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Research paper

TGF-β/SMAD4 mediated UCP2 downregulation contributes to *Aspergillus* protease-induced inflammation in primary bronchial epithelial cells

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ABSTRACT

Elevated levels of mitochondrial reactive oxygen species (ROS) can lead to the development of airway inflammation. In this study, we investigated the role of *Aspergillus* proteases—which contribute to the pathogenesis of *Aspergillus*-induced diseases such as allergic bronchopulmonary aspergillosis, hypersensitivity pneumonitis, and atopic asthma—and their mechanisms of action in airway inflammation using primary human bronchial epithelial cells, and evaluated the inflammatory responses mediated by mitochondrial ROS. We found that *Aspergillus* proteases regulated the expression of multifunctional inflammatory cytokines such as interleukin (IL) − 1β, − 6, and − 8, and transforming growth factor (TGF)-β, which stimulated cytokine production and chemokines involved in leukocyte migration and activated an inflammatory cascade. Expression of these factors and activator protein (AP) − 1 were decreased by treatment with the mitochondrial ROS scavenger Mito-TEMPO, suggesting that mitochondria are important sources of ROS in the context of inflammatory response by *Aspergillus* protease. The regulation of mitochondrial ROS influenced the production of proinflammatory mediators by preventing mitochondrial ROS-induced AP-1 activation in airway epithelial cells. In addition, *Aspergillus* protease-mediated mitochondrial ROS production was associated with downregulation of uncoupling protein (UCP) − 2 expression by TGF-β-SMAD4 signaling, which may play a regulatory role in mitochondrial ROS formation during fungal protease-mediated epithelial inflammation. This improved understanding of the allergenic fungal protease-induced inflammatory mechanism in the bronchial epithelium will help in developing intervention strategies for the regulation of inflammatory response in allergic airway diseases.

1. Introduction

Fungi are omnipresent and the spores of several *Aspergillus* species are inhaled over an individual's lifetime, such that most individuals are colonized with these species. Exposure to infectious fungi can cause the progression of airway disease and lead to death. Fungi have become an ever-increasing threat to human health owing to global climate change [1,2]; sensitization to airborne pathogenic fungi can cause respiratory tract diseases due to components such as chitin, glycan, toxins, and protease [2,3]. *Aspergillus* species are the most common fungi found in the airway and their proteases contribute to diseases such as allergic bronchopulmonary aspergillosis, hypersensitivity pneumonitis, and atopic asthma [2,4]. Among those atopic asthma patient requiring referral to clinicians, 20–25% are known to show reactivity to *Aspergillus* and other fungi in a skin test [5].

The airway epithelium is in continuous contact with an organism's surroundings and is thus the first line of host defense; as such, the barrier functions of the epithelium are tightly regulated [6,7]. Proteolytic allergens such as *Aspergillus* proteases can disrupt the epithelial barrier function and induce an inflammatory response [8]. Additionally, allergenic proteases generate reactive oxygen species (ROS) that have toxic effects on epithelial tissues and affect signaling molecules involved in immune responses [9]. ROS are produced by several enzymes including xanthine oxidase, uncoupling nitric oxide synthase, and nicotinamide adenine dinucleotide phosphate oxidase. Among the several areas in a cell in which ROS are produced, mitochondria constitute the major hub of ROS production [10], and the mitochondrial ROS is especially important because they directly affect the production of inflammatory cytokines by activating retinoic acid-inducible gene-I-like receptors, mitogen-activated protein kinases (MAPKs), and the NACHT, LRR, and PYD domain-containing protein 3 inflammasome [11–13].

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Uncoupling proteins (UCPs) are located in the mitochondrial inner membrane, which are involved in regulation of cell metabolism [14]. UCP2 is ubiquitously expressed in various tissues such as the lung, brain, spleen, and kidney [15] and mediates proton leakage and associated reductions in membrane potential, which lead to superoxide anion production. Thus, UCP2 contributes to the cellular defense against oxidative stress [16,17]. UCP2 has been linked to diabetes, obesity, insulin secretion [18,19], and various inflammatory and infectious diseases including autoimmune encephalomyelitis and *Leishmania* infection via ROS regulation [16,20].

Although mitochondrial ROS and oxidative stress have been implicated in the progression of allergic inflammatory disease such as atopic asthma, their mechanisms of action in the bronchial epithelium are not fully known, although epithelial cells have a crucial role in orchestrating airway inflammation [21]. Thus, we aimed to explore the mechanisms underlying *Aspergillus* protease-induced inflammation in relation to mitochondrial ROS regulation by UCP2. The importance of UCP2 and mitochondrial ROS production in allergenic proteases-induced inflammation were validated further by silencing UCP2, and the effect of down regulation of UCP2 on transforming growth factor (TGF)-β/SMAD4 signaling during *Aspergillus* protease-mediated mitochondrial ROS induction was studied in detail.

