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ORIGINAL ARTICLE



## Mitochondrial ROS and NLRP3 inflammasome in acute ozone-induced murine model of airway inflammation and bronchial hyperresponsiveness

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### ABSTRACT

Oxidative stress is a key mechanism underlying ozone-induced lung injury. Mitochondria can release mitochondrial reactive oxidative species (mtROS), which may lead to the activation of NLRP3 inflammasome. The goal of this study was to examine the roles of mtROS and NLRP3 inflammasome in acute ozone-induced airway inflammation and bronchial hyperresponsiveness (BHR). C57/BL6 mice ( $n=8$ /group) were intraperitoneally treated with vehicle (phosphate buffered saline, PBS) or mitoTEMPO (mtROS inhibitor, 20 mg/kg), or orally treated with VX-765 (caspase-1 inhibitor, 100 mg/kg) 1 h before the ozone exposure (2.5 ppm, 3 h). Compared to the PBS-treated ozone-exposed mice, mitoTEMPO reduced the level of total malondialdehyde in bronchoalveolar lavage (BAL) fluid and increased the expression of mitochondrial complexes II and IV in the lung 24 h after single ozone exposure. VX-765 inhibited ozone-induced BHR, BAL total cells including neutrophils and eosinophils, and BAL inflammatory cytokines including IL-1 $\alpha$ , IL-1 $\beta$ , KC, and IL-6. Both mitoTEMPO and VX-765 reduced ozone-induced mtROS and inhibited caspase-1 activity in lung tissue whilst VX-765 further inhibited DRP1 and MFF expression, increased MFN2 expression, and down-regulated caspase-1 expression in the lung tissue. These results indicate that acute ozone exposure induces mitochondrial dysfunction and NLRP3 inflammasome activation, while the latter has a critical role in the pathogenesis of ozone-induced airway inflammation and BHR.

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### KEYWORDS

Airway inflammation; bronchial hyperresponsiveness; mitochondrial ROS; NLRP3 inflammasome; ozone

## Introduction

Ozone is a secondary atmospheric pollutant produced through photochemical reactions involving sunlight and precursor pollutants, including volatile organic compounds (VOCs), nitrogen oxides (NO<sub>x</sub>), and carbon monoxide (CO), which are released by a wide variety of stationary and mobile sources [1]. Exposure to high ambient ozone has been linked to increased incidence of asthma [2], and increased emergency visits and hospitalisations of patients with asthma and chronic obstructive pulmonary disease (COPD) [3,4]. Experimental studies have demonstrated that high levels of ozone exposure can induce airway inflammation and bronchial hyperresponsiveness (BHR) in both humans [5,6] and mice [7,8]. The mechanism is not fully

elucidated, however, as a potent oxidising gas, ozone can induce oxidative stress, which is likely a key mechanism underlying ozone-induced airway inflammation and BHR.

Mitochondria are double membrane bound organelles that exist in all eukaryotic organisms with multiple functions. The morphology of mitochondria is regulated by fission and fusion process. The former requires the recruitment of dynamin-related protein 1 (DRP1) from the cytosol to receptors including mitochondrial fission protein 1 (FIS1), mitochondrial fission factor (MFF), and mitochondrial elongation factors 1 and 2 (MID51 and MID49), while the latter is mediated by mitofusin 1 and 2 (MFN1/2) at the outer mitochondrial membrane and by optic atrophy 1 (OPA1) at the inner

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mitochondrial membrane [9]. The functions of mitochondria include generation of adenosine triphosphate (ATP) and mitochondrial reactive oxygen species (mtROS), which are produced by the oxidative phosphorylation (OXPHOS) enzyme complexes located in the inner mitochondrial membrane [10]. Under inflammatory conditions, mtROS can initiate downstream inflammatory pathways [11].

Nucleotide binding domain leucine-rich repeat-containing receptor (NLR) families are an important component of signalling by intracellular pattern recognition receptors in the innate immune system, of which the NLRP3 inflammasome is the best characterised member and acts as a potent inducer of inflammation in lung diseases including asthma and COPD. The NLRP3 inflammasome contains a sensor protein, NLRP3, an adaptor protein, apoptosis-associated speck-like protein containing caspase-recruitment domain (ASC), and procaspase-1 [12]. NLRP3 inflammasome can be activated by a variety of exogenous and endogenous agonists, including bacterial toxins, extracellular ATP, and mtROS [13,14]. Stimulation of the NLRP3 inflammasome leads to caspase-1 activation and the subsequent production of proinflammatory cytokines such as IL-1 $\beta$  and IL-18 [12].

We hypothesised that the mtROS–NLRP3 inflammasome nexus is an important pathway in ozone-induced airway inflammation and BHR. Therefore, the goal of this study was to examine the roles of mtROS and NLRP3 inflammasome in acute ozone-induced airway inflammation and BHR using selective inhibitors.

## Materials and methods

### Mice and ozone exposure

Pathogen-free, 8–10-week-old male C57/BL6 mice (Shanghai Super – B&K Laboratory Animal Corp. Ltd., Shanghai, China) were housed in specific-pathogen-free (SPF) conditions under a constant temperature (20 °C) and relative humidity (40–60%) with food and water supplied *ad libitum*. The experimental procedures involving animals and their care were approved by the Laboratory Animal Ethics Committee of the institute.

Mice were exposed to ozone produced from a generator (Model 300, AB Aqua Medick GmbH, Bissendorf, Germany), mixed with air for 3 h at a concentration 2.5 parts per million (ppm) in a sealed Perspex container in the morning. The ozone concentration was continuously monitored and maintained using an ozone switch (OS-4, EcoSensors Division, KWJ Engineering, Inc., Newark, NJ, USA). At the same time, control animals were exposed to filtered air only.

Mice (eight mice in each group) were administered intraperitoneally with vehicle (phosphate buffered saline, PBS), mitoTEMPO (mtROS inhibitor, 20 mg/kg dissolved in PBS), or orally with VX-765 (caspase-1 inhibitor, 100 mg/kg dissolved in distilled water containing 0.5% sodium salt of carboxymethylcellulose and 0.1% Tween 80) 1 h prior to ozone exposure.

### Measurement of bronchial responsiveness

Twenty-four hours after exposure, mice were anaesthetised with an intraperitoneal injection of anaesthetic solution containing ketamine and xylazine and then transferred to a plethysmograph for the measurement of resistance and compliance (EMMS, Hants, UK), and airway responsiveness. Mice were ventilated with Mini ventilator at a rate of 160 breaths/min and at a tidal volume of 220  $\mu$ l, and were monitored with a pneumotachograph connected to a transducer. Transpulmonary pressure was assessed *via* an oesophageal catheter. Instantaneous calculation of pulmonary resistance ( $R_L$ ) was obtained. Increasing concentrations of acetylcholine (ACh) (Sigma-Aldrich, St Louis, MS, USA, 4–256 mg/ml) were administered with an Aeroneb<sup>®</sup> Lab Micropump Nebuliser (EMMS), and  $R_L$  was recorded for a 3-min period following each concentration.  $R_L$  after each concentration was expressed as percentage change from baseline  $R_L$  measured following nebulised PBS. The concentration of ACh required to increase  $R_L$  by 200% from baseline was calculated ( $PC_{200}$ ) and  $-\log PC_{200}$  was taken as a measure of airway responsiveness.

