associated with mitochondrial dysfunction [29].

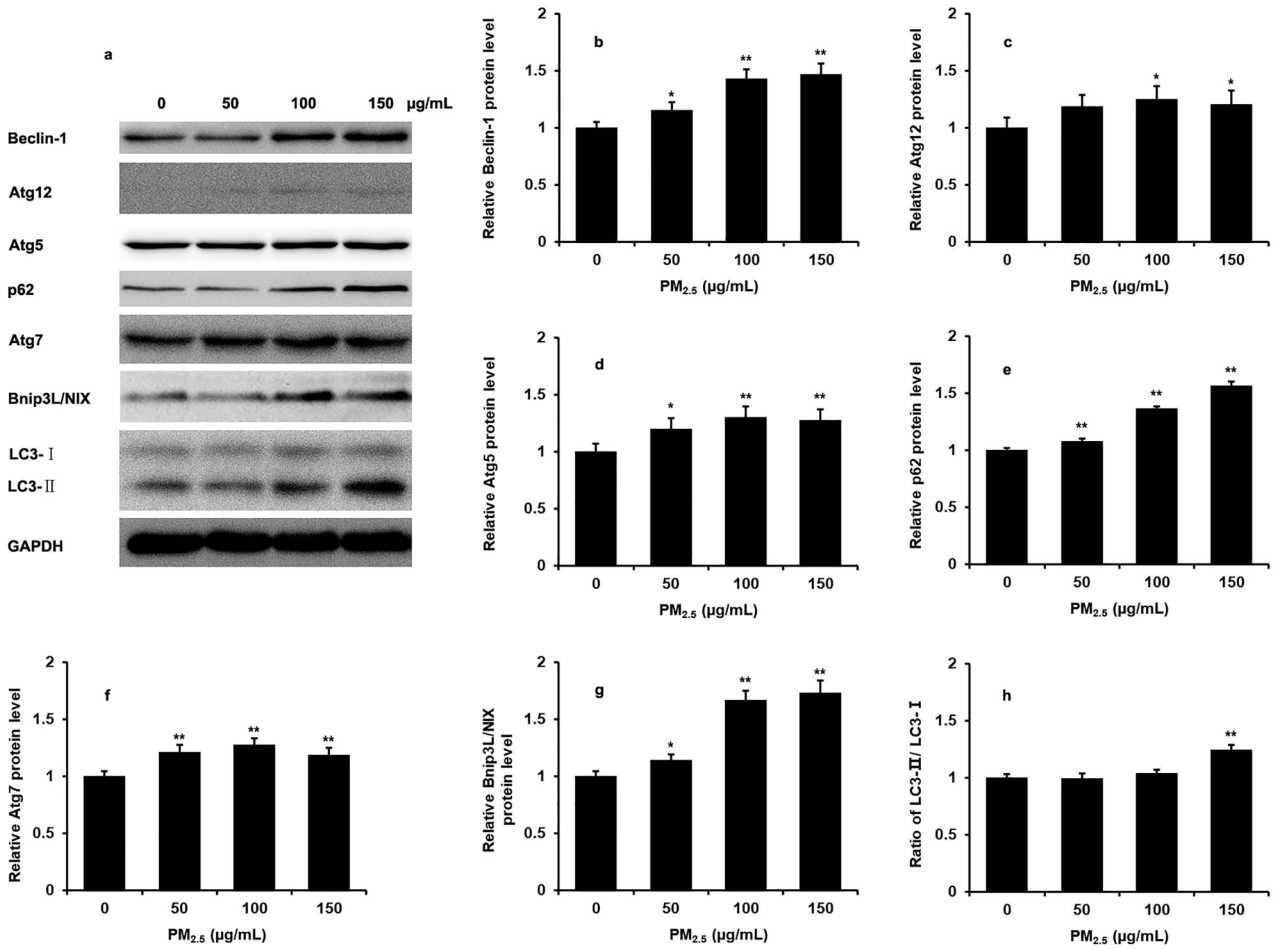

Figure 5. $PM_{2.5}$ induced mitophagy in BEAS-2B cells. (a) Representative western blot of Beclin-1, Atg12, Atg5, p62, Atg7, Bnip3L/NIX, and LC3 I/II protein levels in cells treated with increasing concentrations of $PM_{2.5}$ solution (50, 100, and 150 μg/mL) for 24 h. (b-h) Quantification analysis of protein expression conducted by Image J software. *$P < 0.05$, **$P < 0.01$, versus the control.