2. Materials and methods

2.1. Cell culture

Human primary bronchial epithelial cells purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in bronchial epithelial growth medium (ATCC) at 37 °C in humidified atmosphere of 95% air and 5% CO_2.

2.2. Aspergillus protease and inactive Aspergillus protease treatment

Aspergillus oryzae protease solution (> 500 U/g, cat. no. P6110, lot no. SLBP2675V) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and was dissolved in serum-free medium. *Aspergillus* protease was used at concentrations ranging from 10 to 100 ng/ml; enzyme activity was 0.5 PU mg^{-1}. Inactive protease cDNA was constructed by GeneScript (Township, NJ, USA). Substitutions were made at three active sites in *Aspergillus oryzae* protease [22,23]: aspartic acid was replaced with alanine, histidine was replaced with arginine, and serine was replaced with alanine. The cDNA was subcloned into a pET28a vector for expression into the *E. coli* system. The expressed inactive protease was purified with the Ni^{2+} bead column, and endotoxin was removed. Endotoxin level was measured using the endotoxin quantitation kit (Thermo Fisher Scientific, Waltham, MA, USA), and proteolytic activity of the protease was assessed using the protease activity assay kit (Thermo Fisher Scientific).

2.3. Mito-TEMPO and SB-431542 treatment

Mito-TEMPO (Sigma-Aldrich) was diluted with DMSO (Sigma) to obtain a 5 mM stock solution. The stock solution was further diluted in cell culture medium to achieve different concentrations, and was used immediately. Mito-TEMPO was pretreated for 1 h prior to *Aspergillus* protease stimulation. SB-431542 was purchased from Sigma, and was diluted in cell culture medium to various concentrations; cells were treated with SB-431542 for 4 h, followed by *Aspergillus* protease stimulation for an additional 1 h.

2.4. Monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-1β, −6, and −8, and transforming growth factor (TGF)-β cytokine bead array (CBA)

The culture supernatant was collected from cells after 50 min of *Aspergillus* protease treatment, and was analyzed with a human CBA chemokine and inflammation kit (BD Biosciences, Franklin Lakes, NJ, USA) according to manufacturer's instructions.

2.5. Short interfering (si)RNA transfection

Primary bronchial epithelial cells were transfected for 14 h with human UCP2 siRNA from a siRNA library (Qiagen, Hilden, Germany), using the HiPerFect Transfection Reagent (Qiagen) according to manufacturer's instructions. The medium was refreshed, and cells were cultured for an additional 6 h before the 1 h *Aspergillus* protease treatment in serum-free medium. Proteins and mRNAs were isolated from the cells; UCP2 and SMAD4 levels were assessed; mitochondrial ROS and cytokine levels were also measured.

2.6. RNA extraction and real-time reverse transcription (RT-) PCR

Total RNA was extracted from cultured cells after *Aspergillus* protease treatment for 50 min using the Qiagen RNA extraction kit (Qiagen, Hilden, Germany). RNA concentration and quality were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR was performed on an ABI 7300 sequence detection system using premade TaqMan probes: *MCP-1* probes (#Hs00234140), *ACTIN-β* probes (#Hs01060665), *IL-6* probe (#Hs00985639), *IL-1β* probe (#Hs01555410), *TGF-β* probe (#Hs00998133), *IL-8* probe (#Hs00174103), *UCP-2* probe (#Hs01075227), and *SMAD4* probe (#Hs00929647) (Thermo Fisher Scientific).

2.7. Confocal and fluorescence microscopy

Primary bronchial epithelial cells were cultured in glass-bottomed dishes (Thermo Fisher Scientific). For CellROX confocal microscopy staining, cells were treated with different concentrations of *Aspergillus* protease for 1 h. Following the treatment, cells were washed with PBS, and incubated with culture medium containing 5 μM CellROX orange at 37 °C for 30 min. The medium was then removed, and cells were washed three times with PBS and mounted with PBS containing DRAQ5 (Cell Signaling Technology, Danvers, MA). For MitoSOX confocal microscopy staining, cells were cultured in glass-bottomed dishes (Thermo Fisher Scientific), and were treated with different concentrations of *Aspergillus* protease for 1 h in the presence or absence of Mito-TEMPO. Cells were then washed twice with PBS, and were incubated with serum-free culture medium containing 5 μM MitoSOX at 37 °C for 20 min. Cells were washed again with Hank's Balanced Salt Solution (HBSS), and were mounted with PBS containing DRAQ5. After a final incubation of 5 min, cells were analyzed using an FV10i confocal microscope (Olympus, Tokyo, Japan). For MitoSOX micro plate staining, cells were cultured in 96-well clear bottom black plates (Corning Inc), and were treated with different concentrations of *Aspergillus* protease for 1 h following siRNA transfection. After the treatment, cells were washed twice with PBS, and were incubated with HBSS containing 1 μM MitoSOX at 37 °C for 10 min. The stained cells were then washed with HBSS, and fluorescence was determined using a microplate fluorimeter at 510 nm Ex/580 nm Em (Molecular Devices, Sunnyvale, CA).

