NLRP3 inflammasome activation by mitochondrial ROS in bronchial epithelial cells is required for allergic inflammation

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Abnormality in mitochondria has been suggested to be associated with development of allergic airway disorders. In this study, to evaluate the relationship between mitochondrial reactive oxygen species (ROS) and NLRP3 inflammasome activation in allergic asthma, we used a newly developed mitochondrial ROS inhibitor, NecroX-5. NecroX-5 reduced the increase of mitochondrial ROS generation in airway inflammatory cells, as well as bronchial epithelial cells, NLRP3 inflammasome activation, the nuclear translocation of nuclear factor-κB, increased expression of various inflammatory mediators and pathophysiological features of allergic asthma in mice. Finally, blockade of IL-1/β substantially reduced airway inflammation and hyperresponsiveness in the asthmatic mice. These findings suggest that mitochondrial ROS have a critical role in the pathogenesis of allergic airway inflammation through the modulation of NLRP3 inflammasome activation, providing a novel role of airway epithelial cells expressing NLRP3 inflammasome as an immune responder.

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There is now substantial evidence that an excess of reactive oxygen species (ROS) has an important role in the pathogenesis of airway inflammation and tissue injury observed in asthma, which consists of epithelial cell damage, cell shedding and airway hyperresponsiveness.¹⁻³ In addition, increased oxidative stress is related to severity of asthma, propagation of inflammatory response and reduction of responsiveness to corticosteroids.⁴ Thus, considerable research efforts have been focused on understanding the mechanism of oxidative stress-mediated airway inflammation and finding better antioxidants.

Mitochondria and the Nox family of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase are the two major sources of ROS that are induced by external stimuli, and the mitochondria respiratory chain is considered an important site of ROS production within most cells.⁵ Mitochondria are dynamic double membrane organelles and possess their own genome and proteome.⁶ They are associated with the synthesis and catabolism of metabolites, generation and detoxification of ROS, apoptosis, regulation of cytoplasmic and mitochondrial matrix calcium and generation of adenosine triphosphate by oxidative phosphorylation.⁷ Recently, apart from these classical functions of mitochondria, a new and exciting role for mitochondria has been revealed in various inflammatory disorders such as infectious diseases, neurodegenerative diseases, cerebrovascular diseases and metabolic diseases,⁸⁻¹⁰ especially in the activation and control of innate immune responses. Moreover, recent studies have suggested that abnormality in mitochondria is associated with development of asthma.¹¹⁻¹² However, the precise role of the excess of mitochondrial ROS generation in the development of allergic airway inflammation is not well understood.

Cellular stress or tissue damage is recognized by the pattern recognition receptors (PRRs) of the innate immune system. The allergens such as dust mite and molds contain protease activity and/or innate PRR ligands for the Toll-like receptor (TLR), C-type lectin receptor, and/or nucleotide-binding domain, leucine-rich repeat-containing protein (NLR) families that facilitate their immunogenicity. Among them, several members of the cytosolic NLR family (NLRP1, NLRP3 and NLRC4) act as central components of the multiprotein inflammasome complex.¹³ A number of studies have shown that NLRP3 inflammasome, which consists of NLRP3, apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC), and caspase-1 is related to mitochondrial dysfunction.¹⁴ Activation of NLRP3 inflammasome can be induced by intracellular ROS generation in response to a variety of cellular stress.¹⁵⁻¹⁷ More interestingly,
studies have demonstrated that NLRP3 inflammasome activation is critical for the induction of allergic airway inflammation in bronchial asthma,\textsuperscript{18,19} with increased understanding of how adaptive and innate immunity generate downstream pathology of allergic inflammation.\textsuperscript{20} In fact, although bronchial asthma has been characterized by Mitochondrial ROS in allergic inflammation

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Results

Total cellular ROS and mitochondrial ROS are increased in cells from lung of mice sensitized with OVA and LPS and challenged with OVA (OVA<sub>LPS</sub>-OVA mice). Intensity of fluorescence was significantly higher in bronchoalveolar lavage (BAL) cells (Figure 1a) and primary cultured tracheal epithelial cells (Figure 1e) from OVA<sub>LPS</sub>-OVA mice than those from control mice. The enhancement of fluorescence intensity in BAL cells or primary cultured tracheal epithelial cells from OVA<sub>LPS</sub>-OVA mice was inhibited by treatment with NecroX-5. Additional FACScan analysis also showed that mitochondrial ROS generation as well as total cellular ROS generation was significantly upregulated in BAL cells (Figures 1b–d) and primary cultured tracheal epithelial cells (Figure 1f) from OVA<sub>LPS</sub>-OVA mice. The increases of ROS generations in both cells from OVA<sub>LPS</sub>-OVA mice were significantly decreased by treatment with NecroX-5.