### Bronchoalveolar lavage fluid and cell counting

Following terminal anaesthesia with pentobarbitone 24 h after ozone exposure, mice were lavaged with two 0.8 ml aliquots of PBS *via* a 1 mm diameter endotracheal tube, and bronchoalveolar lavage (BAL) fluid was retrieved. The retrieved lavage aliquots were pooled and centrifuged at 4 °C, 250g, for 10 min, from which supernatant was collected and stored at  $-80$  °C. The cell pellet from the BAL fluid was resuspended in PBS and counted using a haemocytometer. Total cell counts and differential cell counts from cytopsin preparations stained using the Diff-Quick method (Gentauro, Kampenhout, Belgium) were determined under an optical microscope (Olympus BH2, Olympus Optical Company Ltd., Tokyo, Japan). At least 500 cells were counted per mouse and identified as macrophages, neutrophils, lymphocytes, and eosinophils according to standard morphology under  $\times 400$  magnification.

### Malondialdehyde (MDA) and cytokine measurement in BAL fluid

Concentrations of total MDA were measured using high-performance liquid chromatography (HPLC) system with fluorescent detection. An alkaline hydrolysis step described as follows was added before the reaction with TBA: 20  $\mu$ l aliquot of sample was added 40  $\mu$ l of 1 N NaOH and incubated at 60 °C for 30 min in a shaking water bath. After incubation, 40  $\mu$ l of 1 N HCl was added into the mixture and then 500  $\mu$ l phosphoric acid (440 mM) and 100  $\mu$ l thiobarbituric acid (TBA, 42 mM) were added. The mixture was incubated at 80 °C for 1 h, a 20  $\mu$ l aliquot of this final solution was injected into the HPLC system with fluorescence detector set at 532 nm for the excitation wavelength and 553 nm for the emission wavelength. A nova-Pak C<sub>18</sub> column (Waters, Milford, MA, USA) was used with a mobile phase that was composed of 40% methanol and 60% water containing 50 mM KH<sub>2</sub>PO<sub>4</sub> (pH = 6.8) at a flow rate of 0.8 ml/min. The detection limit, extraction recovery, and analytical precision of this method were 1.8 nM, 75.9, and 2.2% (measured as RSD from eight replicate injections), respectively.

Levels of chemokine (C-X-C motif) ligand 1 (CXCL1, KC), tumour necrosis factor alpha (TNF- $\alpha$ ), interleukin-6 (IL-6) and IL-1 $\beta$  in BAL fluid were measured using ELISA DuoSets according to manufacturers' instructions (R & D Systems, Minneapolis, MN, USA).

### Lung mitochondrial ROS analysis and caspase-1 activity

Mitochondria were extracted from fresh lung tissues using a mitochondria Isolation Kit for Tissue (Beyotime, Haimen, Jiangsu, China) with protease inhibitors (Merck Millipore, Darmstadt, Germany) using a Dounce tissue grinder following the manufacturer's instructions, and then quantified by BCA analysis (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of mitochondrial extract were incubated with 5  $\mu$ M MitoSOX<sup>TM</sup> Red (Invitrogen, Carlsbad, CA, USA) for 10 min at 37 °C and protected from the light. Red fluorescence was measured at 510/580 nm using a Flexstation<sup>®</sup>2 fluorescence reader (Molecular Devices, San Jose, CA, USA).

The lung homogenates were extracted using RIPA buffer (Beyotime) with protease inhibitors (Merck Millipore). The levels of caspase-1 activity in mouse lung tissue were detected using a commercial assay kit (Beyotime) and measured at 405 nm by a microplate reader following the manufacturer's instructions.

### Western blot analysis

Equal amounts of protein from mitochondrial and whole lung extracts were separated by SDS-PAGE (Beyotime) and transferred to nitrocellulose membranes by electrophoresis. The membranes were incubated in blocking solution at room temperature for 1 h and then incubated with primary antibodies against total OXPHOS antibody cocktail (Abcam, Cambridge, MA, USA), voltage-dependent anion channel (VDAC, Abcam), DRP1 (Cell Signalling Technologies – CST, Beverly, MA, USA), MFF (CST), MFN2 (CST), OPA1 (CST), GAPDH (CST), NLRP3 (CST), caspase-1 (Abcam) overnight at 4 °C. A horseradish peroxidase-conjugated anti-rabbit secondary antibody (Cell Signalling Technology) was used, and chemiluminescent detection reagent (Merck Millipore) was used for detection. VDAC and tubulin were respectively used as loading controls. The density was quantitated using a densitometer.

### Statistical analysis

All results were expressed as mean  $\pm$  S.E.M. Two-way analysis of variance was performed for comparisons of % change in  $R_L$  between individual groups. One-way analysis of variance (ANOVA) with Bonferroni *post hoc* test (equal variance) or Dunnett T3 *post hoc* test (unequal variance) was performed for comparison between multiple groups.  $p < .05$  was considered significant.

## Results

### Airway responsiveness

There were no significant differences in the baseline lung resistance values following PBS challenge in the four experimental groups. Ozone-exposed mice demonstrated a leftward shift of the concentration–response curve (Figure 1(A)), indicating an increase in airway responsiveness to ACh compared to air-exposed mice ( $-\log PC_{200}$ :  $1.54 \pm 0.05$  vs.  $2.11 \pm 0.15$ ;  $p < .05$ , Figure 1(B)). VX-765 inhibited ozone-induced airway responsiveness to ACh ( $-\log P_{200}$ :  $2.02 \pm 0.12$  vs.  $1.54 \pm 0.05$ ,  $p < .05$ ; Figure 1(B)), while mitoTEMPO showed no effect on airway responsiveness to ACh.

