



Mitochondrial reactive oxygen species regulate fungal protease-induced inflammatory responses

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ABSTRACT

Epidemiological studies have shown that fungal infections are a main cause of respiratory tract diseases, such as asthma, bronchopneumonia, intoxication, and invasive fungal disease. Fungi such as *Aspergillus* and *Candida* species have become increasingly important pathogens as the global climate changes. Accordingly, in this study, we evaluated the toxicological potential of *Aspergillus* protease in the lower respiratory tract. Exposure of *Aspergillus* protease to A549 cells induced upregulation of tumor necrosis factor (TNF)- α , monocyte chemoattractant protein (MCP)-1, and intercellular adhesion molecule (ICAM)-1 mRNAs and increased production of interleukin (IL)-8 and MCP-1 protein through enhanced mitochondrial reactive oxygen species (ROS) generation and activation of mitogen-activated protein kinase (MAPK) and activator protein (AP)-1. Furthermore, the mitochondrial ROS scavenger Mito-TEMPO, which inhibited MAPK and AP-1, significantly reduced MCP-1 and IL-1 β mRNA expression and reduced HL-60 cell migration through the suppression of MCP-1 and IL-8 protein secretion. Thus, our results demonstrated that mitochondria were an important source of *Aspergillus* protease-stimulated ROS and that regulation of mitochondrial ROS modulated inflammatory responses by preventing activation of MAPK and AP-1 in A549 cells.

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1. Introduction

Exposure to airborne fungi is known to contribute to respiratory tract diseases, such as asthma, bronchitis, bronchopneumonia, intoxication, and invasive fungal disease (Dorko et al., 2002; Knutsen et al., 2012). Moreover, fungal sensitization may cause the progression of airway disease symptoms to mortality. Importantly, fungi, such as *Aspergillus* and *Candida* species, have become increasingly important pathogens owing to global climate change (Knutsen et al., 2012; Lass-Flörl, 2009). *Aspergillus* species are major indoor fungi and have enzymes that contribute to the pathogenesis of *Aspergillus*-induced diseases (Knutsen et al., 2012). Additionally, *Aspergillus* protease can cause invasive pulmonary aspergillosis, allergic bronchopulmonary aspergillosis, hypersensitivity pneumonitis, and atopic asthma (Chotirmall et al., 2013), which are involved in the complex interplay between airway epithelium

and host immune responses against inhaled spores. However, the pathogenic mechanisms of *Aspergillus* within the airway are poorly understood (Chotirmall et al., 2013).

The airway epithelium is the primary cellular surface encountering inhaled pathogens and functions as a physical barrier to outer pathogens and in the initiation and maintenance of innate and adaptive immune responses (Bals and Hiemstra 2004; Schleimer et al., 2007). In response to pathogens such as fungi, airway epithelial cells secrete various inflammatory mediators, including cytokines (tumor necrosis factor [TNF]- α , interleukin [IL]-1 β , IL-6, IL-8, IL-25, IL-33, thymic stromal lymphopoietin [TSLP], and granulocyte macrophage colony-stimulating factor [GM-CSF]) and chemokines (eosinophil chemotactic protein [Eotaxin], regulated on activation, normal T cell expressed and secreted [RANTES], thymus and activation-regulated chemokine [TARC], monocyte-chemotactic protein [MCP]-3, and MCP-1), which stimulate the recruitment and activation of T cells, basophils, eosinophils, monocytes, dendritic cells, and macrophages (Bloemen et al., 2007; Brabcova et al., 2014; Fritz et al., 2008; Kim et al., 2008).

Reactive oxygen species (ROS) are constantly generated under physiological conditions but also contribute to pathological conditions, such as cancer, cardiovascular diseases, autoimmune

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diseases, and allergic diseases (Li et al., 2013). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, uncoupling nitric oxide synthase, cytochrome P450, and mitochondrial electron transport chain are known to be major sources of ROS (Li et al., 2013). Although ROS are produced by several sources, mitochondrial ROS are increasingly important because they directly influence the production of inflammatory cytokines and innate immune responses by activation of mitogen-activated protein kinase (MAPK), inflammasomes, and retinoic-acid-inducible gene (RIC)-I-like receptors (West et al., 2011; Wiegman et al., 2015; Zhou et al., 2011).

Although *Aspergillus* protease is known to cause epithelial cell shrinkage, degrading tight junction, and injuring epithelial cells through protease-activated receptor, the mechanisms of inflammation in airway epithelium after the infection process are poorly understood (Denning et al., 2006). In addition, although fungi inhaled into lung alveoli may cause life-threatening diseases, relatively few studies have evaluated airway epithelial responses involved in fungal infection (Osherov, 2012). Thus, we hypothesized that fungal protease might cause inflammation of airway epithelium through signaling involved with mitochondrial ROS which is known to be related with airway inflammatory responses by various pathogens in early stage of airway inflammation.

The present study aimed to investigate the effects of *Aspergillus* protease on airway inflammation using the type II alveolar epithelial cell line A549 and evaluated the inflammatory responses mediated by mitochondrial ROS in these cells. Our results showed that the transcription factor activator protein (AP)-1 was activated during *Aspergillus* protease-associated mitochondrial ROS induction in A549 cells. Furthermore, this *Aspergillus* protease-induced AP-1 activation was mediated by phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). Therefore, our findings provided an important understanding into the molecular mechanisms of respiratory inflammation mediated by fungal-derived *Aspergillus* protease.

2. Materials and methods

2.1. Cell culture

A549 cells, human type II-like lung epithelial cells, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in RPMI medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco BRL), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Rockville, MD, USA). The human promyeloblast cell line HL-60 was purchased from ATCC and maintained in RPMI medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were differentiated into the granulocyte lineage with 1.3% dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA) for 5 days. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

2.2. *Aspergillus* protease and Mito-TEMPO treatment

Aspergillus protease solution (Cat. # P6110, Lot # SLBP2675 V) was purchased from Sigma and was freshly diluted with serum-free RPMI media for immediate use. *Aspergillus* protease was used at concentrations ranging from 10 to 1000 ng/mL. The concentration of *Aspergillus* protease was determined based on in vivo experiments and the amount of household fungi, which was measured in other studies (Kheradmand et al., 2002; Lee et al., 2008; Porter et al., 2009). No live organisms were detected on our own microscopic analysis when the cells with *Aspergillus* protease solution were cultured. *Aspergillus* protease was tested at concentrations

ranging from 10 to 1000 ng/mL. The enzyme activity of *Aspergillus* protease was 0.5 PU mg⁻¹ according to the data sheet of the manufacturer. Mito-TEMPO (Sigma) was dissolved in DMSO (Sigma) to generate 5 mM stock solutions and diluted in cell culture medium at different concentrations for immediate use.