Changes of mitochondrial DNA (mtDNA) in lung tissues of OVA<sub>LPS</sub>-OVA mice. To assess the presence of mtDNA lesions blocking the progress of polymerases, we performed long PCR experiments to concomitantly amplify a long (8642-bp) and a short (316-bp) mtDNA fragment (Figure 2a). The DNA lesions blocking replication are more likely to be present on a long DNA region than on a short fragment. The amplification of mtDNA showed that level of the long mtDNA fragment was decreased in the lung tissues of OVA<sub>LPS</sub>-OVA mice compared with the level of saline-sensitized and -challenged (SAL-SAL) mice, whereas the short mtDNA fragment was not changed significantly in all group tested (Figure 2a). The decrease in amplified products of the long mtDNA fragment was markedly restored by treatment with NecroX-5. Consistent with these results, the ratio of long mtDNA fragment/short mtDNA fragment as an index of mtDNA integrity was significantly decreased in lung tissues of OVA<sub>LPS</sub>-OVA mice compared with the levels of SAL-SAL mice. Interestingly, the decreased ratio was substantially restored by the treatment with NecroX-5 (Figure 2b). In addition, mtDNA content was also reduced in lung tissues of OVA<sub>LPS</sub>-OVA mice compared with that of SAL-SAL mice. The decreased level of mtDNA was markedly restored to the similar levels of SAL-SAL mice by treatment with NecroX-5 (Figure 2c). These results suggest that mitochondrial ROS can affect the integrity and content of mtDNA in lung tissues of an asthmatic murine model.

NecroX-5 reduces protein levels of NLRP3, caspase-1 and IL-1β in primary cultured tracheal epithelial cells from OVA<sub>LPS</sub>-OVA mice. Western blot analyses revealed that levels of NLRP3, caspase-1 and IL-1β in primary tracheal epithelial cells from OVA<sub>LPS</sub>-OVA mice were increased significantly compared with the levels in the cells from SAL-SAL mice (Figures 3a–c). Treatment with NecroX-5 significantly reduced the increases of NLRP3, caspase-1 and IL-1β levels in primary tracheal epithelial cells from OVA<sub>LPS</sub>-OVA mice.

Mitochondrial ROS inhibitor reduces activation of NLRP3 inflammasome in lung tissues of OVA<sub>LPS</sub>-OVA mice. Western blot analysis revealed that protein levels of NLRP3, caspase-1 and IL-1β, which are the hallmarks of inflamma¬sone activation, in lung tissues of OVA<sub>LPS</sub>-OVA mice were greatly increased at 48 h after the last challenge compared with the levels in SAL-SAL mice (Figures 3d–i). The reduction of mitochondrial ROS by administration of NecroX-5 substantially decreased the expression of these proteins in lung tissues of OVA<sub>LPS</sub>-OVA mice.
In addition, enzyme immunoassay showed that the increased IL-1β levels in BAL fluid from OVA_{LPS-}OVA mice were significantly decreased by administration of NecroX-5 (Figure 3l). To ascertain the observations, the expression level of IL-1β in BAL cells was analyzed by immunofluorescence staining. Immunofluorescence of IL-1β in BAL cells from OVA LPS-OVA mice showed the expression of IL-1β was significantly higher in BAL cells from OVA_{LPS-}OVA mice than those from SAL-SAL mice. The enhancement of fluorescent intensity in BAL cells of OVA_{LPS-}OVA mice was inhibited by administration of NecroX-5 (Figure 3m).

**Activation of NLRP3 inflammasome in asthmatic patients.** Western blot analyses showed that the levels of NLRP3 and caspase-1 in BAL fluids from the patients with asthma were significantly higher than the levels in healthy subjects (Figures 3j and k). They were all male persons and their phenotypes were as follows: mean age: 54.0 ± 2.5 (healthy subjects) and 62.0 ± 1.5 (asthmatic patients) (years, mean ± S.E.M., P = 0.066), mean FEV1 of asthmatic patients: 76.0 ± 16.2% (of predictive value, mean ± S.E.M.).

**Effects of NecroX-5 on cellular changes in BAL fluids from OVA_{LPS-}OVA mice.** Numbers of total cells, lymphocytes, neutrophils and eosinophils in BAL fluids of OVA_{LPS-}OVA mice were increased significantly compared with the numbers of SAL-SAL mice. The increase in numbers of these cells, especially neutrophils, in BAL fluids from OVA_{LPS-}OVA mice was significantly reduced by administration of NecroX-5 (Figure 4a).

**NecroX-5 ameliorates pathologic features of OVA_{LPS-}OVA mice.** Histological assessment showed that numerous inflammatory cells infiltrated into the bronchioles and perivascular regions in the lung of OVA_{LPS-}OVA mice (Figure 4c) compared with the BAL-SAL mice (Figure 4b). The OVA_{LPS-}OVA mice treated with NecroX-5 showed marked reduction in the infiltration of inflammatory cells in the peribronchiolar and perivascular regions of the lung (Figures 4d and e).

**Inhibition of mitochondrial ROS decreases myeloperoxidase (MPO) activity in lung tissues of OVA_{LPS-}OVA mice.** MPO is a major constituent of neutrophil cytoplasmic granules, and its activity therefore is a direct measure of the neutrophil presence. As expected, MPO activity was significantly increased in lung tissues of OVA_{LPS-}OVA mice (Figure 4f). The increase of MPO activity in lung tissues of OVA_{LPS-}OVA mice was substantially reduced by administration of NecroX-5.

**NecroX-5 reduces airway hyperresponsiveness of OVA_{LPS-}OVA mice.** Airway responsiveness was assessed through both noninvasive and invasive measurements. In OVA_{LPS-}OVA mice, the dose-response curve of percent enhanced pause (Penh) or respiratory system resistance (Rrs) shifted to the left compared with that of SAL-SAL mice (Figures 4g and h). Administration of NecroX-5 reduced substantially the Penh or Rrs observed at 50 mg/ml of methacholine in OVA_{LPS-}OVA mice.
These results indicate that inhibition of mitochondrial ROS effectively decreases the allergen-induced airway hyperresponsiveness.