2.8. Western blot

Proteins from primary bronchial epithelial cells treated with *Aspergillus* protease were extracted using the radioimmunoprecipitation buffer (LPS Solution, Daejeon, Korea) and the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific). Different proteins were separated by electrophoresis (25 μg/lane), and were then transferred to a polyvinylidene difluoride membrane. Non-specific

binding was blocked with blocking solution for 45 min at 23 °C. The membrane was incubated overnight with gentle agitation at 4 °C with rabbit antibodies against SMAD4 (Abcam, Cambridge, UK)), c-Jun N-terminal kinase (JNK), phosphorylated (p)-JNK, extracellular signal-regulated kinase (ERK)1/2, p-ERK1/2, c-JUN, UCP2, TBP, and β-actin (Cell Signaling Technology, Danvers, MA, USA; 1:1000 dilution). The membrane was washed three times with Tris-buffered saline with 0.1% Tween 20 (TBST), and was then incubated for 1 h at room temperature with biotinylated goat anti-rabbit IgG (Cell Signaling Technology). Following three washes with TBST, immunoreactivity was detected with the Clarity Western ECL Blotting Substrate (Bio-Rad, Hercules, CA, USA); protein bands were visualized with an ImageQuant LAS 4000 mini imager (GE Healthcare Life Science, Chicago, IL, USA).

2.9. Neutrophil isolation

Blood (20 ml) from healthy donor was drawn into an EDTA-containing tube, and was diluted in saline at a 1:1 ratio. Diluted blood was layered over (12 ml) Ficoll (LSM 1077; Lonza, Basel, Switzerland), and was centrifuged at 600*g* for 15 min at room temperature without braking. The upper three layers of plasma, peripheral blood mononuclear cells (PBMCS), and Ficoll were discarded in order to obtain the red blood cell (RBC) pellet containing granulocytes, which was re-suspended in (10 ml) saline. The cell suspension was immediately mixed with 10 ml of 3% dextran-saline solution. After sedimentation, the neutrophil-rich upper layer was collected, and was centrifuged for 3 min at 200*g* and 4 °C. The remaining RBCs were lysed in hypotonic saline (0.2%); neutrophil count was determined, and viability was assessed by morphological examination and the Trypan Blue exclusion test.

2.10. Chemotaxis assay

After incubation in serum-free medium, primary bronchial epithelial cells were stimulated with *Aspergillus* protease for 1 h. The supernatant was collected for chemotaxis assays using a QCM Chemotaxis 24-well (5 μM) Cell Migration kit (Millipore, Billerica, MA, USA). Isolated human primary neutrophils were seeded at a density of 2×10^4/well into the upper well, and culture supernatant was added to the lower well. After a 4 h incubation period, media containing migrated cells in the lower well, as well as detached cells from the bottom of the upper well, were dissolved in lysis buffer with dye. Samples were transferred to 96-well plates, and optical density was measured at 560 nm with a microplate reader (Molecular Devices).

2.11. Statistical analysis

Results except western quantification are shown as mean ± SEM. Quantification results of western blot are shown as mean ± SD. Multiple comparisons between three or more experimental groups were evaluated by one-way ANOVA. Two-sided differences between two samples were performed using the unpaired *t*-test. A P value < 0.05 was considered to be significant. All statistical analyses were performed using the Prism v.6.0 software (GraphPad Inc., San Diego, CA, USA).

3. Results

3.1. Effects of Aspergillus protease on inflammatory cytokine production and gene expression

To determine whether the proteolytic activity of *Aspergillus* protease induces inflammatory responses, we first examined proteolytic activity and endotoxin levels of active and inactive *Aspergillus* protease. Proteolytic activity of inactive protease was significantly decreased as compared with that of the active *Aspergillus* protease (Fig. 1A); both active and inactive *Aspergillus* proteases showed no endotoxin levels (Fig. 1B). We examined whether *Aspergillus* protease is associated with inflammation by evaluating gene expression and protein level of inflammatory cytokines. The levels of IL-1β, −6, and −8, TGF-β, MCP-1, and other chemokines were evaluated in *Aspergillus* protease-stimulated human primary bronchial epithelial cells. IL-1β, −6, and −8, and TGF-β levels were higher in cells treated with 100 ng/ml *Aspergillus* protease for 50 min as compared with those in untreated cells. MCP- 1 was also increased; however, this difference was not statistically significant (Fig. 1C). However, chemokine (C–C motif) ligand (CCL) 5 (also known as RANTES), CCL11 (also known as eotaxin 1), and CCL17 (also known as TARC) levels were unaffected by the treatment (data not shown). This suggested that *Aspergillus* protease induces inflammatory cytokines in airway epithelium-derived cells.