As a lysosomal degradation process that could eliminate and recycle dysfunctional organelles, mitophagy is reported to be crucial for the maintenance of mitochondrial quality and cellular homeostasis [34–36]. In our current study, $PM_{2.5}$ treatment resulted in autophagosome formation and significant upregulation of typical biomarkers at different stages of autophagy (i.e. Beclin-1, Atg12, Atg5, and Atg7) and Bnip3L/NIX, a special mitophagy receptor, in BEAS-2B cells. Increases in these autophagy initiation markers were accompanied by accumulation of autolysosomes with undegraded cargo and persistently increased expression of p62 and LC3-II, both of which should have been theoretically degraded at the late phase of autophagy. These results indicated that mitophagy was induced by $PM_{2.5}$, but, simultaneously, the autophagic flux was inhibited by the blockade of autolysosome degradation [37].

Several studies have reported that mitophagy dysfunction, whether increased or decreased, was closely associated with $PM_{2.5}$ toxicity in different cells and tissues [7, 29]. Evidence also indicated that accumulation of damaged mitochondria and the accompanying oxidative stress resulted from defective mitophagy caused an exaggerated inflammatory response [38–40]. In the present work, activation of the Nrf-2/TXNIP-mediated NF-κB signaling pathway and increased production of inflammatory cytokines (i.e. IL-6 and IL-8) were induced in BEAS-2B cells by $PM_{2.5}$. A 6-h 3-MA pretreatment clearly increased the oxidative stress and inflammatory response as indicated by increasing protein level of HO-1, TXNIP, and gene expression of *IL-8*. Additionally, Bnip3L/NIX protein was also significantly upregulated by 3-MA. Taken together, these data demonstrated that internalization of $PM_{2.5}$ particles into cells initially triggered oxidative stress and mitochondrial dysfunction, which then resulted in the initiation of mitophagy. Although induction of Bnip3L/NIX-mediated mitophagy in BEAS-2B cells seemed to confer protection in response to $PM_{2.5}$ in this study, dysfunction of autophagic flux may be a crucial contributor to defective mitophagy and cellular inflammatory injury. Some previous studies reported that cellular responses to $PM_{2.5}$ is closely related with its particle size, chemical and biological composition, and exposure scenario [41,