Effects of NecroX-5 on serum level of OVA-specific IgE, IgG1, and IgG2a in OVA, LPS-OVA mice. Enzyme immunoassays showed the significant increases in OVA-specific IgE,
IgG1 and IgG2 levels in the serum of OVA\textsubscript{LPS-OVA} mice compared with the levels in the control mice (Figures 4i–k). The administration of NecroX-5 substantially lowered the serum level of OVA-specific IgE compared with the levels of OVA\textsubscript{LPS-OVA} mice treated with drug vehicle only. In addition, the serum level of OVA-specific IgG1, the Th2-type immunoglobulin was decreased by treatment with NecroX-5, whereas the level of OVA-specific IgG2a was not affected by NecroX-5, although the level of IgG2a showed the increased tendency on the treatment with 30 mg/kg of NecroX-5 in OVA\textsubscript{LPS-OVA} mice.

NecroX-5 lowers the production of inflammatory cytokines in OVA\textsubscript{LPS-OVA} mice. To investigate whether mitochondrial ROS evokes the inflammatory responses in allergic airway inflammation, we examined the effects of NecroX-5 on the levels of inflammatory cytokines in the lung of OVA\textsubscript{LPS-OVA} mice. Western blot analyses showed that levels of IL-4, IL-5, IL-13, TNF-\(\alpha\), IFN-\(\gamma\), IL-17 and KC protein in lung tissues of OVA\textsubscript{LPS-OVA} mice were significantly increased compared with the levels of SAL-SAL mice (Figures 5a–n). Administration of NecroX-5 reduced significantly the OVA-induced increases of IL-4,
NecroX-5 prevents nuclear translocation of NF-κB p65 in lung tissues of OVA LPS-OVA mice. Western blotting data showed that the level of nuclear NF-κB (NF-κB) p65 in lung tissues of OVA LPS-OVA mice was significantly increased compared with the level in SAL-SAL mice (Figures 5o and p). The increase of nuclear NF-κB p65 was significantly reduced by administration of NecroX-5.

NecroX-5 reduces allergic airway inflammation, bronchial hyperresponsiveness, activation of NLRP3 inflammasome and generation of mitochondrial ROS in the lung of house dust mite (HDM)-instilled mice. Numbers of total cells, eosinophils, neutrophils and lymphocytes in BAL fluids of HDM-instilled mice were increased significantly compared with the numbers of saline-instilled mice administered drug vehicle (SV). The increase in the numbers of these cells, especially eosinophils, in BAL fluids from HDM-instilled mice was significantly reduced by administration of NecroX-5 (Figure 6a). Histological assessment showed that numerous inflammatory cells infiltrated into the bronchioles and perivascular regions in the lung of HDM-instilled mice (Figure 6c) compared with the SV mice (Figure 6b). The HDM-instilled mice treated with NecroX-5 showed marked reduction in the infiltration of inflammatory cells in the peribronchiolar and perivascular regions of the lung (Figures 6d and e). Airway responsiveness was assessed by Rrs measurement. In HDM-instilled mice, the methacholine dose-response curve of Rrs shifted to the left compared with that in SV mice (Figure 6f). Administration of NecroX-5 reduced substantially the Rrs observed at 10–50 mg/ml of methacholine in HDM-instilled mice. Western blot analysis revealed that protein levels of NLRP3, caspase-1 and IL-1β in lung tissues of HDM-instilled mice were greatly increased at 48 h after the last challenge compared with the levels in SV mice (Figures 6g–l). The administration of NecroX-5 substantially decreased the expression of these proteins in lung tissues of HDM-instilled mice. Intensity of fluorescence was significantly higher in BAL cells from HDM-stilled mice than those from SV mice. The enhancement of fluorescence intensity in BAL cells from HDM-instilled mice was inhibited by treatment with NecroX-5.

Effects of blockade of IL-1β on cellular changes in BAL fluids, airway hyperresponsiveness and levels of inflammatory cytokines in the lung of OVA_LPS-OVA mice and...
OVA<sub>LPS</sub>-OVA IL-1R knock-out (KO) mice. In order to verify the action mechanism of NecroX-5, that is, whether or not the anti-asthmatic effect of NecroX-5 works via suppression of inflammasome activation, we evaluated the role of IL-1β on changes in BAL cells, histologic features, airway hyperresponsiveness and protein levels of pro-inflammatory cytokines using an anti-IL-1β-neutralizing antibody or IL-1R KO mice. The increased numbers of total cells, lymphocytes, neutrophils and eosinophils in OVA<sub>LPS</sub>-OVA mice were substantially reduced by neutralization of IL-1β with the antibody (Figure 7a). In addition, the OVA<sub>LPS</sub>-OVA mice treated with the anti-IL-1β-neutralizing antibody showed a marked reduction in infiltration of numerous inflammatory cells into peribronchiolar and perivascular regions on histologic examination (Figures 7b–d).
Airway responsiveness assessed by both noninvasive and invasive measurements, a percent increase of Penh or $R_{rs}$ in response to increasing doses of methacholine, was revealed as follows: in OVA_LPS-OVA mice, the percent Penh or $R_{rs}$ produced by methacholine was significantly increased compared with the levels of SAL-SAL mice (Figures 7e and f). Treatment with the anti-IL-1β-neutralizing antibody significantly reduced the Penh and the $R_{rs}$ at 50 mg/ml of methacholine compared with that of OVA_LPS-OVA mice.