mRNA levels of IL-1β, −6, and −8, TNF-α, TGF-β intercellular adhesion molecule (ICAM)-1, and MCP-1 were assessed in *Aspergillus* protease-stimulated human primary bronchial epithelial cells. Treatment with 10 and 100 ng/ml protease for 50 min resulted in increased IL-1β, −6, and −8, TGF-β, and MCP-1 mRNA expression as compared with that in control cells. (Fig. 1D). On the other hand, ICAM-1 was unaltered by the treatment (data not shown). Our overall results showed that *Aspergillus* protease induces inflammatory gene expression in primary bronchial epithelial cells.

3.2. Effects of Aspergillus protease on MAPK activation, intracellular, and mitochondrial ROS levels

It has been reported that AP-1 and MAPKs such as p38, ERK1/2, and JNK regulate the expression of cytokines and other factors during inflammation [24,25]. We therefore investigated whether *Aspergillus* protease modulates MAPK and AP-1 activation. JNK and ERK phosphorylation and AP-1 expression were increased in cells treated with protease as compared to that in the control (Fig. 2A), indicating that *Aspergillus* protease induces MAPK activation. ROS act as second messengers in intracellular signaling cascades (e.g., MAPK signaling) to regulate the expression of inflammatory mediators and cytokines [25]. We examined intracellular ROS levels in *Aspergillus* protease-treated human primary bronchial epithelial cells by CellROX staining. ROS production was increased by *Aspergillus* protease treatment in a concentration-dependent manner (Fig. 2B). Mitochondria are a major source of intracellular ROS [11]; mitochondrial dysfunction is associated with airway inflammation, and ROS function as second messengers in inflammatory signaling cascades [26,27]. We found here that cells treated with *Aspergillus* protease had higher levels of mitochondrial ROS than untreated cells and that the observed increase was concentration-dependent (Fig. 2C).

3.3. Effects of Aspergillus protease on UCP2 expression and role of UCP2 in ROS and inflammatory cytokine production

To clarify the mechanism underlying the increase in mitochondrial ROS production induced by *Aspergillus* protease treatment, UCP2 protein level and UCP2 mRNA level in primary bronchial epithelial cells were examined. UCP2 protein expression and mRNA level were reduced by *Aspergillus* protease treatment as compared to that in untreated control cells (Fig. 3A, B). Moreover, siRNA-mediated UCP2 knockdown stimulated the production of mitochondrial ROS as compared to that in cells transfected with control siRNA (Fig. 3D). These results indicate that *Aspergillus* protease induces mitochondrial ROS production by inhibiting UCP2 protein expression.

3.4. Effects of Aspergillus protease on SMAD4 expression and roles of SMAD4 and TGF-β in UCP2 expression following Aspergillus protease treatment

To clarify the mechanism underlying the down regulation of UCP2 expression by *Aspergillus* protease treatment, SMAD4 protein level and