### BAL fluid cells and cytokine levels

Ozone exposure increased total BAL cell counts including that of neutrophils and eosinophils compared with air-exposed mice ( $p < .001$ ,  $p < .001$ , and  $p < .05$ , respectively, Figure 2(A)). Treatment with VX-765, not

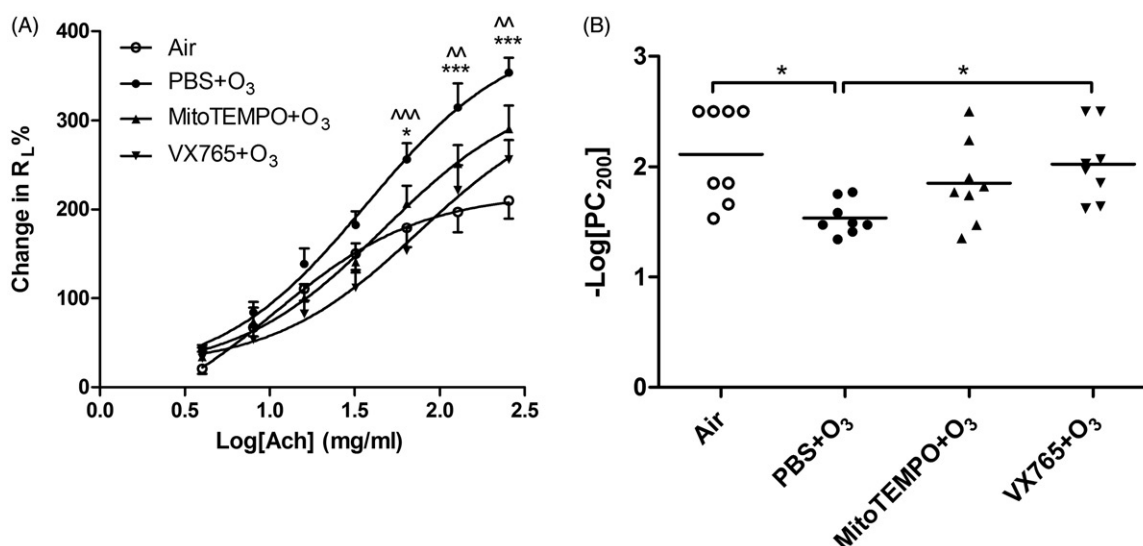


**Figure 1.** Effects of mitoTEMPO and VX765 on acute ozone exposure induced mean percentage increase in lung resistance ( $R_L$ ) to increasing concentrations of acetylcholine (ACh) (A) and  $-\log PC_{200}$  (B). \* $p < .05$ , \*\*\* $p < .001$  compared to air-exposed mice; °° $p < .01$ , °°° $p < .001$  compared to VX-765-treated ozone-exposed mice.

mitoTEMPO, decreased the numbers of total cells, neutrophils, and eosinophils (all  $p < .05$ , Figure 2(A)). Ozone exposure increased BAL concentrations of IL-1 $\alpha$ , IL-1 $\beta$ , KC, and IL-6 in comparison with air-exposed mice ( $p < .001$ ,  $p < .001$ ,  $p < .001$ , and  $p < .05$ , respectively, Figure 2(B-E)). VX-765 treatment significantly decreased the concentrations of IL-1 $\alpha$ , IL-1 $\beta$ , KC, and IL-6 ( $p < .01$ ,  $p < .001$ ,  $p < .001$ , and  $p < .01$ , respectively, Figure 2(B-E)). MitoTEMPO treatment significantly reduced the level of KC ( $p < .001$ ) (Figure 2(D)).

### Oxidative stress in lung

Ozone exposure enhanced total MDA levels in BAL and mtROS levels in lung tissue compared to the control mice (both  $p < .001$ , Figure 3(A,B)). MitoTEMPO treatment reduced total MDA and mitochondrial ROS ( $p < .05$  and  $p < .01$  respectively, Figure 3(A,B)) whilst VX-765 significantly reduced mtROS ( $p < .001$ , Figure 3(B)).

### Expression of mitochondrial OXPHOS in lung tissue

There was decreased expression of mitochondrial OXPHOS complexes II, III, and IV in the ozone-exposed mice compared with the control mice ( $p < .05$ ,  $p < .01$ , and  $p < .001$ , respectively, Figure 4(A,C,D,E)). MitoTEMPO increased the expression of mitochondrial complex II and IV in the lung of the ozone-exposed mice ( $p < .05$  and  $p < .05$ , respectively, Figure 4(A,C,E)).

VX-765 had no effect on the expression of OXPHOS complex proteins.

### Mitochondrial related protein expression

The expression of DRP1 and MFF was increased in the ozone-exposed mice compared to the control mice ( $p < .01$  and  $p < .05$ , respectively, Figure 5(A,B)), VX765 treatment inhibited the expression of DRP1 and MFF ( $p < .01$  and  $p < .05$ , respectively, Figure 5(A,B)). The expression of OPA1 was unchanged and MFN2 was decreased in the ozone-exposed mice compared to the control mice ( $p < .01$ , Figure 5(C,D)) and VX765 treatment increased MFN2 expression ( $p < .01$ , Figure 5(D)).

### Expression of NLRP3&caspase-1 and caspase-1 activity in lung tissue

The expression of NLRP3 was unchanged by ozone exposure when compared with the control mice. Neither mitoTEMPO nor VX-765 affected NLRP3 expression (Figure 6(A)). However, the expression and activity of caspase-1 were increased in ozone-exposed mice when compared with the control mice ( $p < .01$  and  $p < .05$ , respectively, Figure 6(B,C)). VX-765 significantly inhibited the expression of caspase-1 in the lung tissue (both  $p < .01$ , Figure 6(B)), furthermore, both VX-765 and mitoTEMPO reduced the activity of caspase-1 ( $p < .01$ , Figure 6(C)).