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The protein levels of IL-4, IL-5, IL-13, TNF-α, IFN-γ, IL-17 and KC protein in lung tissues of OVA LPS-OVA mice were markedly increased compared with the levels in SAL-SAL mice (Figures 8a–n). Administration of the anti-IL-1β-neutralizing antibody significantly reduced the increases of IL-4, IL-5, IL-13, TNF-α, IFN-γ, IL-17 and KC protein in lung tissues.

Consistent with these findings, OVA LPS-OVA IL-1R KO mice showed the significantly reduced asthmatic manifestations including the number of airway inflammatory cells, pathologic

**Figure 8** Effects of blockade of IL-1β on the protein levels of various inflammatory mediators in lung tissues of OVA LPS-OVA mice and OVA LPS-OVA IL-1R KO mice. Sampling was performed at 48 h after the last challenge in SP OLC-Ab, OLA-IL-1β Ab, WT OL, IL-1R KO CON, and IL-1R KO OL. Representative western blots of IL-4 (a and o), IL-5 (c and q), IL-13 (e and s), TNF-α (g), IFN-γ (i), IL-17 (k and u) and KC (m and w) in lung tissues. (b, d, f, h, j, l, n, p, r, t, v, x). Densitometric analysis of the bands on films is presented as the relative ratio of each protein to actin. The relative ratio of each protein in the lung tissues of SP or WT CON is arbitrarily presented as 1. Bars represent mean ± S.E.M. from five or seven mice per group.*P < 0.05 versus SP or WT CON; #P < 0.05 versus OLC-Ab or WT OL.
changes and airway hyperresponsiveness compared with the levels of OVALPS-OVA wild-type (WT) mice (Figures 7g–l). In addition, the IL-1R KO mice showed the lower levels of pro-inflammatory mediators such as IL-4, IL-5, IL-13, IL-17 and KC in lung tissues than the levels of OVALPS-OVA WT mice after OVA challenges (Figures 8o–x).

These results indicate that blockade of IL-1β action attenuates inflammatory responses and airway hyperresponsiveness in OVALPS-OVA mice.

Discussion

In this study, we demonstrate for the first time the role of mitochondrial ROS in the development of allergic inflammation using an OVA and LPS-induced mouse model of neutrophil-dominant allergic airway disease and HDM-induced allergic asthmatic murine model. Moreover, the molecular bases of action of a novel mitochondrial ROS inhibitor, NecroX-5, were evaluated in this pathologic situation, in which NecroX-5 attenuated the asthmatic features including airway inflammation and hyperresponsiveness through the modulation of NLRP3 inflammasome activation that leads to the production of IL-1β.

It is well known that one of the causative mechanisms of airway inflammation and airway obstruction is oxidative stress or tilt in the delicate balance of the cellular redox state. Moreover, allergen-activated and recruited inflammatory cells such as eosinophils, macrophages, monocytes and neutrophils from asthmatic patients produce more ROS than those from normal subjects do. The constitutive airway cells such as epithelial cells are also a potential source of ROS. In addition to cellular sources of ROS, several asthma mediators including lipid mediators, chemokines, adhesion molecules and eosinophil granule proteins are potential stimuli of ROS production. Thus, antioxidant treatment of allergic asthmatic inflammation has long been a subject of therapeutic strategy. Despite plentiful data from animal studies using antioxidants, previous human studies have yielded disappointing results with the effects of antioxidant supplementation in allergic airway inflammation, such as vitamin C, vitamin E and flavonoids. However, as these compounds do not significantly accumulate within mitochondria, their effectiveness remains limited. Although endogenous sources of intracellular ROS include the NADPH oxidases (NOXs), the Ero1–DPI oxidative folding system in the endoplasmic reticulum, and the mitochondrial electron transport chain, there is no available evidence to date that shows for which source has a major role in the pathogenesis of bronchial asthma. In this study, our results showed that OVA challenge increases cellular ROS generation in BAL cells. More intriguingly, we also found that mitochondrial ROS generation is significantly increased in BAL cells and primary cultured tracheal epithelial cells from OVALPS-OVA mice or HDM-instilled mice and that a ROS inhibitor, NecroX-5, markedly reduces total and mitochondrial ROS generation in the mice. Moreover, our data revealed that enhanced airway hyperresponsiveness and inflammation in OVALPS-OVA mice or HDM-instilled mice were substantially suppressed by administration of NecroX-5. These findings indicate that bronchial asthma can be aggravated, at least in part, by the induction of mitochondrial ROS generation in airway inflammatory cells and epithelial cells and that a novel antioxidant, NecroX-5, works well targeting mitochondrial ROS in airway inflammation.