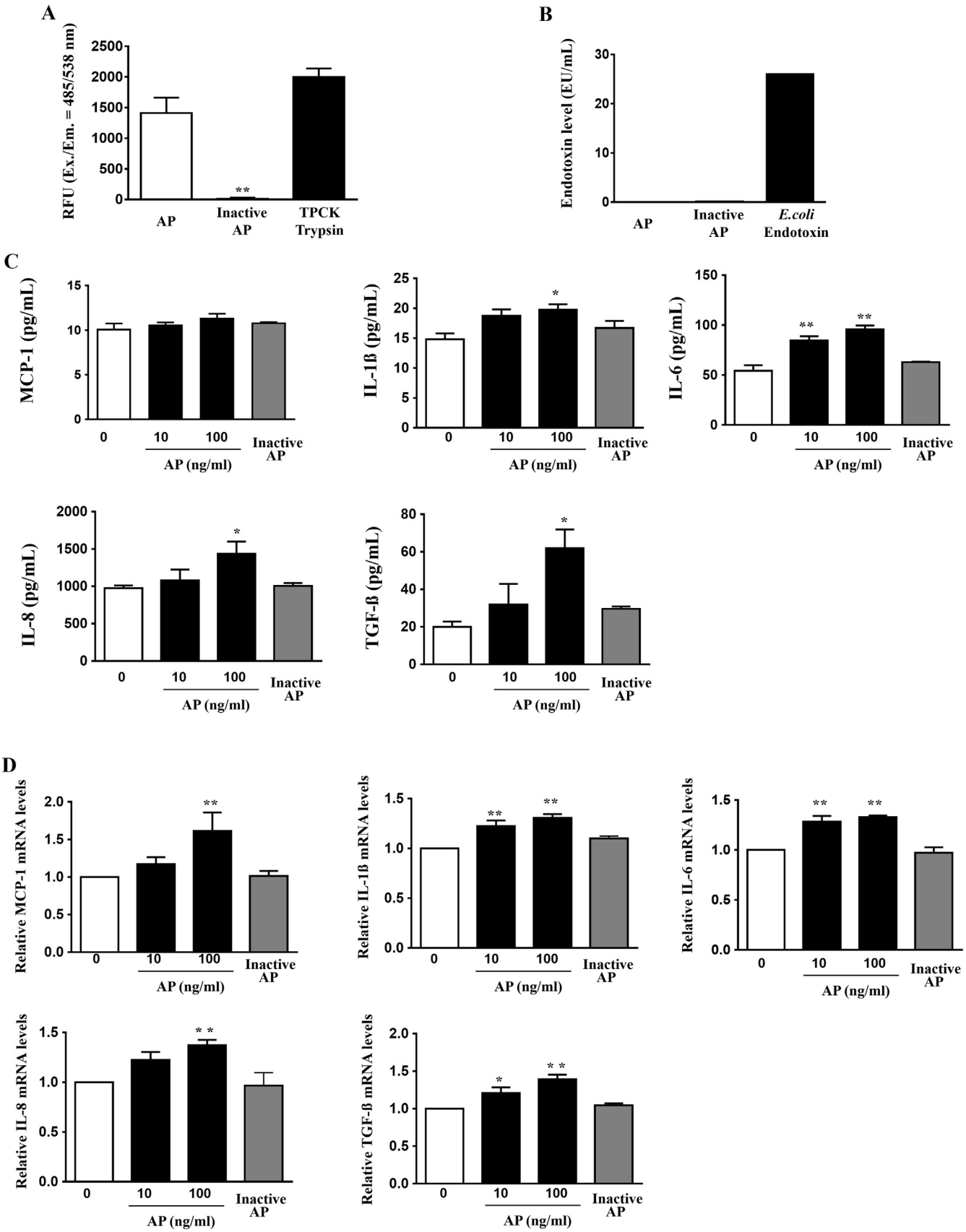

Fig. 1. *Effects of Aspergillus protease on inflammatory cytokine production and gene expression in primary bronchial epithelial cells.* (A) The activity of protease (10 μg/ml) was assessed using protease activity kit. TPCK trypsin (10 μg/ml) was used as positive control group. (B) Endotoxin level was assessed using endotoxin quantitation kit. Endotoxin of *E.coli* was used as positive control group. (C) Levels of MCP-1 and IL-1β, −6, −8, and TGF- β were measured with a cytometric bead array. (D) Levels of MCP-1, IL-1β, −6, −8, and TGF- β mRNA. *P < 0.05, * *P < 0.01 vs. control (0) group.