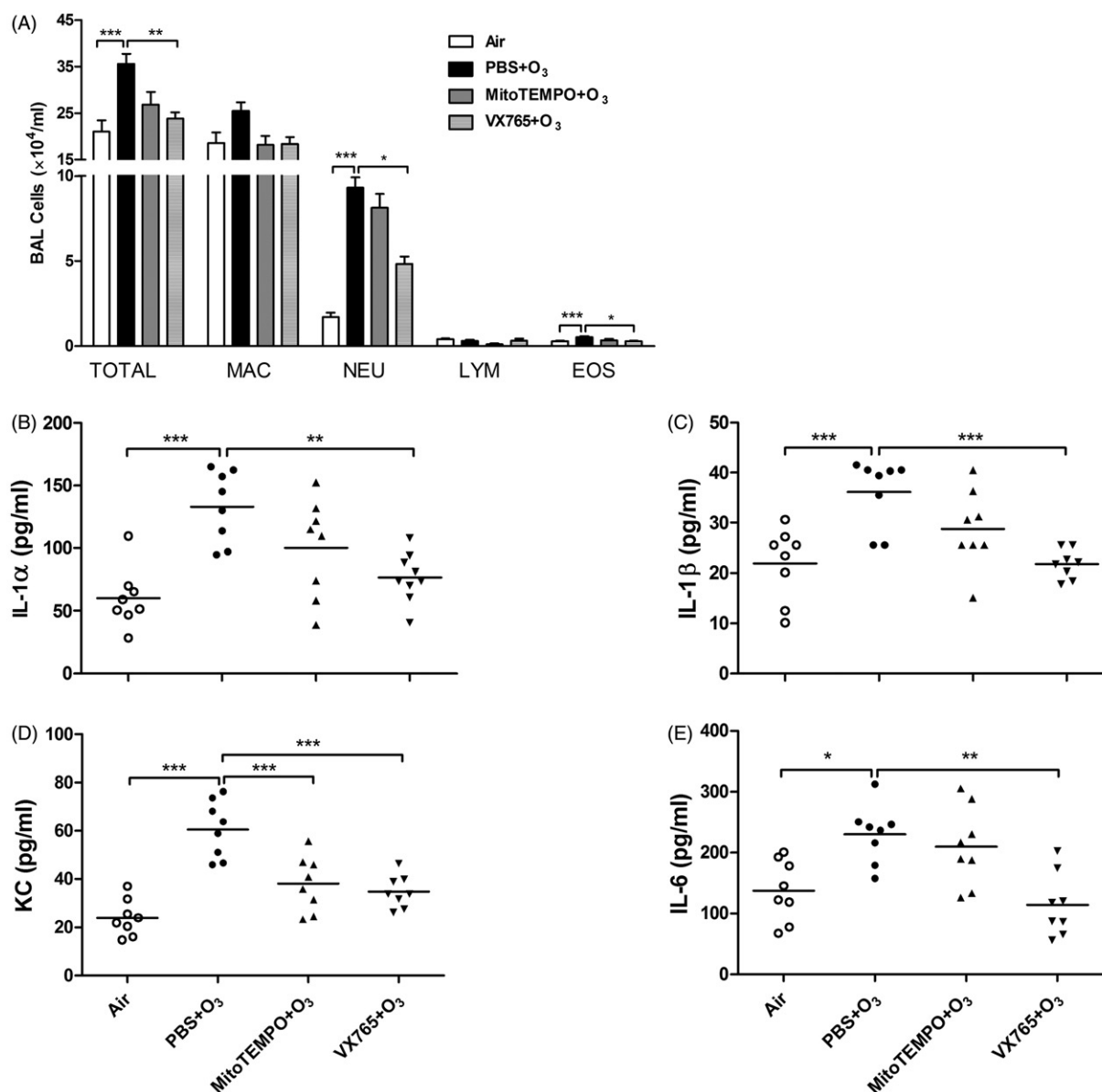


**Figure 2.** Effects of mitoTEMPO and VX765 on acute ozone exposure induced airway inflammation. (A) Effects of mitoTEMPO and VX765 on acute ozone exposure induced increased cell numbers in bronchoalveolar lavage (BAL) fluid, including total cells (TOTAL), macrophages (MAC), lymphocytes (LYM), neutrophils (NEU), and eosinophils (EOS). Each bar represents the mean  $\pm$  SEM. (B–E). Effects of mitoTEMPO and VX765 on acute ozone exposure induced increased concentrations of cytokines in BAL fluid, including IL-1 $\alpha$  (B), IL-1 $\beta$  (C), KC (D), and IL-6 (E). \* $p$  < .05, \*\* $p$  < .01, \*\*\* $p$  < .001.

## Discussion

The present study demonstrated that in an acute ozone exposure model, VX-765 (caspase-1 inhibitor) inhibited airway inflammation and BHR, which was associated with suppression of ozone-induced BAL cell counts and cytokine levels, suppression of ozone-induced mitochondrial dysfunction, and increased caspase-1 activity and expression in lung by VX-765. MitoTEMPO (mtROS inhibitor) decreased BAL levels of MDA and increased the expression of mitochondrial OXPHOS in the lung. These data suggest that ozone exposure induces airway

inflammation and BHR at least in part through activating the NLRP3 inflammasome in lung tissue.

The present research is consistent with previous studies which show that a single ozone exposure causes airway inflammation [7,8]. Ozone exposure increased the numbers of total cells, neutrophils and eosinophils, and elevated levels of IL-1 $\alpha$ , IL-1 $\beta$ , KC and IL-6 in BAL fluid. Both IL-1 $\alpha$  and IL-1 $\beta$  belong to IL-1 family and play an important role in modulating innate and adaptive immune response. In vitro, ozone exposure (0.1 ppm, 1-hour) stimulated the secretion of IL-1 $\alpha$

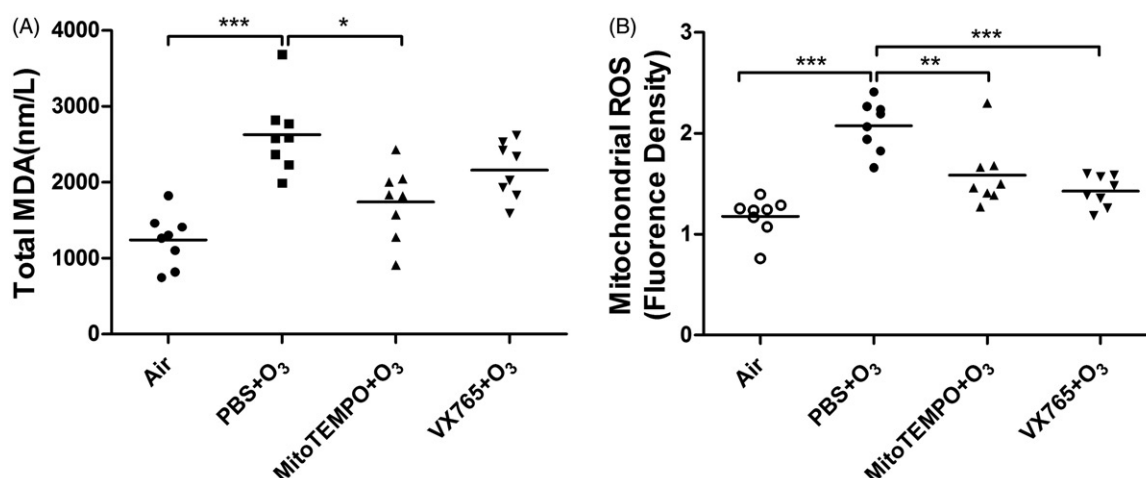


**Figure 3.** Effects of mitoTEMPO and VX765 on acute ozone exposure induced increases in malonaldehyde in BAL fluid (A) and mitochondrial ROS (B) in lung tissue. \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .

and IL-1 $\beta$  from rat alveolar macrophages [15] and in vivo, ozone exposure (0.3 ppm, 72-hour) increased IL-1 $\alpha$  and IL-1 $\beta$  mRNA levels in lung tissue [16]. The production of IL-1 $\alpha$  and IL-1 $\beta$  requires caspase-1 activation within the NLRP3 inflammasome complex to enable conversion from their inactive precursor forms [17]. KC and IL-6 are partially dependent on IL-1 $\beta$  as both cytokines are reduced in an IL-1 $\alpha/\beta$  double knock-out mice [18]. The present study showed that VX-765 reduced the presence of BAL cells and cytokines and indicated that the NLRP3 inflammasome is directly related to inflammation in this acute ozone-challenge model. MitoTEMPO reduced ozone-induced BAL KC expression and only had a trend towards suppression of inflammatory cell infiltration.