Inflammasomes are a group of protein complexes that recognize a diverse set of inflammation-inducing stimuli, pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs) and that control production of important pro-inflammatory cytokines such as IL-1β and IL-18. Whereas the absent in melanoma 2 and NLRC4 inflammasomes are activated only by specific PAMPs, double-stranded DNA and specific bacterial proteins, respectively, NLRP3 is activated by a large variety of signals, including PAMPs, DAMPs and bacterial toxins. Two common events that are required for these activators of the NLRP3 inflammasome are potassium efflux and ROS generation. Scavenging of ROS blocks NLRP3 inflammasome activation in response to a wide variety of stimuli. It was initially postulated that the source of ROS is from the phagosome-associated NADPH oxidase. However, a number of subsequent studies have demonstrated that the NADPH oxidase is dispensable for NLRP3 inflammasome activation, suggesting that the source of the ROS may be of mitochondrial origin. In addition, treatment of macrophages with Mito-TEMPO, a scavenger of mitochondrial ROS, resulted in inhibition of NLRP3 inflammasome activation. Furthermore, mtDNA is very sensitive to oxidative stress up to 50-fold more compared with nuclear DNA. Severe damaged and not properly repaired mtDNA are known to be released to cytoplasm from mitochondria, which function as a coactivator in the NLRP3-dependent activation of caspase-1. These damaged mtDNA fragments accumulated in cytoplasm have been called as mitochondrial DAMPs. In our OVALPS-OVA mice or HDM-instilled mice, the protein expression levels of NLRP3 and caspase-1 were significantly increased in lung tissues and the primary cultured tracheal epithelial cells. In addition, active form of IL-1β was highly expressed in lung tissues and primary cultured tracheal epithelial cells, as well as various BAL cells from OVALPS-OVA mice. Furthermore, we revealed that the protein expression levels of NLRP3 and caspase-1 were also increased in BAL fluids from asthmatic patients compared with the levels of healthy subjects. Treatment with NecroX-5 substantially reduced the increases in protein levels of NLRP3, caspase-1 and IL-1β in the lung tissues of OVALPS-OVA mice and HDM-instilled mice. In addition, our data showed that mtDNA was significantly damaged and reduced in lung tissues of OVALPS-OVA mice and that the integrity and content of mtDNA were restored by the treatment with NecroX-5. These results suggest that NLRP3 inflammasome assembly is activated under airway inflammatory conditions, which consequently induces IL-1β production and release. They also suggest that mitochondrial ROS generation and/or the mtDNA damage is closely associated with NLRP3 inflammasome activation in bronchial asthma. In addition, airway epithelial cells can be one of source cells in which the inflammasome activation occurs.

IL-1 family cytokines, such as IL-1α and IL-1β, are involved in multiple aspects of immune and inflammatory responses. There is increasing evidence that IL-1 contributes to allergic
diseases by promoting mast cell activation and Th2 cytokine production. Indeed, elevated levels of IL-1β have been found in the BAL fluid from patients with asthma. Classically, IL-1β is synthesized as a precursor of IL-1β (pro-IL-1β) by blood monocytes, tissue macrophages and dendritic cells after activation by pro-inflammatory stimuli such as IL-1, TNF or TLR ligands and needs to be processed to be active. Pro-IL-1β is cleaved by the protease caspase-1, which is activated through an inflammasome. Importantly, recent studies have revealed that NLRP3 inflammasome activation, leading to IL-1β production, is critical in allergic lung inflammation and that exposure of Nirp3−/− and WT mice to urban particulate matter demonstrates NLRP3-dependent production of IL-1β in the lung and airway neutrophilia. Our current data from OVA-PS-OVA-induced asthmatic mice in vivo and primary cultured epithelial cells in vitro showed that both cleaved caspase-1 and mature IL-1β levels were increased, indicating that NLRP3 inflammasome is functional. We also observed the presence of IL-1β in various BAL cells, in situ positive IL-1β staining from BAL fluid specimens. These results support our contention that NLRP3 inflammasome is active in response to inflammatory stimuli, subsequently producing mature IL-1β in airway inflammatory cells and epithelial cells in a classic manner. In addition, for the confirmation whether the mature IL-1β contributes to the development of asthmatic symptoms, that is, airway inflammation and hyperresponsiveness, we evaluated the effects of blockade of IL-1β on the asthmatic features. Consequently, blockade of IL-1β by treatment with an anti-IL-1β-neutralizing antibody or the use of IL-1R KO mice successfully inhibited the increased airway inflammation and hyperresponsiveness in our current animal model. NF-κB, a multiprotein complex, is involved in the early cellular defense reactions in higher organisms and has a pivotal role in immune and inflammatory responses. Development of oxidant/antioxidant imbalance in asthma leads to the activation of this redox-sensitive transcription factor, NF-κB. ROS have also been directly implicated as second messengers in the activation of NF-κB, based on the ability to activate NF-κB by oxidation of a cysteine-SH group or by ubiquitination and proteolysis of IκB. Consistent with these observations, our results showed that NF-κB levels in nuclear protein extracts from lung tissues are substantially increased in the OVA-PS-OVA-induced neutrophilic allergic airway disease. In addition, the increase of various pro-inflammatory mediators including Th2 cytokines, IL-17, KC, TNF-α and IL-1β was observed in our OVA-PS-OVA-induced murine model of allergic airway disease. Administration of NecroX-5 resulted in a significant reduction of NF-κB translocation into the nucleus, accompanying reduction in the expression of Th2 cytokines, IL-17, KC, TNF-α and IL-1β in the lung of OVA-PS-OVA mice, which are linked to asthmatic symptoms. These results indicate that inhibition of mitochondrial ROS reduces the traits of asthmatic symptoms through modulation of NF-κB activation as well as control of NLRP3 inflammasome.