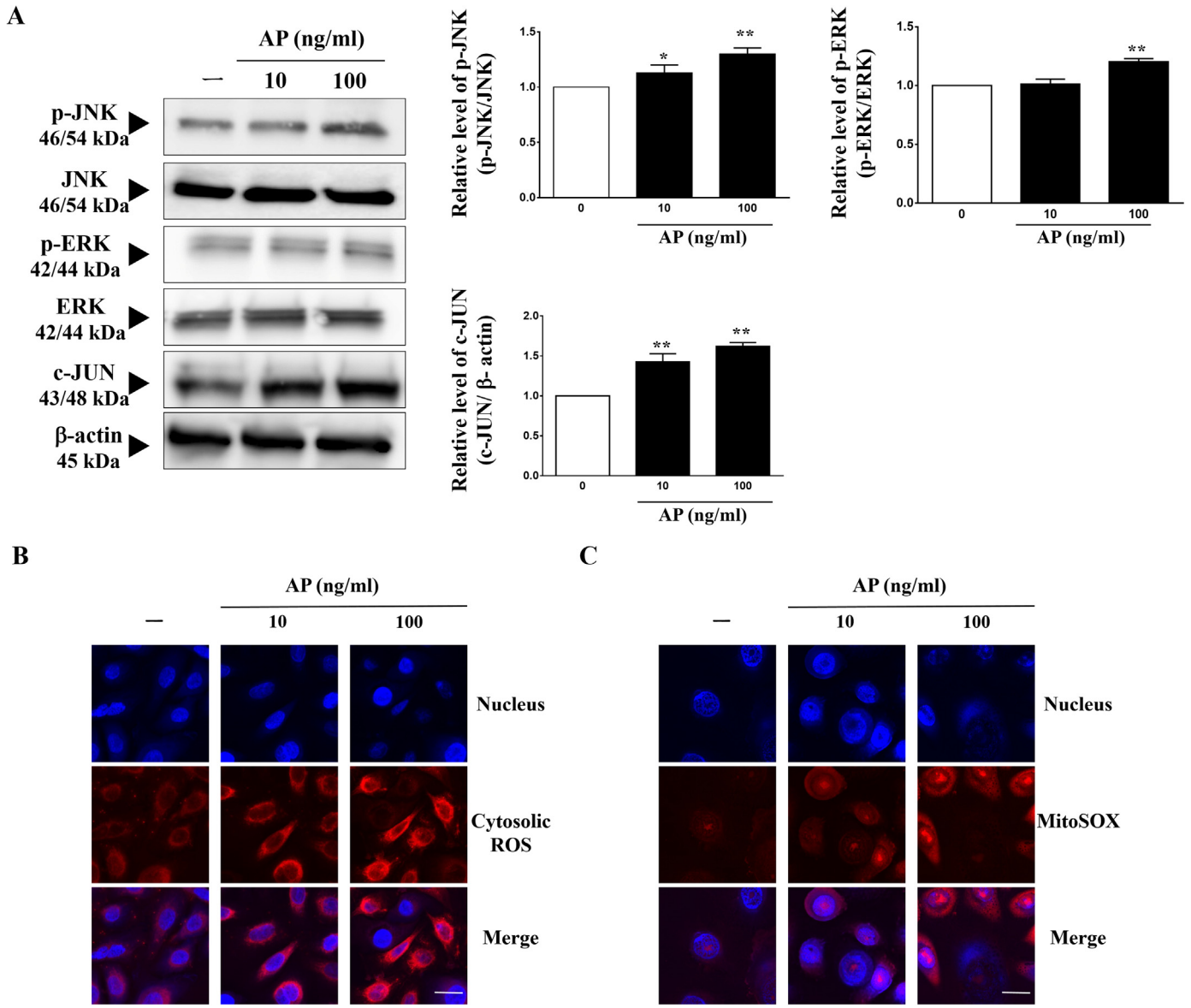

Fig. 2. *Effects of Aspergillus protease on MAPK activation, intracellular ROS production, and induction of mitochondrial ROS.* (A) Phosphorylation of JNK, ERK1/2, and c-JUN were determined by western blotting following pretreatment with *Aspergillus* protease (AP) for 1 h. Images were acquired with LAS software and quantification was performed using ImageJ software. β-Actin was used as a loading control. (B) Levels of intracellular ROS were evaluated in *Aspergillus* protease (AP)-treated primary bronchial epithelial cells using CellROX and by confocal microscopy (magnification: 130 ×; scale bar = 20 μm). (C) Levels of mitochondrial ROS were measured using MitoSOX and by fluorescence spectroscopy (magnification 130 ×; scale bar = 20 μm). *P < 0.05, **P < 0.01 vs. control (0) group.

SMAD4 translocation from the cytosol to the nucleus in primary bronchial epithelial cells were examined. SMAD4 expression and translocation to nucleus were increased by *Aspergillus* protease treatment as compared to those in control cells (Fig. 4A), and SMAD4 mRNA level was increased by *Aspergillus* protease treatment (Fig. 4B). Moreover, siRNA-mediated SMAD4 knockdown increased UCP2 levels as compared to that in control siRNA transfected cells with or without *Aspergillus* protease treatment (Fig. 4C). In the case of treatment of TGF-*β* receptor inhibitor, SB-431542 reduced SMAD4 expression and recovered UCP2 expression (Fig. 4D). These results indicate that *Aspergillus* protease affects UCP2 expression via TGF-*β* and SMAD4.

3.5. *Aspergillus protease-induced mitochondrial ROS activate MAPK and AP-1*

Mito-TEMPO is an antioxidant that targets mitochondria [28,29]. We tested various concentrations of Mito-TEMPO (20, 50, 100, and 200 μM) and found that 100 and 200 μM Mito-TEMPO effectively scavenged ROS in human primary bronchial epithelial cells without cytotoxic effects. We then assessed the levels of mitochondrial ROS in *Aspergillus* protease-stimulated cells pretreated with Mito-TEMPO for 1 h. Mitochondrial ROS levels were reduced by the pretreatment as compared to that in cells treated with *Aspergillus* protease only (Fig. 5A). To investigate the effect of reduced mitochondrial ROS production on signaling pathways involved in inflammation, we assessed JNK, ERK, and AP-1 activation following ROS scavenging by western blotting and found that ERK phosphorylation was reduced by Mito-TEMPO pretreatment, with a corresponding decrease in AP-1 activation (Fig. 5B).