2.3. MTS assays for analysis of cell viability and cellular ROS detection assays

A549 cells were plated at a density of 1×10^4 cells/well in flat-bottomed 96-well plates containing RPMI complete media. The cells were incubated for 24 h at 37 °C under a humidified atmosphere of 95% air and 5% CO₂. After incubation, cells were treated with different concentrations of *Aspergillus* protease diluted in RPMI media. After another 1 h of incubation, cell viability was determined by reduction of MTS (Promega, Madison, WI, USA) to its formazan product according to manufacturer's instructions. Briefly, after removing the *Aspergillus* protease-stimulated medium, 200 µL RPMI with MTS solution was added to each well, and plates were reacted at 37 °C for 4 h. The absorbance of the reaction at 490 nm was determined using a microplate fluorimeter (Molecular Devices, Sunnyvale, CA, USA).

For DCFDA assays (Abcam, Cambridge, UK), after stimulation with varying concentrations of *Aspergillus* protease for 1 h, cells were washed and treated with 25 µM DCFDA at 37 °C for 35 min. After another wash with phosphate-buffered saline (PBS), the levels of DCFDA were analyzed with a microplate fluorimeter (Molecular Devices).

2.4. MCP-1 and IL-8 cytokine bead assays (CBAs)

Cells were plated at a density of 0.5×10^6 cells/well in flat-bottomed 6-well plates (Corning, Corning, NY, USA). Upon reaching confluence, the medium was changed to serum-free RPMI, and the plates were incubated for an additional 24 h. The cells were stimulated with *Aspergillus* protease in presence or absence of a 1-h pretreatment with Mito-TEMPO. The culture supernatants were collected and quantified using human CBA kits (BD Bioscience, Franklin Lakes, NJ, USA) according to the manufacturer's instructions.

2.5. RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cultured cells using a Qiagen RNA extraction kit (Qiagen, Hilden, Germany). The concentration and quality of the RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was synthesized using a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR was performed using an ABI 7300 sequence detection system employing premade TaqMan probes (Thermo Fisher Scientific).

2.6. Immunofluorescence confocal microscopy

A549 cells were cultured on glass-bottomed dishes (Thermo Fisher Scientific) and treated with different concentrations of *Aspergillus* protease for 1 h with or without a 1-h pretreatment with Mito-TEMPO. After treatment, the cells were fixed in 4% paraformaldehyde/PBS for 20 min and washed with PBS containing 0.1% Tween-20. Cells were then permeabilized with 0.1% Triton X-100/PBS for 15 min, washed again, and incubated with MitoSOX (Thermo Fisher Scientific) at 37 °C for 30 min. The cells were then washed again with PBST and mounted with PBS containing DRAQ5

(Cell Signaling Technology, Danvers, MA, USA). After a final incubation for 10 min, the cells were analyzed using an FV10i confocal microscope (Olympus, Tokyo, Japan).

2.7. Western blot analysis

After treatment with *Aspergillus* protease and washing with PBS, A549 cells were lysed in RIPA buffer (LPS Solution, Daejeon, Korea). The cell lysates (25 µg/lane) were separated by electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with blocking solution for 50 min at 23 °C. After blocking, the membranes were incubated with rabbit anti-JNK, anti-phospho (p)-JNK, anti-ERK1/2, anti-p-ERK1/2, anti-c-FOS, anti-c-JUN, and anti-β-actin antibodies (Cell Signaling Technology; 1:1000 dilution) with gentle agitation overnight at 4 °C. After incubation with the primary antibodies, the membranes were washed three times with TBST and incubated with biotinylated goat anti-rabbit IgG antibodies (Cell Signaling) for 2 h at room temperature. The membranes were washed three times with the TBST, developed using Clarity Western ECL Blotting Substrate (Bio-Rad, Hercules, CA, USA), and visualized using an ImageQuant LAS 4000 mini (GE Healthcare Life Science, Chicago, IL, USA).

2.8. Chemotaxis assay

After incubation with serum-free medium with 0.2% bovine serum albumin for 24 h, A549 cells were stimulated with *Aspergillus* protease for 1 h. The supernatants were collected for chemotaxis assays using a QCM Chemotaxis 24-well (5 µM) Cell Migration Kit (Millipore, Billerica, MA, USA). HL-60 cells at a density of 2×10^4 cells/well were seeded in the upper wells of the kit, and culture supernatants were added to the lower wells. Following incubation for 18 h, medium containing migrated cells in lower wells and detached cells from the bottom of the upper wells was dissolved in lysis buffer with dye. These medium samples were transferred to 96-well plates, and the optimal density was measured using a microplate reader at 560 nm (Molecular Devices).

2.9. Statistical analysis

Statistical analysis was conducted using Prism 6.0 software with one-way analysis of variance (ANOVA) for multiple comparisons (Graphic Pad Software Inc., San Diego, CA, USA). The results are shown as the means ± standard errors of the means (SEMs) of three independent experiments. In all cases, differences with *P* values of less than 0.05 were considered significant.

3. Results

3.1. Effects of *Aspergillus* protease on intracellular ROS production

Prior to evaluating the effects of *Aspergillus* protease on airway epithelial cells, we first examined the concentration-dependent cytotoxic effects of *Aspergillus* protease on A549 cells using MTS assays. Cell viability was not affected except when 1000 ng/mL *Aspergillus* protease was used after 1 h of treatment (Fig. 1A). Next, we examined the levels of *Aspergillus* protease-induced intracellular ROS production by staining cells with DCFDA. Intracellular ROS levels increased in response to *Aspergillus* protease treatment in a concentration-dependent manner (Fig. 1B). Although intracellular ROS levels were highest when *Aspergillus* protease was used at a concentration of 1000 ng/mL, cytotoxicity was significant; thus, we treated cells with 10 and 100 ng/mL *Aspergillus* protease in subsequent experiments.

3.2. Effects of *Aspergillus* protease on the production of inflammatory mediators

To investigate the effects of *Aspergillus* protease on inflammatory responses, the production of IL-1β, TNF-α, IL-8, ICAM-1, and MCP-1 mRNAs was assessed in *Aspergillus* protease-stimulated A549 cells. After stimulation with *Aspergillus* protease (10 and 100 ng/mL), A549 cells exhibited increased expression of TNF-α, MCP-1, and ICAM-1 mRNAs. Moreover, IL-1β gene expression was increased; however, this difference was not significant (Fig. 2A), and IL-8 mRNA levels were not changed (data not shown). Next, we quantified the protein expression of IL-1β, TNF-α, IL-8, and MCP-1. Although the levels of IL-1β and TNF-α were not changed (data not shown), MCP-1 and IL-8 production was significantly increased with 100 ng/mL *Aspergillus* protease treatment (Fig. 2B). In addition, eotaxin, MARC, and TARC levels were not changed (data not shown). Therefore, these results indicated that *Aspergillus* protease induced inflammatory mediator expression from the airway epithelium.