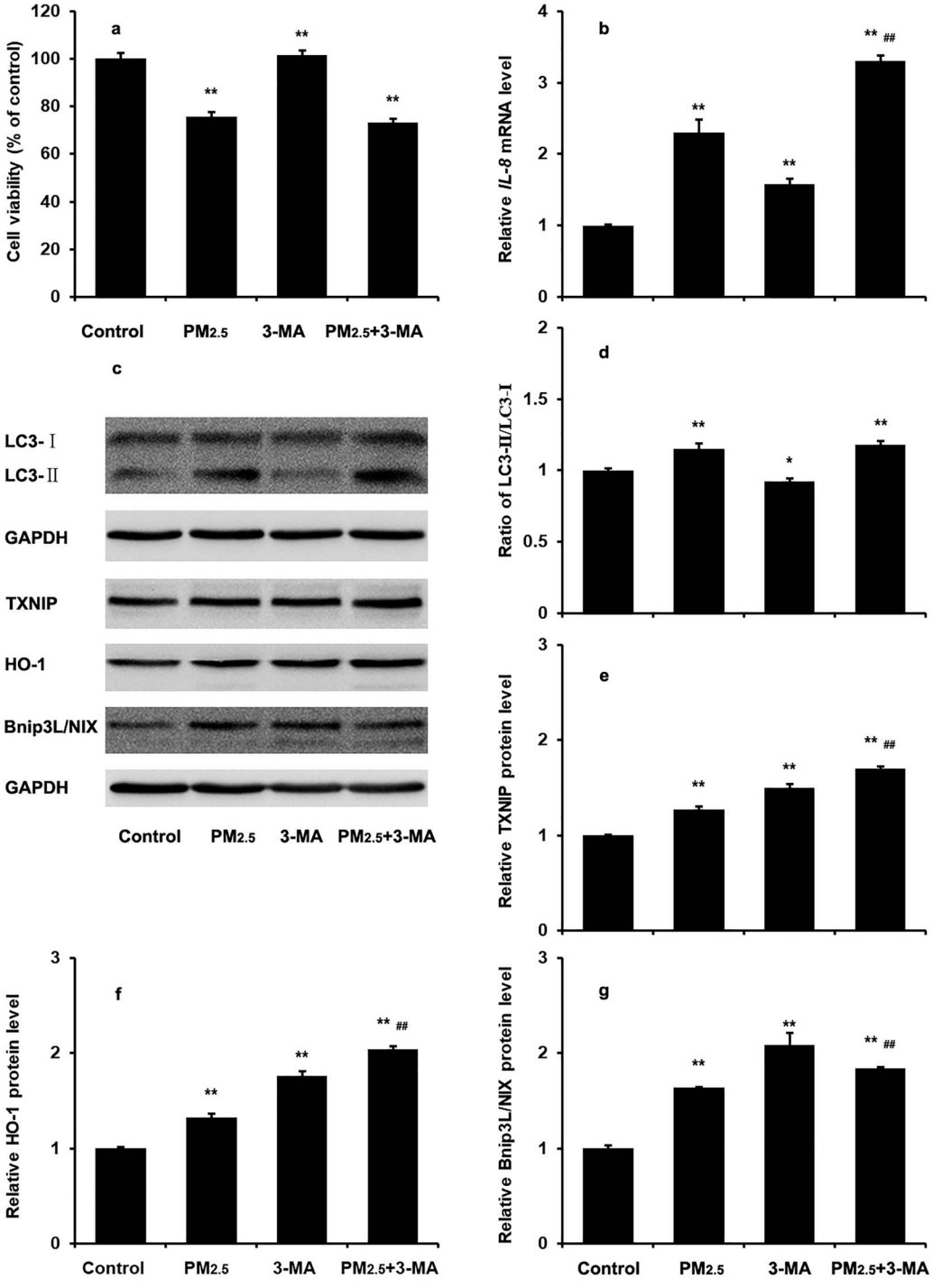

Figure 6. Crosstalk between mitophagy and oxidative stress-mediated inflammation during $PM_{2.5}$ exposure. After pretreatment with 2 mM 3-MA for 6 h, BEAS-2B cells were treated with 150 μg/mL $PM_{2.5}$ for another 24 h. (a) Cell viability determined by CCK8 assay kits. (b) Levels of *IL-8* mRNA measured by real-time polymerase chain reaction. (c) Representative western blot of LC3A/B, TXNIP, HO-1, and Bnip3L/NIX protein level in BEAS-2B cells. (d-g) Quantification analysis of protein expression conducted by Image J software. $^{*}P < 0.05$, $^{**}P < 0.01$, versus the control. $^{\#\#}P < 0.01$, versus the $PM_{2.5}$-treated group.

42]. However, the relative importance of the interplay between oxidative stress, mitochondrial dysfunction, mitophagy, and inflammation in different cell types induced by $PM_{2.5}$ samples with different physicochemical and biological characterizations needs to be further investigated.

Conclusion

Ambient $PM_{2.5}$ collected in Suzhou, China, induced inflammatory responses resulting from oxidative stress, altered mitochondrial redox homeostasis, and initiation of mitophagy in BEAS-2B cells. Inhibition of autophagic flux aggravated BEAS-2B cell injury, suggesting that

induction of Bnip3L/NIX-mediated mitophagy conferred protection against $PM_{2.5}$-induced cellular damage. Together, our results indicated that oxidative stress and dysfunction of autophagic flux contributed to the inflammatory response in BEAS-2B cells induced by $PM_{2.5}$. Additionally, these findings in the present study could provide theoretical evidence for the prevention and therapeutic strategy of $PM_{2.5}$-induced lung injury.

Data availability

Data are available from corresponding author upon request.

Authors' Contributions

L.L.X. conceived of the study. X.D.Z., J.S.W., and J.J.S. performed data collection, data analysis, and produced the figures and tables, with overall guidance from L.L.X. All authors wrote the manuscript. X.D.Z. and L.L.X. deposited the data.

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Conflict of interest

There are no conflict of interest to declare.

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