3.6. *Aspergillus protease-induced mitochondrial ROS modulate inflammation and neutrophil migration*

We evaluated the effects of mitochondrial ROS scavenging on

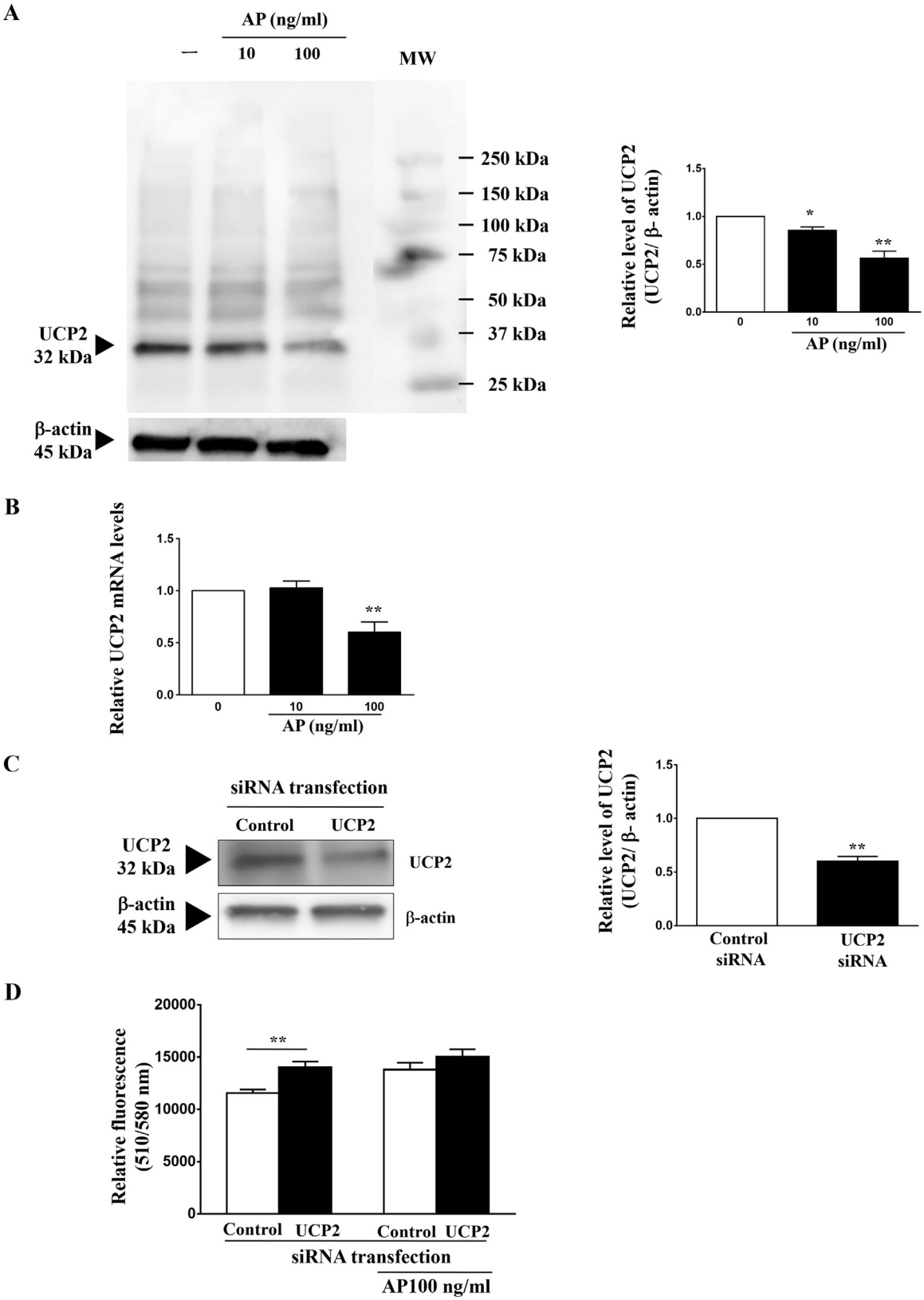

Fig. 3. *Effects of Aspergillus protease on UCP2 expression level, and effects of UCP2 gene silencing on ROS production.* (A, B) UCP2 expression level was evaluated by western blotting (A) and UCP2 mRNA level (B) after stimulating primary bronchial epithelial cells with *Aspergillus* protease (AP). (C) UCP2 expression level was evaluated by western blotting after cell transfection with UCP2 or negative control (NC) siRNA. Images were acquired with LAS image analysis software and quantification was performed with ImageJ software. β-Actin was used as a loading control. (D) Effect of UCP2 gene silencing on mitochondrial ROS. * *P < 0.05, * *P < 0.01 vs. control (0) group.