3.3. Effects of *Aspergillus* protease on the induction of mitochondrial ROS and activation of JNK, ERK, and AP-1

Because *Aspergillus* protease mediated the induction of inflammatory mediators by lung epithelial cells, we attempted to elucidate the mechanisms underlying these inflammatory responses. Because mitochondrial dysfunction is known to be related to airway inflammation (Aguilera-Aguirre et al., 2009),

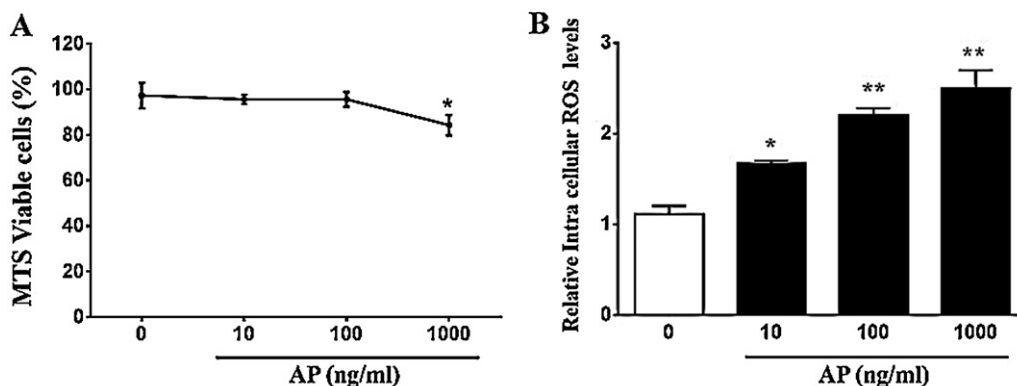


Fig. 1. Effects of *Aspergillus* protease on cell viability and intracellular ROS production. A. The viability of A549 cells was assessed after treatment with various concentrations of *Aspergillus* protease for 1 h. B. The level of intracellular ROS in *Aspergillus* protease-treated cells was evaluated using DCFDA and fluorescence spectroscopy. **P* < 0.05 and ***P* < 0.01 compared with the control (0) group.

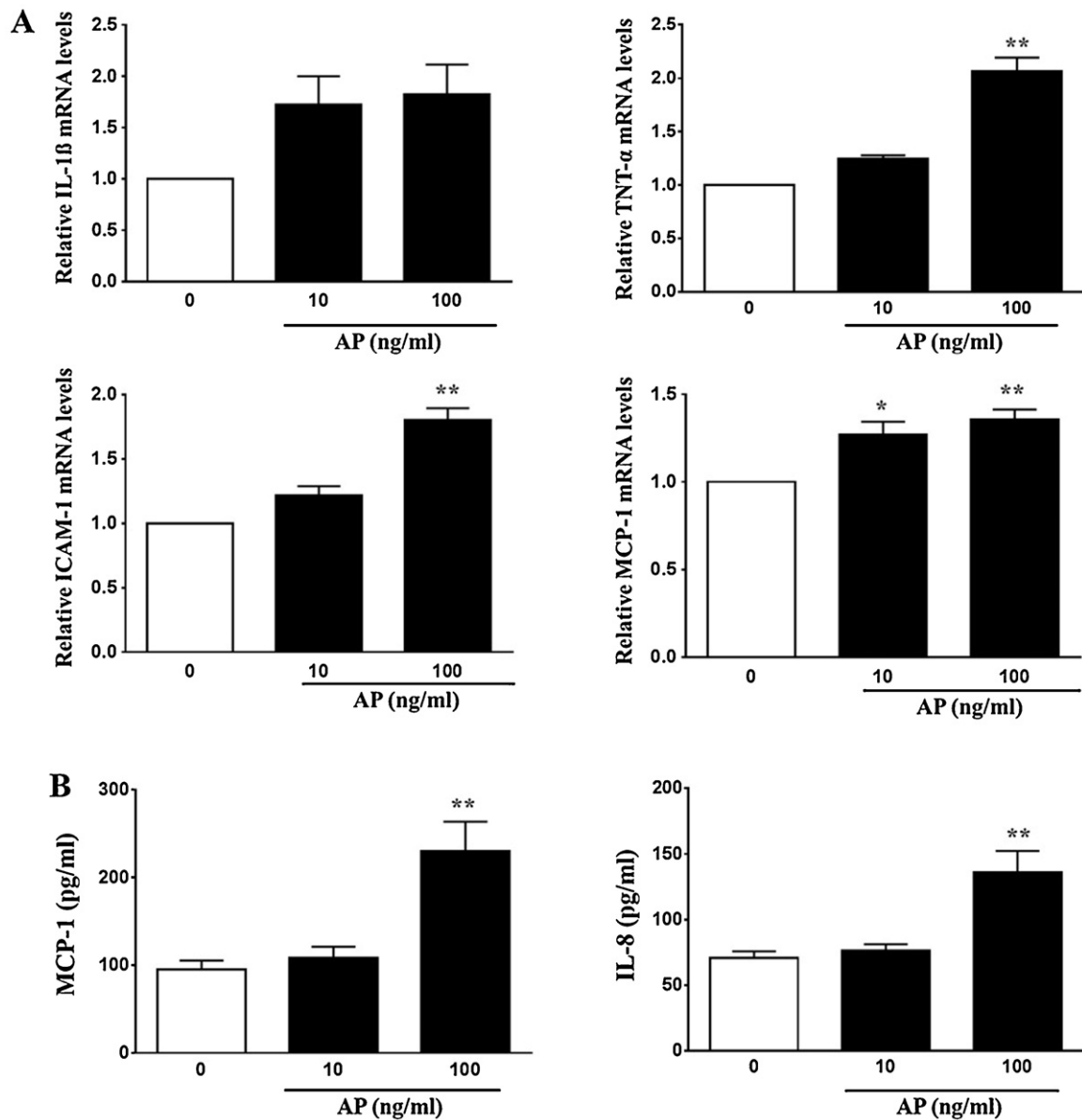


Fig. 2. Effects of *Aspergillus* protease on the production of inflammatory mediators. A. The levels of IL-1 β , TNF- α , ICAM-1, and MCP-1 mRNA expression in A549 cells treated with *Aspergillus* protease for 1 h were measured. The expression levels were normalized to the expression of β -actin mRNA as a control (0: control group; without *Aspergillus* protease treatment). B. IL-8 and MCP-1 cytokine levels were quantified with CBA after treatment with *Aspergillus* protease. * $P < 0.05$ and ** $P < 0.01$ compared with the control (0) group.

we investigated the levels of mitochondrial ROS production following *Aspergillus* protease stimulation. *Aspergillus* protease-treated A549 cells produced higher levels of mitochondrial ROS in a concentration-dependent manner (Fig. 3A). Additionally, because mitochondrial ROS function as second messengers for intracellular signaling cascades and AP-1 and MAPKs, such as p38, ERK1/2, and JNK, regulate the expression of inflammatory mediators and cytokines during inflammatory responses (Hess et al., 2004; Kaminska 2005), we investigated whether *Aspergillus* protease mediated mitochondrial ROS production and MAPK and AP-1 activation. As shown in Fig. 3B, *Aspergillus* protease increased the phosphorylation of JNK and ERK and enhanced the expression of AP-1. Taken together, these data indicated that *Aspergillus* protease induced mitochondrial ROS and MAPK activation.