BHR is a characterised effect of ozone exposure and this occurrence of excessive bronchoconstriction in response to a variety of inhaled stimuli is an important characteristic of both asthma and COPD [19]. Eosinophils can release a number of different mediators with the capacity to cause BHR in asthma [20], whereas lung neutrophilia is strongly associated with BHR in COPD probably as a result of their production of ROS and proinflammatory cytokines [21]. In the present study, NLRP3 inhibition attenuated ozone-induced BHR and inhibited BAL neutrophils and eosinophils. In contrast, mitoTEMPO, an inhibitor of mtROS, did not ameliorate ozone-induced BHR or reduce inflammatory cells in BAL fluid. This result is different from a previous study, in which Necrox-5, the other mitochondrial ROS inhibitor, reduced BHR in an ovalbumin-induced asthma model [22]. The data may indicate BAL inflammation, not mtROS, is associated with BHR.

Oxidative stress is an important mechanism implicated in the pathogenesis of both asthma and COPD

and results from increases in free radicals and other reactive oxygen/nitrogen species and/or decreases in endogenous antioxidant defences [23,24]. The exogenous sources of oxidative stress include cigarette smoking, environmental pollution such as particulate matter and ozone [22,23]. The endogenous sources of oxidative stress mainly come from inflammatory cells and epithelial cells *via* intracellular enzymes and organelles such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, nitric oxide synthase, peroxidase, and mitochondria [23,24]. Ozone exposure can directly lead to the development of oxidised intermediates, which are either formed in the epithelial lining fluid or on cell surface proteins and lipids [25]. Consistent with previous reports [7], we confirmed that a single ozone exposure increased MDA levels in BAL fluid. MDA is a reliable marker of oxidative damage *via* lipid peroxidation [26] and this up-regulation seen with ozone was prevented by the treatment with mitoTEMPO.

Mitochondria are sensitive to even mild oxidative stress [27], and thus they represent a potential target for ozone. Ozone causes alterations in mitochondrial respiratory chain enzymes [28]. A low concentration (0.5 ppm, 8 h/day) of ozone for 1 or 5 days induced oxidant stress and mitochondrial DNA damage (mtDNA) in lung and aortic tissues of C57/BL6 mice [29]. In contrast, a high concentration (3 ppm, 3 h, twice a week) of ozone for 1 or 6 weeks led to mitochondrial dysfunction represented by decreased mitochondrial membrane potential ( $\Delta\Psi_m$ ), increased mitochondrial oxidative stress, and reduced mitochondrial complex I, III, and V expression in lung of C57/BL6 mice [30]. In the present study, we found that mtROS was increased in lung tissue 24 h after a single ozone exposure. Both mitoTEMPO and VX-765 reduced ozone-induced

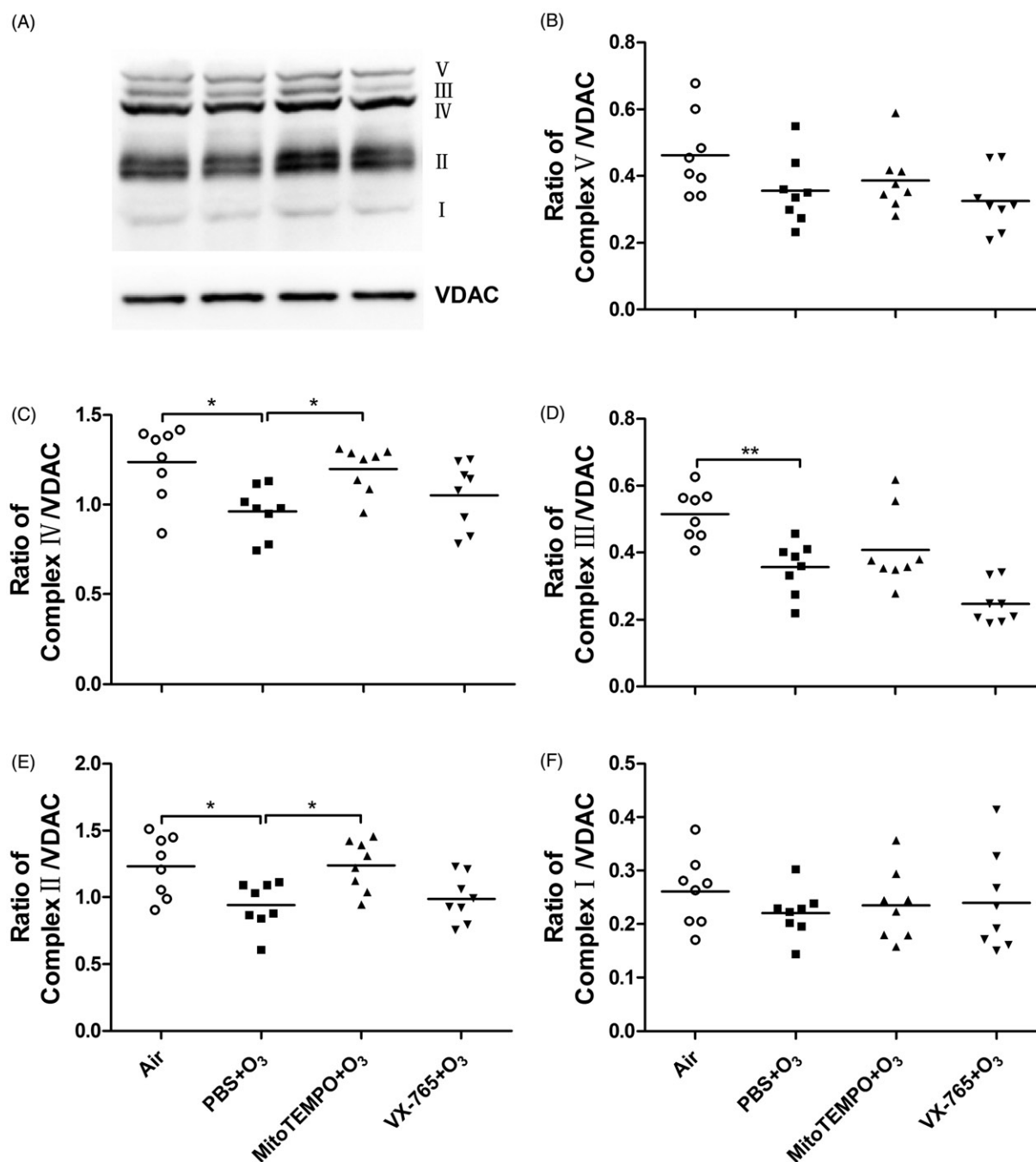


**Figure 4.** Representative Western blot images of mitochondrial OXPHOS and VDAC in lung tissue (A). (B–F) Effects of mitoTEMPO and VX765 on the protein expression of OXPHOS complex V (B), IV (C), III (D), II (E), and I (F) in lung tissue after acute ozone exposure. \* $p < .05$ , \*\* $p < .01$ .

mitochondrial ROS and caspase-1 activity in lung. This indicates that cross-talk occurs between mitochondrial function and NLRP3 inflammasome activation [31] although a linear pathway relationship is unlikely. The effect of the acute ozone exposure on the expression of mitochondrial OXPHOS complex proteins II, III, and IV was different from what have previously reported in chronic ozone-exposed mice [29,32], which may reflect temporal differences.