Conclusively, this study using both murine models of allergic asthma, that is, OVA-PS-OVA mice and HDM-instilled mice suggests that mitochondrial ROS have a critical role in the pathogenesis of allergenic airway inflammation through the modulation of NLRP3 inflammasome activation, especially in bronchial epithelial cells as an immune responder, providing a novel concept of therapeutic strategy for allergen-induced airway disorders.

Materials and Methods

Animals and experimental protocol. Female C57BL/6 mice, 8 to 10 weeks of age and free of murine-specific pathogens, were obtained from the Orient Bio Inc. (Seongnam, Korea). In addition, female C57BL/6 IL-1R KO mice and WT mice, 8 to 10 weeks of age and free of murine-specific pathogens, were obtained from the Jackson Laboratory (Sacramento, CA, USA). They were housed throughout the experiments in a laminar flow cabinet, and were maintained on standard laboratory chow ad libitum. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University. For the neutrophilic asthma model, mice were sensitized intranasally with OVA and LPS (OVA/LPS: 10 μg of OVA combined with 1 μg of LPS in saline; Sigma-Aldrich, St. Louis, MO, USA) on days 1, 2, 3 and 14. On days 21, 22 and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (wt/vol) OVA in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U17, Omron, Kyoto, Japan). In case of HDM-induced asthma models, female C57BL/6 mice (Orient Bio Inc.) were sensitized intracheally with HDM extract (100 μg, Demarphagoids pteronyssius, GREER Laboratories, Lenoir, NC, USA) on days 1 and 7. On day 14 after the initial sensitization, the mice were challenged intracheally with 100 μg of HDM extract in saline (or with saline as a control). BAL was performed at 48 h after the last challenge with OVA or HDM in mice. At the time of lavage, the mice were killed by cervical dislocation. The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter secured with ligatures. Prewarmed 0.9% NaCl solution was slowly instilled into the lung and withdrawn. The collected solutions were pooled and then kept at 4 °C. A part of each pool was then centrifuged, and the supernatants were kept at −70 °C until use. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared by cytospin (Thermo Electron, Waltham, MA, USA) and stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico Inc., Aguada, Puerto Rico) in order to examine cell differentials. Two independent, blinded investigators counted the cells using a microscope. Approximately 400 cells were counted in each of four different random locations. Inter-investigators variation was <5%. The mean number from the two investigators was used to estimate the cell differentials.

Administration of a mitochondrial ROS inhibitor, NecroX-5 and an anti-IL-1β-neutralizing antibody. NecroX-5 (3 or 30 mg/kg body wt/day, Enzo Life Sciences, Farmingdale, NY, USA; Figure 9) diluted with distilled water was administered by intraperitoneal injection two times to each treated animal, once at 1 h before the first challenge with OVA and the second time at 6 h after the last challenge in OVA-PS-OVA mice. Anti-IL-1β-neutralizing antibody or isotype control monoclonal antibody (100 μg/kg body wt per day, eBioscience, San Diego, CA, USA) was administered intravenously two times to each animal, once at 1 h before the first challenge with OVA and the second time at 6 h after the last challenge in OVA-PS-OVA mice.

Measurement of total cellular ROS and mitochondrial ROS. Total cellular ROS were measured as described previously with a modification. To measure intracellular ROS level, cells were incubated for 10 min at room temperature with PBS containing 3,3′,7,7′-dichlorofluorescein-diacetate (DCFDA) and 2′,7′-dichlorofluorescin-diacetate (DCHDA) to form the ROS-sensitive fluorescent product, 2′,7′-dichlorofluorescein (DCF).

Figure 9 Structure of NecroX-5

| Structure of NecroX-5 |
For the measurement of the content of mtDNA, mitochondria were isolated by differential centrifugation in lung tissues of mice. mtDNA was isolated using the Mitochondrial DNA isolation kit (Biovision Inc., Mountain view, CA, USA) and differential centrifugation in lung tissues of mice. mtDNA was isolated using a CellQuest Pro software (LSM 510 META, Carl Zeiss, Jena, Germany).

**Measurement of integrity and content of mtDNA.** First, to perform long PCR experiments, total DNA was extracted from lung tissue samples using the NucleoZOL reagent according to manufacturer's protocol (GibcoLife Technologies, Carlsbad, CA, USA). This long PCR technique is based on the amplification of a long (8642-bp) and a short (316-bp) mtDNA fragment. The primers used were as follows: short mtDNA fragment, sense: 5′-CGACAGCTAGACGCCAACCTGGG-3′; antisense: 5′-CTCATTTTCTCCATTTTGTGGTGC-3′; and long mtDNA fragment, sense: 5′-TACAGTCGCGGACCTTTCAAGGAC-3′; antisense: 5′-GGGTGATCTTGTGTTCCGGGT-3′. PCR reactions were performed in a thermocycler (GeneAmps PCR System 2400, Applied Biosystems, Foster City, CA, USA). The thermocycler profile for short mtDNA fragment included initial denaturation at 94 °C for 2 min, denaturation at 95 °C for 45 s, annealing at 61 °C for 10 s and extension at 72 °C for 8 min, and final extension at 72 °C for 7 min. For long mtDNA fragment, the profile is follows: initial denaturation at 75 °C for 2 min and 94 °C and 1 min, denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s and extension at 65 °C for 12 min, and final extension at 72 °C for 10 min. Amplified PCR products were electrophoresed using 1.6% agarose gels stained with ethidium bromide. DNA bands were visualized using Fuji film LAS-3000 (Fuji film, Tokyo, Japan) under ultraviolet transillumination.