3.4. *Aspergillus* protease-induced mitochondrial ROS modulated inflammatory mediator production in A549 cells

We showed that *Aspergillus* protease treatment induced the production of inflammatory mediators and activated mitochondrial ROS and MAPK in A549 cells. To elucidate whether mitochondrial ROS directly induced the *Aspergillus* protease-mediated increase in inflammatory mediators, we scavenged mitochondrial ROS using Mito-TEMPO during *Aspergillus* protease treatment; Mito-TEMPO targets a mitochondrial superoxide dismutase (SOD) and has been used for the analysis of mitochondrial ROS (Trnka et al., 2009). First, we treated cells with various concentrations of Mito-TEMPO (20, 50, 100, and 200 μ M) and found that 100 and 200 μ M Mito-TEMPO scavenged ROS without exerting cytotoxic effects (data not shown). We next assessed the levels of mitochondrial ROS

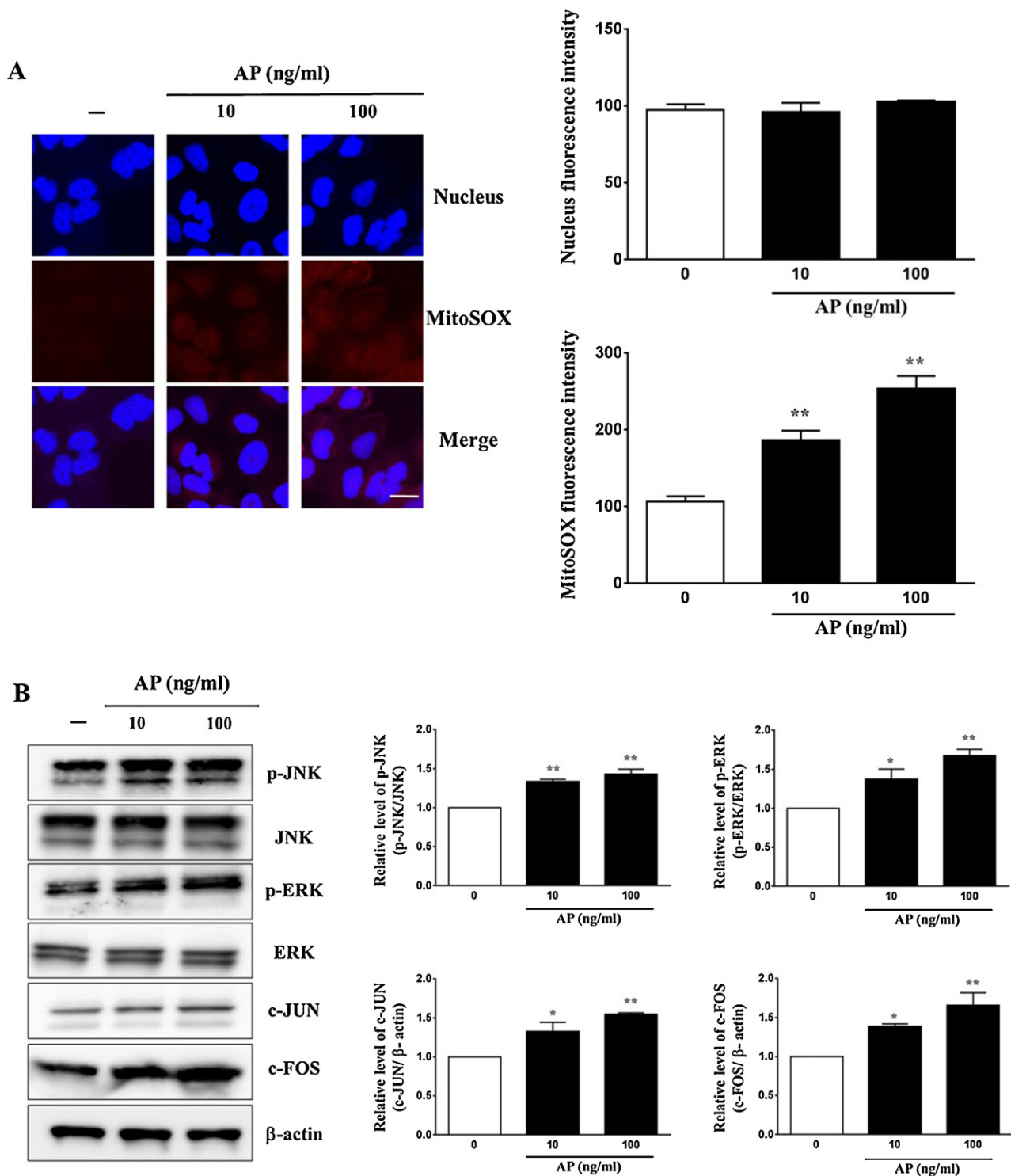


Fig. 3. Effects of *Aspergillus* protease on the induction of mitochondrial ROS and activation of JNK, ERK1/2 and AP-1. A. A549 cells were incubated with a mitochondrial indicator, MitoSOX and analyzed with confocal microscopy (magnification 60×2.5 , scale bar = $20 \mu\text{m}$) after stimulation with *Aspergillus* protease. Images were quantified using ImageJ. B. Phosphorylation of JNK and ERK1/2 and expression of c-FOS and c-JUN were determined by western blot analysis after stimulation of A549 cells with *Aspergillus* protease. Images were acquired using LAS and quantified with ImageJ. β -Actin was used as an internal control. ** $P < 0.05$ and *** $P < 0.01$ compared with the control (0) group.

in *Aspergillus* protease-stimulated A549 cells after pretreatment with Mito-TEMPO for 1 h. The levels of mitochondrial ROS were decreased by pretreatment with Mito-TEMPO compared with that in cells treated with *Aspergillus* protease only (Fig. 4A).

Next, we evaluated the effects of mitochondrial ROS scavenging on inflammatory mediators. In the presence of Mito-TEMPO,

production of IL-1 β and MCP-1 mRNA was significantly reduced, and ICAM-1 and TNF- α mRNA levels were also decreased, although the differences were not significant (Fig. 4B). In addition, secretion of IL-8 and MCP-1 protein was significantly diminished by Mito-TEMPO pretreatment (Fig. 4C). Taken together, these data suggested that mitochondrial ROS induction by *Aspergillus*