Mitochondria are regulated through cycles of fusion and fission, which influence mitochondrial mixing, mitochondrial DNA integrity, mitochondrial respiration, and energy metabolism. Upon exposure to stress (inflammation, environment insults), mitochondria undergo fission and display in fragmented morphology. Cigarette smoke extract (CSE) induced mitochondrial fragmentation and damaged their morphology by increased expression of DRP1 and decreased MFN2 in human

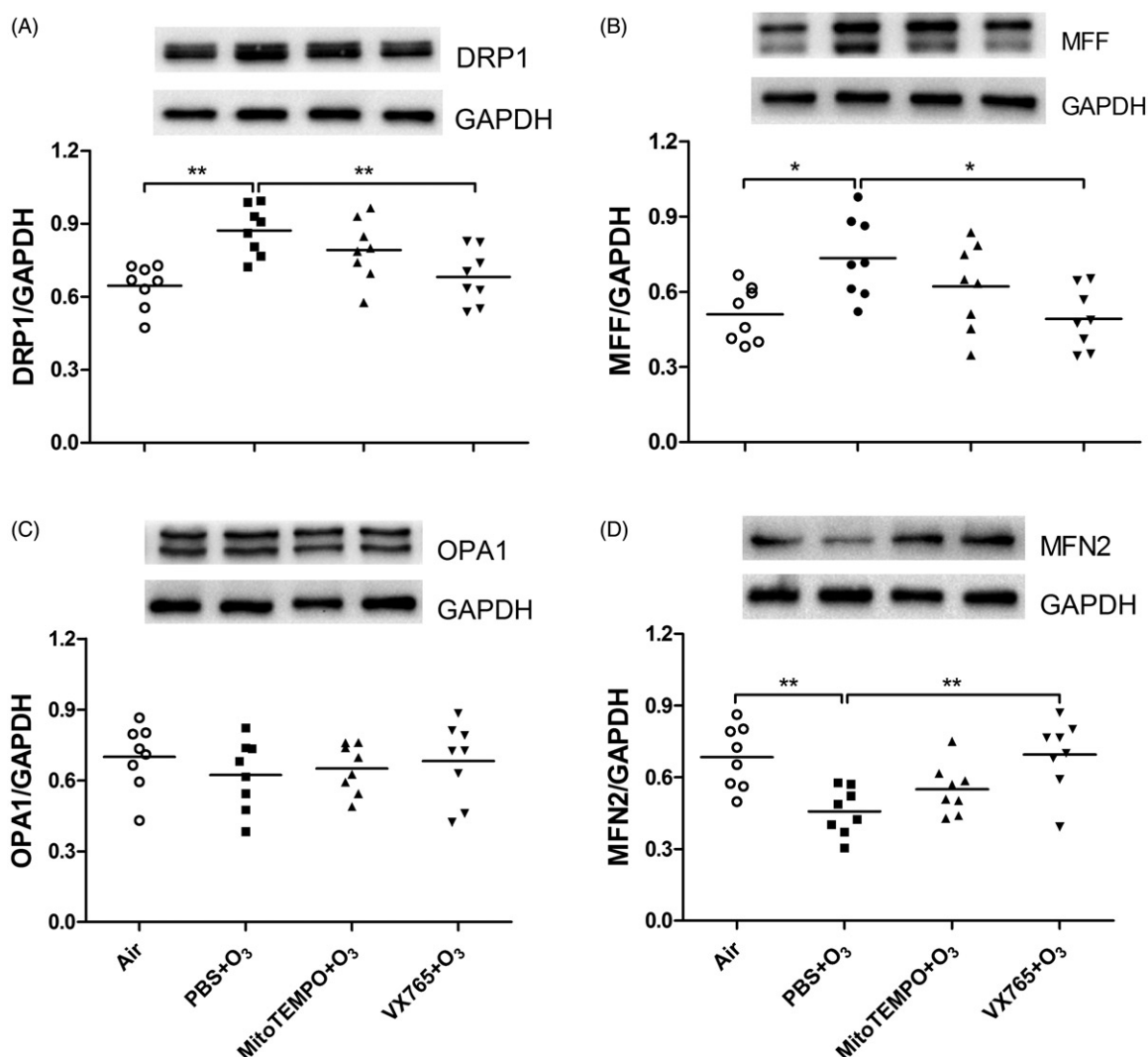


**Figure 5.** Effects of mitoTEMPO and VX765 on the protein expression of mitochondrial fission/fusion-related proteins, including DRP1 (A), MFF (B), OPA1 (C), and MFN2 (D), in lung tissue after acute ozone exposure. Each panel shows representative Western blot. \* $p < .05$ , \*\* $p < .01$ .

airway smooth muscle cells [33]. Similarly, CSE enhanced DRP1 and MFF and decreased OPA1 and MFN2 in airway epithelial cell lines (A549 cells and Beas-2b cells) [34]. Acute PM<sub>2.5</sub> intranasal instillation increased DRP1 and MFF and reduced OPA1 and MFN1 in lung tissue in C57/BL6 mice [35]. The present study showed that acute ozone exposure resulted in elevated DRP1 and MFF and reduced MFN2 in lung tissue, while VX765 treatment inhibited the expression of DRP1 and MFF. The imbalance of mitochondrial fission and fusion proteins may contribute to mitochondrial dysfunction, such as decreased ATP synthesis and increased ROS production, leading to cell damage, apoptosis, inflammation, and ultimately cell death.

Ozone-induced oxidant stress modifies several known cell signalling mechanisms such as the innate immune signalling pathways, which lead to the up-regulation of antioxidant genes and the enhanced release of damage-

associated molecular pattern molecules (DAMPs) [36]. There is much evidence to indicate the involvement of NLRP3 inflammasome in the pathogenesis of airway diseases [12,37]. Consistent with our previous study in chronic ozone-exposed animals [38], we showed here that the NLRP3 inflammasome was activated by single ozone exposure evidenced by increased IL-1 $\alpha$  and IL-1 $\beta$  concentration in BAL fluid and increased caspase-1 activity and expression in lung tissue. We initially hypothesised that mtROS acts as a trigger of NLRP3 activation, but the effects of VX-765 and mitoTEMPO are not consistent with this linear model and we suggest that these pathways are activated in parallel although cross-talk does occur between the two.