For the measurement of the content of mtDNA, mitochondria were isolated by differential centrifugation in lung tissues of mice, mtDNA was isolated using the Mitochondrial DNA isolation kit (Biovision Inc., Mountain view, CA, USA) and dissolved in a Tris-EDTA (10 mmol/l Tris pH 8.0 and 1 mmol/l EDTA) buffer. Samples were quantified to a final concentration of 1 ng/μl. Protein levels were determined using Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA).

**BAL procedure.** Three asthmatic patients and three healthy subjects were pretreated intramuscularly with atropine (0.5 mg), and 4% lidocaine was used as a local anesthetic immediately before the BAL procedure, which was performed in the segmental bronchus of the right middle lobe. Fifty milliliters of warm 0.9% NaCl solution was instilled into the bronchial tree five times, followed by gentle suction with a negative pressure 80–100 mm Hg using a flexible fiberoptic bronchoscope (Olympus BF-260; Olympus Optical Co. Ltd, Tokyo, Japan). No complications occurred during the BAL procedures. The total volume of recovered fluids was measured. The aspirated fluid was collected into sterile siliconized containers at 4 °C and then filtered through sterile gauze and separated into its fluid and cellular components by centrifugation at 400 × g for 10 min at 4 °C. The study was approved by Institutional Review Board of Choukoub National University Hospital (IRB file No. 2009-02-01).

**Western blot analysis.** Lung tissues or BAL cells were homogenized in the presence of protease inhibitor cocktail (Sigma-Alrich), and protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories). Samples were loaded onto a SDS-PAGE gel. After electrophoresis at 120 V for 90 min, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories) at 250 mA for 90 min by a wet transfer method. Non-specific sites were blocked with 5% non-fat dry milk in Tris-buffered saline Tween 20 (25 mmol/l Tris pH 7.5, 150 mmol/l NaCl, 0.1% Tween 20) for 1 h, and the blots were then incubated overnight at 4 °C with an anti-NLRP3 antibody (Adipogen, San Diego, CA, USA), anti-caspase-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IL-4 antibody (Serotec Ltd, Oxford, UK), anti-IL-5 antibody (Santa Cruz Biotechnology), anti-IL-13 antibody (R&D Systems, Minneapolis, MN, USA), anti-IL-17 antibody (R&D Systems), anti-KC antibody (BioVision, Mountain View, CA, USA), anti-IFN-γ antibody (Santa Cruz Biotechnology), anti-IL-1β antibody (Thermo Scientific, Waltham, MA, USA), anti-TNF-α antibody (Thermo Scientific) and anti-actin antibody (Sigma-Alrich). Antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. For histological examination, 4-μm sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica Microsystems Nussloch GmbH, Wetzlar, Germany), placed on glass slides, deparaffinized and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI, USA). Stained slides were analyzed using a light microscope (Axio Imager M1, Carl Zeiss) under identical conditions, including magnification (×20), gain, camera position, and background illumination.51

**Measurement of IL-1β in BAL fluids.** Levels of IL-1β in BAL fluids were quantified by enzyme immunoassay according to the manufacturer's protocol (R&D Systems). Sensitivity for IL-1β assays was 3.0 pg/ml.

**Measurement of OVA-specific IgE, IgG1 and IgG2a in serum.** Levels of OVA-specific IgE, IgG1 and IgG2a in serum were quantified by enzyme immunoassay according to the manufacturer's protocol (eBioscience). Sensitivities for IgE, IgG1 and IgG2a assays were 4.0, 3.13 and 3.90 ng/ml, respectively.

**Immunofluorescence staining for IL-1β.** Smeared BAL cells were fixed with ice cold methanol, permeabilized in PBS containing 0.25% Triton X-100 for 10 min at room temperature and washed three times with PBS. Subsequently, after antigen retrieval for 15 min at 37 °C in proteinase K (Dako, Glostrup, Denmark), non-specific bindings were blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS containing 0.05% Tween 20 (0.05% BSA-T) for 1 h. Specimens were then incubated in a humidified chamber for 2 h at room temperature with an anti-IL-1β antibody (Santa Cruz Biotechnology). For the detection of binding antibody to IL-1β, Alexa Fluor 488 (green) labeled donkey anti-goat IgG (Invitrogen) in 1% BSA were loaded for 1 h at room temperature in the dark. After the specimens were washed, nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Stained cells were mounted on slides using fluorescent mounting medium (Golden Bridge International, Inc., Mukilteo, WA, USA) and visualized using a confocal microscope (Zeiss LSM 510 Meta, Carl Zeiss) equipped with a C-Apochromat 63 ×/1.20 W Korr UV-VIS-IR M27 water immersion objective. Phase contrast microscopy of each group was used for morphological analysis of BAL cells.