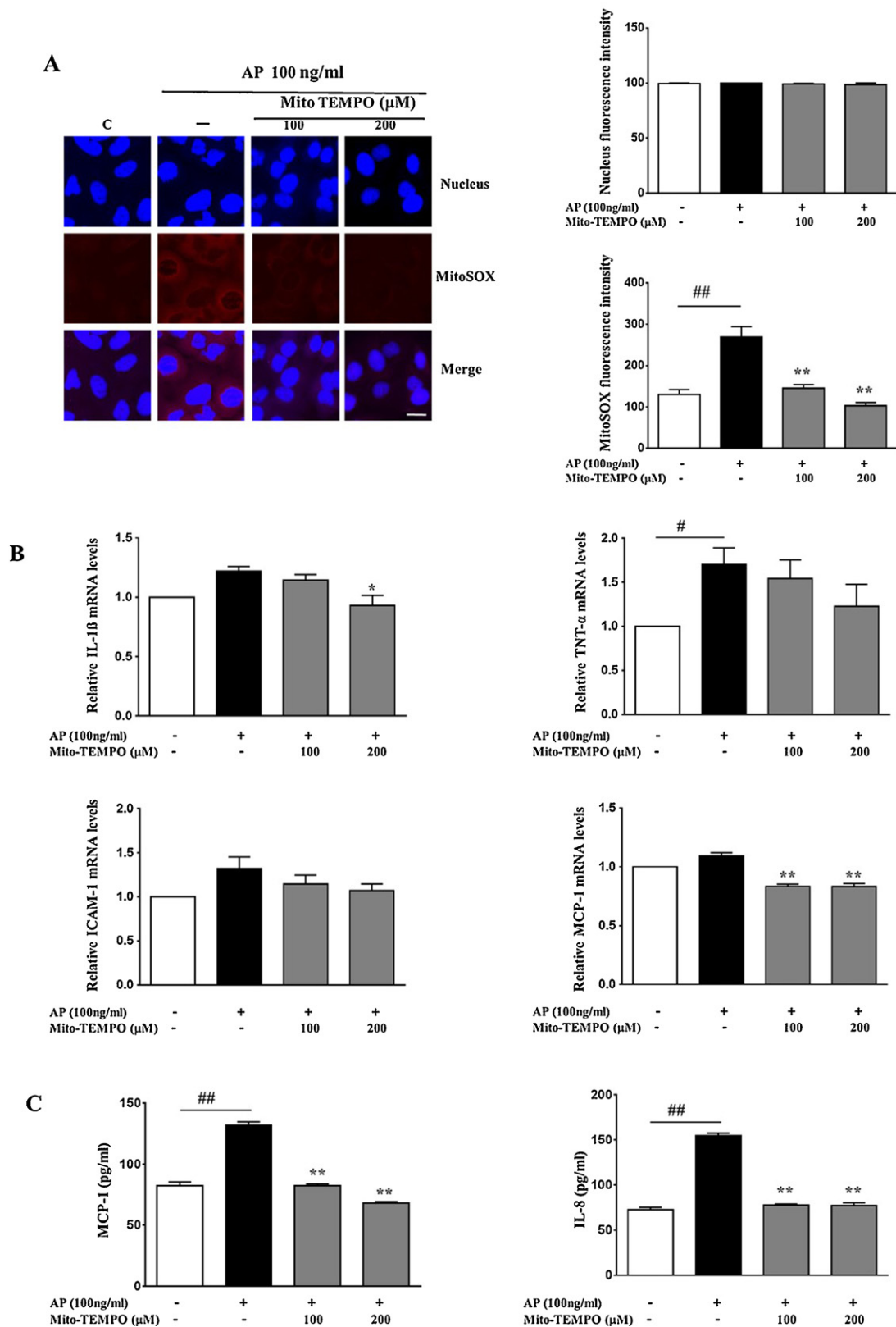


Fig. 4. *Aspergillus* protease-induced mitochondrial ROS controlled inflammatory mediator production in A549 cells. **A.** *Aspergillus* protease-induced mitochondrial ROS production was measured by confocal microscopy with or without Mito-TEMPO in A549 cells (magnification 60×2.5). Images were quantified using ImageJ. Representative data are presented as means $\pm$ SEMs of three independent experiments. **B.** The levels of IL-1 β , TNF- α , ICAM-1, and MCP-1 mRNAs were quantified after *Aspergillus* protease treatment. **C.** IL-8 and MCP-1 levels were quantified with CBA after treatment with *Aspergillus* protease. * $P < 0.05$ and ** $P < 0.01$ compared with *Aspergillus* protease stimulation without Mito-TEMPO. # $P < 0.05$ and ## $P < 0.01$ compared with the control group (control group; neither *Aspergillus* protease stimulation nor Mito-TEMPO pretreatment).