There are several limitations in the study which could be addressed in the future studies. First, mitoTEMPO was failed in inhibiting airway inflammation and BHR in the present study. Considering

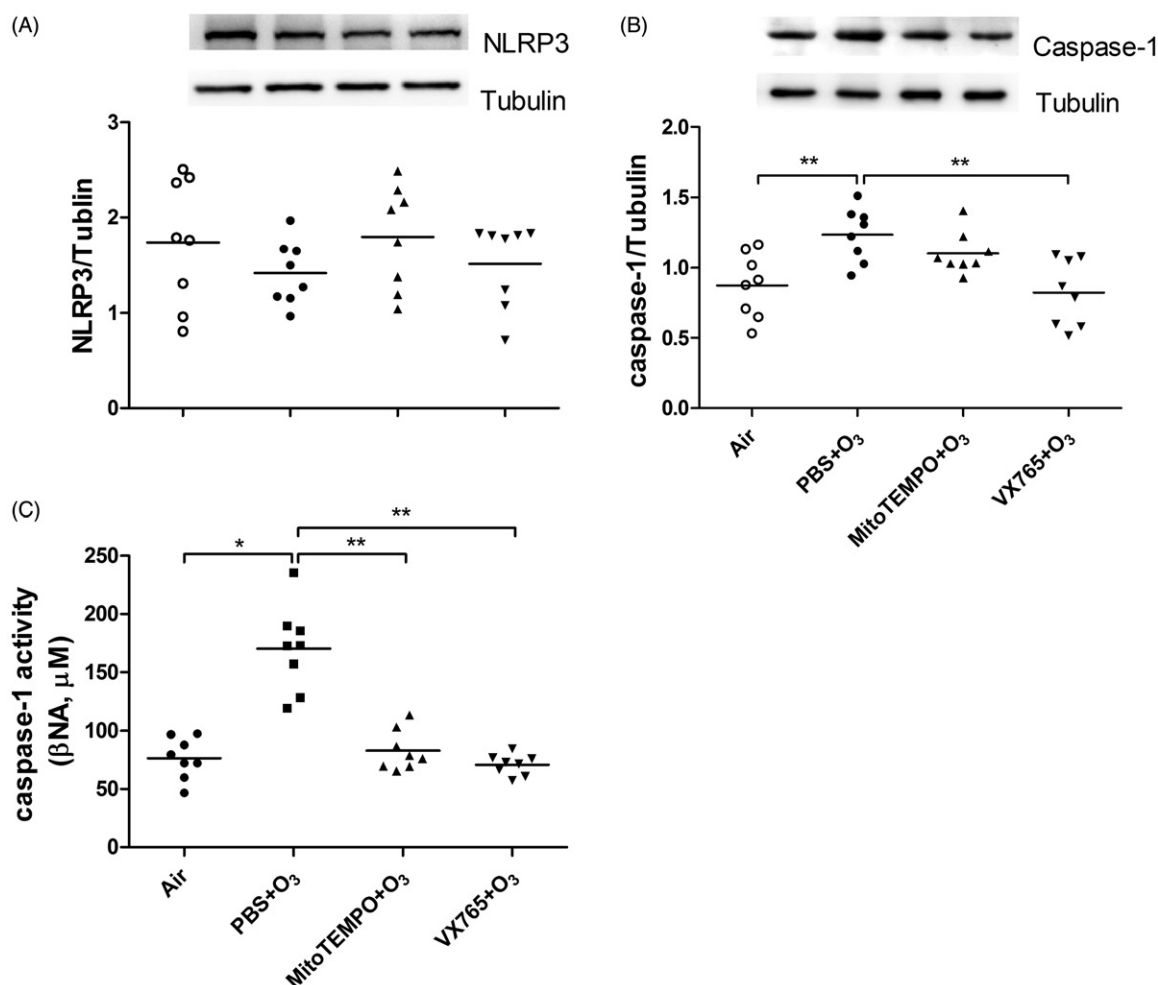


**Figure 6.** Effects of mitoTEMPO and VX765 on the protein expression of NLRP3 (A) and caspase-1 (B), and the activity of caspase-1 (C) in lung tissue after acute ozone exposure. Each panel shows representative Western blot. \* $p < .05$ , \*\* $p < .01$ .

mitochondria are the major source of endogenous ROS and mitochondrial dysfunction plays an important role in COPD, new compounds preventing or reversing mitochondrial dysfunction can be investigated *in vivo* or *in vitro*. Second, more *in vivo* cell lines studies can be conducted to further examine the effect and mechanism of mitochondrial dysfunction–NLRP3 pathway in inflammation. Third, to elucidate the role of NLRP3 inflammasome in ozone-exposed model, NLRP3<sup>−/−</sup> mouse can be used in later studies.

In summary, this study suggests that acute ozone exposure induces mitochondrial dysfunction and NLRP3 inflammasome activation. NLRP3 inflammasome can be activated by non-mtROS mechanism, has a critical role in the pathogenesis of ozone induced airway inflammation and BHR, providing a novel therapeutic strategy for treating ozone-induced airway disorders.

### Disclosure statement

No potential conflict of interest was reported by the authors.

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### References

- [1] US Environmental Protection Agency. Final report: integrated science assessment of ozone and related photochemical oxidants. Washington (DC): United States Environmental Protection Agency; 2013, EPA/600/R-10/076F. Available from: <http://cfpub.epa.gov/ncea/isa/recordisplay.cfm?deid=247492>