**MPO assay.** MPO was extracted from each homogenized lung tissue sample by suspending the sample in 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, MO, USA) in 50 mmol/l potassium phosphate buffer, pH 6.0, before sonication in an ice bath for 20 s. The samples were freeze-thawed two times, after which sonication was repeated. Suspensions were then centrifuged at 13,200 r.p.m. for 15 min, and the supernatant was assayed. MPO activity was determined by mixing 1 μl of supernatant with 299 μl of the above potassium phosphate buffer containing 0.167 mg/ml diiodosalicylic acid (Sigma Chemical Co.) and 0.0005% hydrogen peroxide (Sigma Chemical Co.). The change in absorbance at 450 nm was measured. MPO activity was then determined from the observed change in absorbance per minute. The activity was expressed as the observed change in absorbance per minute per milligram of protein.

**Isolation and primary culture of murine tracheal epithelial cells.** Murine tracheal epithelial cells were isolated under sterile conditions as described previously.52 The epithelial cells were seeded onto 80-mm collagen-coated dishes for submerged culture. The growth medium, DMEM (Invitrogen), containing 10% fetal bovine serum, penicillin, streptomycin and amphotericin B was used.
supplemented with insulin, transferrin, hydrocortisone, phosphoethanolamine, cholela toxin, ethanolamine, bovine pituitary extract and BSA. The cells were maintained in a humidified 5% CO2 incubator at 37 °C until they adhered.

**NecroX-5 treatment on murine tracheal epithelial cells from OVA{sub}-OVA mice.** Cells were seeded in culture dishes and grown until 70% confluence. The medium was then replaced with a new medium containing vehicle (DMSO) or NecroX-5 (10 μM, Enzo Life Science) and incubated overnight at 37 °C.25

**Cytosolic or nuclear protein extractions.** Lungs were removed and homogenized in two volumes of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.5 mM DTI, 5 mM MgCl2 and 1 mM PMSF) containing protease inhibitor cocktails. The homogenates were centrifuged at 1000 × g for 15 min at 4 °C. The supernatants collected were incubated on ice for 10 min and centrifuged at 100 000 × g for 1 h at 4 °C to obtain cytosolic proteins for analysis of NF-κB p65. The pellets were washed twice in buffer A, resuspended in buffer B (13.2 mM sucrose, 1.0 mM MgCl2, and 10 mM potassium phosphate buffer, pH 6.8) and then pelleted at 1000 × g for 15 min. The pellets were suspended in buffer B with a final sucrose concentration of 2.2 mol/l and centrifuged at 100 000 × g for 1 h. The resulting pellets were washed once with a solution containing 0.25 mol/l sucrose, 0.5 mM MgCl2, and 20 mM Tris-HCl, pH 7.2, and centrifuged at 1000 × g for 10 min. The pellets were solubilized with a solution containing 50 mM Tris-HCl (pH 7.2), 0.3 M sucrose, 150 mM NaCl, 2 mM EDTA, 20% glycerol, 2% Triton X-100, 2 mM PMSF and protease inhibitor cocktail. The mixture was kept on ice for 1 h with gentle stirring and centrifuged at 12 000 × g for 30 min. The resulting supernatant was used as soluble nuclear proteins for analysis of NF-κB p65. The protein levels were analyzed by western blotting using anti-NF-κB p65 antibody (Upstate Biotech, Lake Placid, NY, USA) as described above.

**Determination of airway responsiveness to methacholine.** Both noninvasive and invasive measurements of airway responsiveness were used in this study. For noninvasive measurement, airway responsiveness was assessed in unrestrained and conscious mice at 48 h after the last challenge with OVA, as described previously.25 Mice were placed in a barometric plethysmographic chamber (All Medicus Co., Seoul, Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine in increasing concentrations (2.5–50 mg/ml) were nebulized into an inlet of the main chamber for 3 min. Readings were taken and averaged for 3 min after each nebulization. Penh, calculated as (expiratory time/relaxation time–1) × (peak expiratory flow/peak inspiratory flow), according to the manufacturers’ protocol, is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. Penh was used as a measure of airway resistance to methacholine. Results were expressed as the percentage increase of Penh following challenge with each concentration of methacholine, where the baseline Penh (after saline challenge) was expressed as 100%. Penh values averaged for 3 min after each nebulization were evaluated.

Invasive measurement of airway responsiveness was performed as described elsewhere.26,27 Anesthesia was achieved through intraperitoneal injection of 45 mg/kg body weight of sodium pentobarbital. The trachea was then exposed through a midcervical incision, tracheostomized and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small animal ventilator (flexiVent, Scireq, Montreal, Canada). The mouse was quasi-sinusoidally ventilated with nominal tidal volume of 10 ml/kg body weight at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H2O to achieve a mean lung volume close to that during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to water column. Methacholine aerosol was generated with an in-line nebulizer and administered directly through the ventilator. To determine the differences in airway response to methacholine, each mouse was challenged with methacholine aerosol in increasing concentrations (5–50 mg/ml in saline). After each methacholine challenge, the data of calculated Rn were continuously collected. Maximum values of Rn were selected to express changes in airway function, which was represented as a percentage change from the baseline after saline aerosol.

**Conflict of Interest**

The authors declare no conflict of interest.

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**Author contributions**

SR Kim designed research, interpreted data and wrote manuscript; DI Kim performed experiments and analyzed data; SH Kim reviewed the manuscript; H Lee analyzed data and reviewed the manuscript; KS Lee performed experiments; SH Cho reviewed the manuscript; YC Lee designed research, interpreted data and edited the manuscript.

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