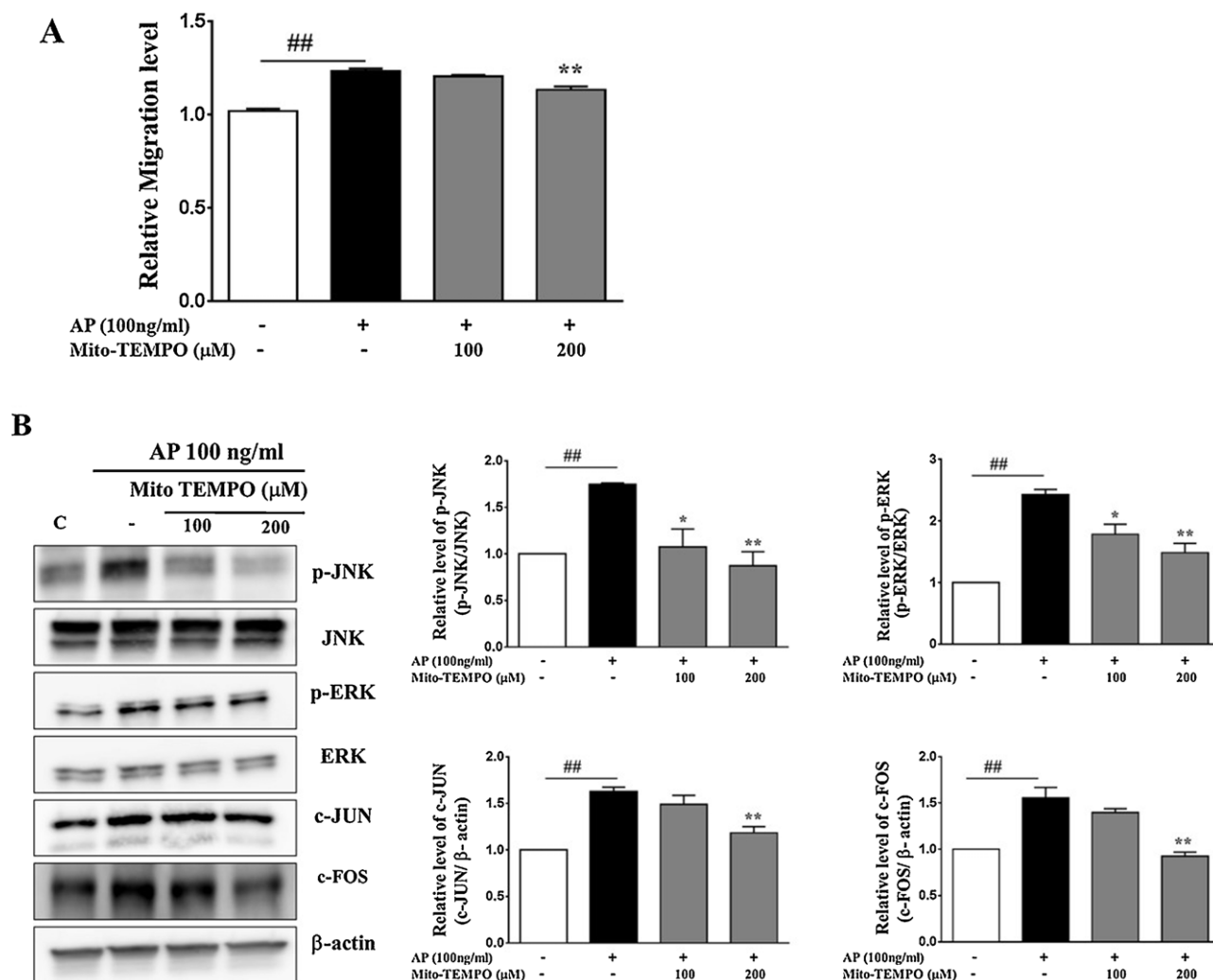


Fig. 5. Effects of *Aspergillus* protease-induced mitochondrial ROS on HL60 cell migration. **A.** Migration of HL60 cells induced by mitochondrial ROS production in A549 cells stimulated with *Aspergillus* protease. Representative data are presented as means $\pm$ SEMs of three independent experiments. ^{##} $P < 0.01$ compared with the control group (control group; neither *Aspergillus* protease stimulation nor Mito-TEMPO pretreatment). ^{**} $P < 0.01$ compared with *Aspergillus* protease stimulation without Mito-TEMPO. **B.** Phosphorylation of JNK and ERK1/2 and expression of c-FOS and c-JUN were determined by western blot analysis. Images were acquired using LAS and quantified with ImageJ. β -Actin was used as an internal control. ^{##} $P < 0.01$ compared with the control group (control group; neither *Aspergillus* protease stimulation nor Mito-TEMPO pretreatment). ^{*} $P < 0.05$, ^{**} $P < 0.01$ compared with *Aspergillus* protease stimulation without Mito-TEMPO.

protease stimulation was strongly involved in the production of inflammatory mediators.

3.5. *Aspergillus* protease-induced mitochondrial ROS modulated HL60 cell migration through activation of MAPK and AP-1 in A549 cells

So far we have shown that AP treatment enhanced the production of mitochondrial ROS-mediated inflammatory mediators including IL-8 by A549 cells. Because IL-8 is a well-known chemoattractant for neutrophils (Kunkel et al., 1991), we next evaluated the migration HL60 human neutrophils using supernatants from *Aspergillus* protease-treated A549 cells. Compared with the *Aspergillus* protease-stimulated group, the Mito-TEMPO-pretreated group showed significantly reduced migratory capacity (Fig. 5A). To elucidate the molecular mechanisms underlying these effects, we evaluated changes in JNK, ERK, and AP-1 activation following ROS scavenging. Western blot analyses showed that phosphorylation of JNK and ERK was significantly decreased, resulting in reduction of AP-1 activation with Mito-TEMPO pretreatment (Fig. 5B). Taken together, these data demonstrated that

Aspergillus protease-induced mitochondrial ROS enhanced inflammatory responses through induction of inflammatory mediators via MAPK- and AP-1-dependent mechanisms in the airway epithelium.

4. Discussion

In the present study, we evaluated the airway inflammatory potential of *Aspergillus* protease on the epithelium of lower respiratory tract and investigated the mechanisms underlying *Aspergillus* protease-mediated inflammatory responses in the epithelium. We showed that *Aspergillus* protease directly caused inflammatory responses in airway epithelium by inducing the production of ROS and inflammatory mediators, and the possibility of stimulating the inflammatory cascade through recruitment of leukocytes, such as monocytes and neutrophils. Our results also demonstrated that mitochondrial ROS were responsible for *Aspergillus* protease-mediated inflammation of airway epithelial cells because inhibition of mitochondrial ROS by Mito-TEMPO treatment decreased the secretion of IL-8 and MCP-1 and suppressed the expression of IL-1 β , TNF- α , ICAM-1, and MCP-1 genes in a MAPK-dependent manner. Therefore, our findings indicated that *Aspergillus* protease is likely

to induce airway inflammation and secrete chemokines, such as IL-8 and MCP-1 for the recruitment of leukocyte via a mitochondrial ROS/MAPK axis-dependent inflammatory signaling pathway.

Fungi are known to be associated with lower airway diseases, which cause inflammation and tissue damage (Holgate 2007; Knutsen et al., 2012). To elucidate the pathogenesis and mechanism of fungal infection in the lower respiratory tract, we used A549 lung epithelial cells and acute *Aspergillus* protease exposure. The concentration of *Aspergillus* protease for this study was determined considering in vivo experimental concentration and the amount of proteinase-secreting household fungi, in which *Aspergillus* protease from household dust of Houston area showed 3.82 ± 0.02 PU mg^{-1} hyphae protease activity in the previous studies (Kheradmand et al., 2002; Lee et al., 2008; Porter et al., 2009). In addition, the enzyme activity of *Aspergillus* protease (1000 ng/mL) was higher than that of trypsin (1000 ng/mL) when their activity was compared with protease assay kit (350,000 relative units of trypsin versus 450,000 relative units of *Aspergillus* protease). It is also noted that single band was detected when *Aspergillus* protease was subject to zymogram indicating the purity is high at least in terms of protease activity.

Aspergillus protease regulated multifunctional pro-inflammatory cytokines, such as IL-1 β , TNF- α , ICAM-1, and MCP-1, at the gene level. In addition, the levels of MCP-1 and IL-8 protein were significantly increased by *Aspergillus* protease stimulation. ICAM-1 is responsible for recruitment of leukocytes into regions of inflammation (Fritz et al., 2008), and MCP-1 functions to stimulate migrating macrophages and monocytes (Sierra-Filardi et al., 2014). MCP-1 and IL-8 also function as chemoattractants and activate neutrophils (Kunkel et al., 1991). Therefore, our data suggested that *Aspergillus* protease induced the expression of inflammatory mediators involved in the migration of leukocytes, thereby stimulating the inflammatory cascade and persistent inflammation. However, considering the enzyme activity of *Aspergillus* protease is 0.5 PU mg^{-1} , it needs to clarify whether the amounts we used in this study is corresponding to the in vivo exposure to the organism although our results showed *Aspergillus* protease induced inflammatory responses.