- [2] Zmirou D, Gauvin S, Pin I, et al. Traffic related air pollution and incidence of childhood asthma: results of the Vesta case-control study. *J Epidemiol Commun Health*. 2004;58(1):18–23.
- [3] Galán I, Tobías A, Banegas JR, et al. Short-term effects of air pollution on daily asthma emergency room admissions. *Eur Respir J*. 2003;22(5):802–808.
- [4] Ko FW, Tam W, Wong TW, et al. Temporal relationship between air pollutants and hospital admissions for chronic obstructive pulmonary disease in Hong Kong. *Thorax*. 2007;62(9):780–785.
- [5] Blomberg A, Mudway I, Svensson M, et al. Clara cell protein as a biomarker for ozone-induced lung injury in humans. *Eur Respir J*. 2003;22(6):883–888.
- [6] Que LG, Stiles JV, Sundry JS, et al. Pulmonary function, bronchial reactivity, and epithelial permeability are response phenotypes to ozone and develop differentially in healthy humans. *J Appl Physiol* (1985). 2011; 111(3):679–687.
- [7] Zhang P, Li F, Wiegman CH, et al. Inhibitory effect of hydrogen sulfide on ozone-induced airway inflammation, oxidative stress, and bronchial hyperresponsiveness. *Am J Respir Cell Mol Biol*. 2015;52(1):129–137.
- [8] Yang Q, Ge MQ, Kokalari B, et al. Group 2 innate lymphoid cells mediate ozone-induced airway inflammation and hyperresponsiveness in mice. *J Allergy Clin Immunol*. 2016;137(2):571–578.
- [9] Mishra P, Chan DC. Mitochondrial dynamics and inheritance during cell division, development and disease. *Nat Rev Mol Cell Biol*. 2014;15(10):634–646.
- [10] Aravamudan B, Thompson MA, Pabelick CM, et al. Mitochondria in lung diseases. *Exp Rev Respir Med*. 2013;7(6):631–646.
- [11] Schumacker PT, Gillespie MN, Nakahira K, et al. Mitochondria in lung biology and pathology: more than just a powerhouse. *Am J Physiol Lung Cell Mol Physiol*. 2014;306(11):L962–L974.
- [12] Lee S, Suh GY, Ryter SW, et al. Regulation and function of the nucleotide binding domain leucine-rich repeat-containing receptor, pyrin domain-containing-3 inflammasome in lung disease. *Am J Respir Cell Mol Biol*. 2016;54(2):151–160.
- [13] Zhou R, Yazdi AS, Menu P, et al. A role for mitochondria in NLRP3 inflammasome activation. *Nature*. 2011; 469(7329):221–225.
- [14] Nakahira K, Haspel JA, Rathinam VA, et al. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol*. 2011;12(3): 222–230.
- [15] Manzer R, Dinarello CA, McConville G, et al. Ozone exposure of macrophages induces an alveolar epithelial chemokine response through IL-1 $\alpha$ . *Am J Respir Cell Mol Biol*. 2008;38(3):318–323.
- [16] Johnston RA, Mizgerd JP, Flynt L, et al. Type I interleukin-1 receptor is required for pulmonary responses to subacute ozone exposure in mice. *Am J Respir Cell Mol Biol*. 2007;37(4):477–484.
- [17] Gross O, Yazdi AS, Thomas CJ, et al. Inflammasome activators induce interleukin-1 $\alpha$  secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity*. 2012;36(3): 388–400.
- [18] Savage CD, Lopez-Castejon G, Denes A, et al. NLRP3-inflammasome activating DAMPs stimulate an inflammatory response in glia in the absence of priming which contributes to brain inflammation after injury. *Front Immunol*. 2012;3:288.
- [19] Borak J, Lefkowitz RY, Linde B. Bronchial hyperresponsiveness: a technical update. *Occup Med (Lond)*. 2018;68(8):519–522.
- [20] McBrien CN, Menzies-Gow A. The biology of eosinophils and their role in asthma. *Front Med (Lausanne)*. 2017;4:93.
- [21] van den Berge M, Vonk JM, Gosman M, et al. Clinical and inflammatory determinants of bronchial hyperresponsiveness in COPD. *Eur Respir J*. 2012;40(5): 1098–1105.
- [22] Kim SR, Kim DI, Kim SH, et al. NLRP3 inflammasome activation by mitochondrial ROS in bronchial epithelial cells is required for allergic inflammation. *Cell Death Dis*. 2014;5:e1498.
- [23] Holguin F. Oxidative stress in airway diseases. *Ann Am Thorac Soc*. 2013;10(Suppl):S150–S157.
- [24] Thomson NC. Targeting oxidant-dependent mechanisms for the treatment of respiratory diseases and their comorbidities. *Curr Opin Pharmacol*. 2018;40:1–8.
- [25] Li Z, Tighe RM, Feng F, et al. Genes of innate immunity and the biological response to inhaled ozone. *J Biochem Mol Toxicol*. 2013;27(1):3–16.
- [26] Hulo S, Tieset H, Lancel S, et al. AMP-activated protein kinase deficiency reduces ozone-induced lung injury and oxidative stress in mice. *Respir Res*. 2011; 12:64.
- [27] Mailloux RJ, Jin X, Willmore WG. Redox regulation of mitochondrial function with emphasis on cysteine oxidation reactions. *Redox Biol*. 2014;2:123–139.
- [28] Madej P, Plewka A, Madej JA, et al. Ozone therapy in induced endotoxemic shock. II. The effect of ozone therapy upon selected histochemical reactions in organs of rats in endotoxemic shock. *Inflammation*. 2007;30(3–4):69–86.
- [29] Chuang GC, Yang Z, Westbrook DG, et al. Pulmonary ozone exposure induces vascular dysfunction, mitochondrial damage, and atherogenesis. *Am J Physiol Lung Cell Mol Physiol*. 2009;297(2):L209–L216.
- [30] Wiegman CH, Michaeloudes C, Haji G, et al. Oxidative stress-induced mitochondrial dysfunction drives inflammation and airway smooth muscle modeling in patients with chronic obstructive pulmonary disease. *J Allergy Clin Immunol*. 2015;136(3):769–780.
- [31] Yu J, Nagasu H, Murakami T, et al. Inflammasome activation leads to caspase-1-dependent mitochondrial damage and block of mitophagy. *Proc Natl Acad Sci USA*. 2014;111(43):15514–15519.
- [32] Li F, Xu M, Wang M, et al. Roles of mitochondrial ROS and NLRP3 inflammasome in multiple ozone-induced lung inflammation and emphysema. *Respir Res*. 2018; 19(1):230.
- [33] Aravamudan B, Kiel A, Freeman M, et al. Cigarette smoke-induced mitochondrial fragmentation and dysfunction in human airway smooth muscle. *Am*

- J Physiol Lung Cell Mol Physiol. 2014;306(9): L840–L854.
- [34] Wang M, Zhang Y, Xu M, et al. Roles of TRPA1 and TRPV1 in cigarette smoke-induced airway epithelial cell injury model. *Free Radic Biol Med*. 2019;134: 229–238.
- [35] Xu M, Li F, Wang M, et al. Protective effects of VGX-1027 in PM2.5-induced airway inflammation and bronchial hyperresponsiveness. *Eur J Pharmacol*. 2019;842: 373–383.
- [36] Liu X, Chen Z. The pathophysiological role of mitochondrial oxidative stress in lung diseases. *J Transl Med*. 2017;15(1):207.
- [37] Pinkerton JW, Kim RY, Robertson AAB, et al. Inflammasomes in the lung. *Mol Immunol*. 2017;86:44–55.
- [38] Li F, Zhang P, Zhang M, et al. Hydrogen sulfide prevents and partially reverses ozone-induced features of lung inflammation and emphysema in mice. *Am J Respir Cell Mol Biol*. 2016;55(1):72–81.