To identify the mechanisms of chemokine secretion mediated by *Aspergillus* protease stimulation, we measured mitochondrial ROS levels. ROS are produced as byproducts of cellular metabolism and have roles in various physiological processes, such as killing pathogens and tissue repair (Bhattacharyya et al., 2014). In contrast, disproportionate formation of ROS causes tissue damage (Bhattacharyya et al., 2014). The role of multiple source-derived ROS, such as NADPH oxidase-derived ROS (Kim et al., 2012), XO generating ROS (Heunks et al., 1999; Ward, 2010) and NO generating ROS (Dotsch et al., 1996; Thomassen et al., 1999) in airway inflammation, have been massively studied. Although mitochondria are known to be one of major sources of intracellular ROS (Dikalov, 2011), functional significance of mitochondrial ROS has not been fully examined due to the lack of information for regulation of mitochondria ROS comparing to other ROS. In addition, recent studies have indicated that mitochondrial ROS play crucial roles in immune responses and cytokine production and moreover immune modulation in innate immune system (Bhattacharyya et al., 2014; Naik and Dixit 2011; Park et al., 2015; Tait and Green 2012; West et al., 2011). Besides, a previous study showed that *Aspergillus* protease induced allergic immune response and mitochondrial ROS of T cells in NADPH oxidase 2 deficient mice (Kwon et al., 2016). Interestingly, our results showed that *Aspergillus* protease induced mitochondrial ROS production in A549 cells. Thus, our findings suggested that *Aspergillus* protease may cause disproportionate ROS formation in cells and that these ROS may contribute to excessive inflammatory responses and oxidative cellular damage.

To evaluate the effects of mitochondrial ROS on *Aspergillus* protease-induced inflammatory mediators, we examined changes in the expression and secretion of various cytokines in the presence of the mitochondrial ROS scavenger Mito-TEMPO. Mito-TEMPO has been shown to be useful in studies of mitochondrial ROS due to its function as a scavenger of mitochondria-targeted SOD with low toxicity (Trnka et al., 2009). In our study, IL-1 β and MCP-1 gene expression levels were significantly reduced by pretreatment with Mito-TEMPO under *Aspergillus* protease-stimulated condition. Additionally, the expression levels of the TNF- α and ICAM-1 genes were also decreased, although these differences were not statistically significant. Secretion of IL-8 and MCP-1 protein, which was stimulated by *Aspergillus* protease treatment, was significantly decreased with Mito-TEMPO pretreatment. Therefore, these results suggested that mitochondrial ROS regulated the gene expression levels of inflammatory mediators and the protein production of IL-8 and MCP-1 directly.

ROS-induced MAPK activation is known to play a critical role in several signal transduction pathways and functions as a transcription factor during inflammation and cytokine stimulation in response to various ligands, including lipopolysaccharide (LPS) (Huang et al., 2009; Hwang et al., 2004; Park et al., 2015; Seger and Krebs 1995). Similar to LPS stimulation, *Aspergillus* protease activated MAPK in our study. The total amounts of cellular ROS and mitochondrial ROS were also increased by *Aspergillus* protease treatment. Thus, MAPK activation may represent one potential mechanism mediating the effects of mitochondrial ROS on the regulation of chemokine production in response to *Aspergillus* protease stimulation in A549 cells. Interestingly, we found that decreased mitochondrial ROS reduced JNK and ERK activation and AP-1 expression in the context of *Aspergillus* protease stimulation. Therefore, these data indicated that mitochondrial ROS may have a crucial role in various signaling cascades and that mitochondrial ROS signaling may be mediated through MAPK in *Aspergillus* protease-stimulated A549 cells. However, it is possible that these results cannot adequately reflect the disease pathogenesis because we used *in vitro* models in this study. Although we used epithelial cell line and neutrophil cell line, the airway inflammation and asthmatic airway immune reaction are the consequences of interplay among multiple cells, such as dendritic cells, neutrophils, eosinophils, B cells, and T cells (Schleimer et al., 2007). For the evaluation of the mechanism of airway epithelium inflammation and the role of mitochondrial ROS in asthmatic airway epithelium by *Aspergillus* protease considering disease pathogenesis, further studies through in vivo models should be warranted.

In conclusion, our results demonstrated that mitochondria were an important source of ROS in the context of *Aspergillus* protease stimulation and that regulation of mitochondrial ROS modulated the production of pro-inflammatory mediators by preventing mitochondrial ROS-induced MAPK activation in A549 cells. Thus, these findings indicated that targeted suppression of mitochondrial ROS in airway epithelium may, at least in part, provide a useful means to develop effective therapeutics for blocking inflammatory responses in the airways caused by fungal infections and fungal proteases.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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References

- Aguilera-Aguirre, L., Bacsí, A., Saavedra-Molina, A., Kurosky, A., Sur, S., Boldogh, I., 2009. Mitochondrial dysfunction increases allergic airway inflammation. *J. Immunol.* 183, 5379–5387.
- Bals, R., Hiemstra, P.S., 2004. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur. Respir. J.* 23, 327–333.
- Bhattacharyya, A., Chattopadhyay, R., Mitra, S., Crowe, S.E., 2014. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol. Rev.* 94, 329–354.
- Bloemen, K., Verstraalen, S., Van Den Heuvel, R., Witters, H., Nelissen, I., Schoeters, G., 2007. The allergic cascade: review of the most important molecules in the asthmatic lung. *Immunol. Lett.* 113, 6–18.
- Brabcova, E., Kolesar, L., Thorburn, E., Striz, I., 2014. Chemokines induced in human respiratory epithelial cells by IL-1 family of cytokines. *Folia Biol. (Praha)* 60, 180–186.
- Chotirmall, S.H., Al-Alawi, M., Mirkovic, B., Lavelle, G., Logan, P.M., Greene, C.M., McElvaney, N.G., 2013. Aspergillus-associated airway disease, inflammation, and the innate immune response. *BioMed Res. Int.* 2013, 723129.
- Denning, D., O'driscoll, B., Hogaboam, C., Bowyer, P., Niven, R., 2006. The link between fungi and severe asthma: a summary of the evidence. *Eur. Respir. J.* 27, 615–626.
- Dikalov, S., 2011. Cross talk between mitochondria and NADPH oxidases. *Free Radic. Biol. Med.* 51, 1289–1301.
- Dorko, E., Pilipinec, E., Tkacikova, L., 2002. Fungal diseases of the respiratory tract. *Folia Microbiol.* 47, 302–304.
- Dotsch, J., Demirakca, S., Terbrack, H., Huls, G., Rascher, W., Kuhl, P., 1996. Airway nitric oxide in asthmatic children and patients with cystic fibrosis. *Eur. Respir. J.* 9, 2537–2540.
- Fritz, J.H., Le Bourhis, L., Magalhaes, J.G., Philpott, D.J., 2008. Innate immune recognition at the epithelial barrier drives adaptive immunity: APCs take the back seat. *Trends Immunol.* 29, 41–49.
- Hess, J., Angel, P., Schorpp-Kistner, M., 2004. AP-1 subunits: quarrel and harmony among siblings. *J. Cell Sci.* 117, 5965–5973.
- Heunks, L.M., Viña, J., van Herwaarden, C.L., Folgering, H.T., Gimeno, A., Dekhuijzen, P.R., 1999. Xanthine oxidase is involved in exercise-induced oxidative stress in chronic obstructive pulmonary disease. *American Journal of Physiology-Regulatory, Integr. Comp. Physiol.* 277, R1697–R1704.
- Holgate, S.T., 2007. Epithelium dysfunction in asthma. *J. Allergy Clin. Immunol.* 120, 1233–1244 (quiz 1245–1236).
- Huang, G., Shi, L.Z., Chi, H., 2009. Regulation of JNK and p38 MAPK in the immune system: signal integration, propagation and termination. *Cytokine* 48, 161–169.
- Hwang, Y.S., Jeong, M., Park, J.S., Kim, M.H., Lee, D.B., Shin, B.A., Mukaida, N., Ellis, L.M., Kim, H.R., Ahn, B.W., 2004. Interleukin-1 β stimulates IL-8 expression through MAP kinase and ROS signaling in human gastric carcinoma cells. *Oncogene* 23, 6603–6611.
- Kaminska, B., 2005. MAPK signalling pathways as molecular targets for anti-inflammatory therapy—from molecular mechanisms to therapeutic benefits. *Biochim. Biophys. Acta (BBA)—Proteins Proteomics* 1754, 253–262.
- Kheradmand, F., Kiss, A., Xu, J., Lee, S.-H., Kolattukudy, P.E., Corry, D.B., 2002. A protease-activated pathway underlying Th cell type 2 activation and allergic lung disease. *J. Immunol.* 169, 5904–5911.
- Kim, H., Hwang, J.S., Woo, C.H., Kim, E.Y., Kim, T.H., Cho, K.J., Kim, J.H., Seo, J.M., Lee, S.S., 2008. TNF-alpha-induced up-regulation of intercellular adhesion molecule-1 is regulated by a Rac-ROS-dependent cascade in human airway epithelial cells. *Exp. Mol. Med.* 40, 167–175.
- Kim, S.Y., Moon, K.-A., Jo, H.-Y., Jeong, S., Seon, S.-H., Jung, E., Cho, Y.S., Chun, E., Lee, K.-Y., 2012. Anti-inflammatory effects of apocynin, an inhibitor of NADPH oxidase, in airway inflammation. *Immunol. Cell Biol.* 90, 441–448.
- Knutsen, A.P., Bush, R.K., Demain, J.G., Denning, D.W., Dixit, A., Fairs, A., Greenberger, P.A., Kariuki, B., Kita, H., Kurup, V.P., Moss, R.B., Niven, R.M., Pashley, C.H., Slavin, R.G., Vijay, H.M., Wardlaw, A.J., 2012. Fungi and allergic lower respiratory tract diseases. *J. Allergy Clin. Immunol.* 129, 280–291 (quiz 292–283).
- Kunkel, S.L., Standiford, T., Kasahara, K., Strieter, R.M., 1991. Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. *Exp. Lung Res.* 17, 17–23.
- Kwon, B.I., Kim, T.W., Shin, K., Kim, Y.H., Yuk, C.M., Yuk, J.M., Shin, D.M., Jo, E.K., Lee, C.H., Lee, S.H., 2016. Enhanced Th2 cell differentiation and function in the absence of Nox2. *Allergy*.
- Lass-Flörl, C., 2009. The changing face of epidemiology of invasive fungal disease in Europe. *Mycoses* 52, 197–205.
- Lee, S.-H., Prince, J.E., Rais, M., Kheradmand, F., Ballantyne, C.M., Weitz-Schmidt, G., Smith, C.W., Corry, D.B., 2008. Developmental control of integrin expression regulates Th2 effector homing. *J. Immunol.* 180, 4656–4667.
- Li, X., Fang, P., Mai, J., Choi, E.T., Wang, H., Yang, X.-f., 2013. Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers. *J. Hematol. Oncol.* 6, 1.
- Naik, E., Dixit, V.M., 2011. Mitochondrial reactive oxygen species drive proinflammatory cytokine production. *J. Exp. Med.* 208, 417–420.
- Oshero, N., 2012. Interaction of the pathogenic mold *Aspergillus fumigatus* with lung epithelial cells. *Front. Microbiol.* 3, 346.
- Park, J., Min, J.-S., Kim, B., Chae, U.-B., Yun, J.W., Choi, M.-S., Kong, I.-K., Chang, K.-T., Lee, D.-S., 2015. Mitochondrial ROS govern the LPS-induced pro-inflammatory response in microglia cells by regulating MAPK and NF- κ B pathways. *Neurosci. Lett.* 584, 191–196.
- Porter, P., Susarla, S., Polikepahad, S., Qian, Y., Hampton, J., Kiss, A., Vaidya, S., Sur, S., Ongeri, V., Yang, T., 2009. Link between allergic asthma and airway mucosal infection suggested by proteinase-secreting household fungi. *Mucosal Immunol.* 2, 504–517.
- Schleimer, R.P., Kato, A., Kern, R., Kuperman, D., Avila, P.C., 2007. Epithelium: at the interface of innate and adaptive immune responses. *J. Allergy Clin. Immunol.* 120, 1279–1284.
- Seger, R., Krebs, E.G., 1995. The MAPK signaling cascade. *FASEB J.* 9, 726–735.
- Sierra-Filardi, E., Nieto, C., Dominguez-Soto, A., Barroso, R., Sanchez-Mateos, P., Puig-Kroger, A., Lopez-Bravo, M., Joven, J., Ardavin, C., Rodriguez-Fernandez, J.L., Sanchez-Torres, C., Mellado, M., Corbi, A.L., 2014. CCL2 shapes macrophage polarization by GM-CSF and M-CSF: identification of CCL2/CCR2-dependent gene expression profile. *J. Immunol. (Baltimore, Md. 1950)* 192, 3858–3867.
- Tait, S.W., Green, D.R., 2012. Mitochondria and cell signalling. *J. Cell Sci.* 125, 807–815.
- Thomassen, M.J., Raychaudhuri, B., Dweik, R.A., Farver, C., Buhrow, L., Malur, A., Connors, M.J., Drazba, J., Hammel, J., Erzurum, S.C., 1999. Nitric oxide regulation of asthmatic airway inflammation with segmental allergen challenge. *J. Allergy Clin. Immunol.* 104, 1174–1182.
- Trnka, J., Blaikie, F.H., Logan, A., Smith, R.A., Murphy, M.P., 2009. Antioxidant properties of MitoTEMPOL and its hydroxylamine. *Free Radic. Res.* 43, 4–12.
- Ward, P.A., 2010. Oxidative stress: acute and progressive lung injury. *Ann. N. Y. Acad. Sci.* 1203, 53–59.
- West, A.P., Shadel, G.S., Ghosh, S., 2011. Mitochondria in innate immune responses. *Nat. Rev. Immunol.* 11, 389–402.
- Wiegman, C.H., Michaeloudes, C., Haji, G., Narang, P., Clarke, C.J., Russell, K.E., Bao, W., Pavlidis, S., Barnes, P.J., Kanerva, J., 2015. Oxidative stress-induced mitochondrial dysfunction drives inflammation and airway smooth muscle remodeling in patients with chronic obstructive pulmonary disease. *J. Allergy Clin. Immunol.* 136, 769–780.
- Zhou, R., Yazdi, A.S., Menu, P., Tschopp, J., 2011. A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469, 221–225.