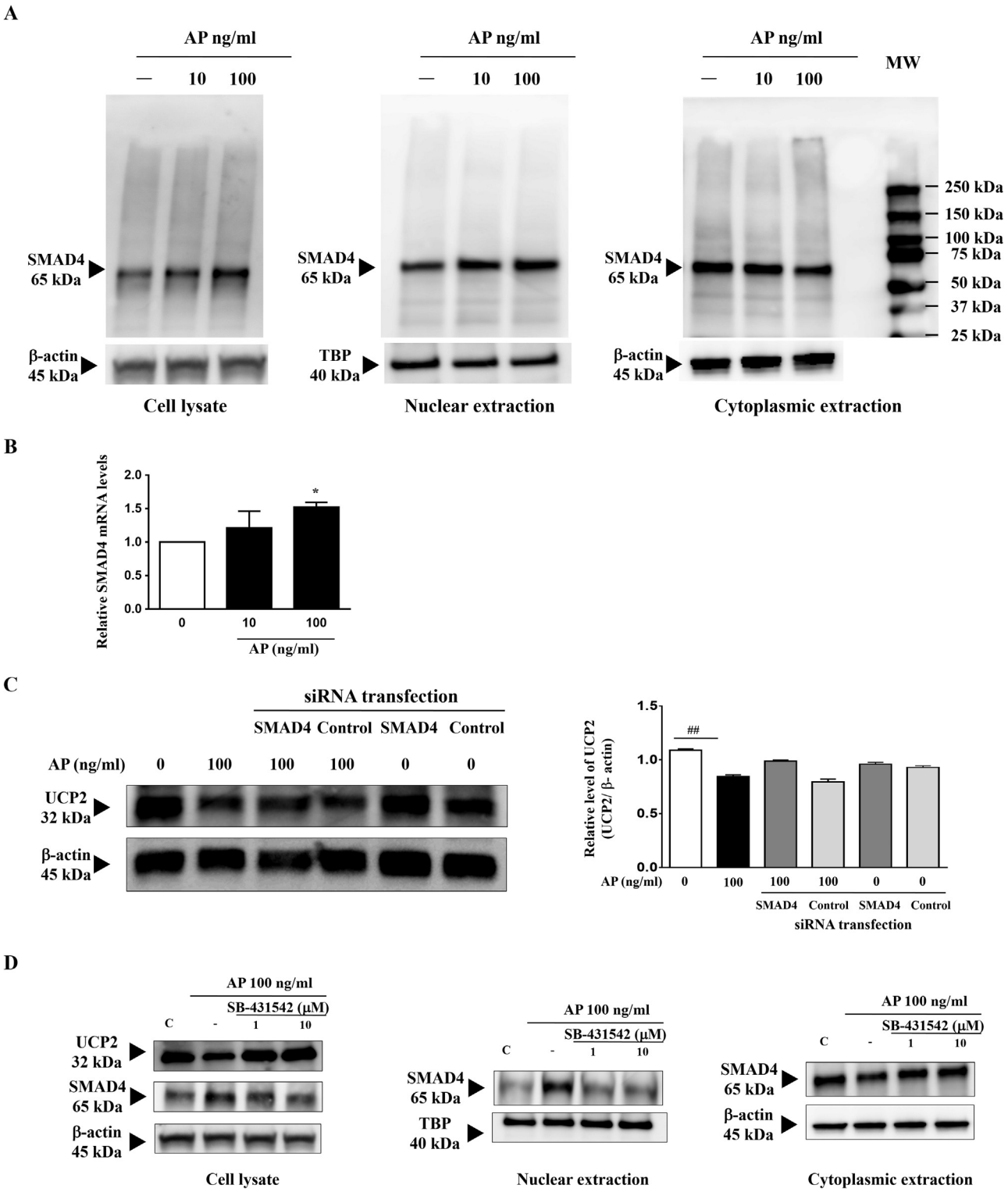

Fig. 4. *Effects of Aspergillus protease on SMAD4 expression, and roles of SMAD4 and TGF-β in UCP2 expression under Aspergillus protease treatment.* (A) SMAD4 expression and translocation levels in primary bronchial epithelial cells were evaluated by western blot and (B) SMAD4 mRNA level following stimulation with *Aspergillus* protease (AP). (C) Effect of SMAD4 gene silencing on UCP2 protein expression. (D) Effect of SB-431542 treatment on UCP2 and SMAD4 protein levels. Images were acquired using the LAS image analysis software. β-Actin was used as a loading control, and TBP was used as a loading control for nuclear extracts. * *P < 0.05, * *P < 0.01 vs. control (0) group.

inflammation and found that Mito-TEMPO inhibited the secretion of IL-6 and IL-8 in human primary bronchial epithelial cells. IL-1β and TGF-β release were also decreased; however, this differences were not statistically significant (Fig. 6A). In addition, IL-1β, −6, and −8, and MCP-1 mRNA levels were downregulated in these cells (Fig. 6B), suggesting that *Aspergillus* protease-induced mitochondrial ROS production is involved in the production of inflammatory mediators. IL-8 is a well-known chemoattractant for neutrophils; we therefore examined the migration of human primary neutrophils using culture supernatants from human primary bronchial epithelial cells stimulated with

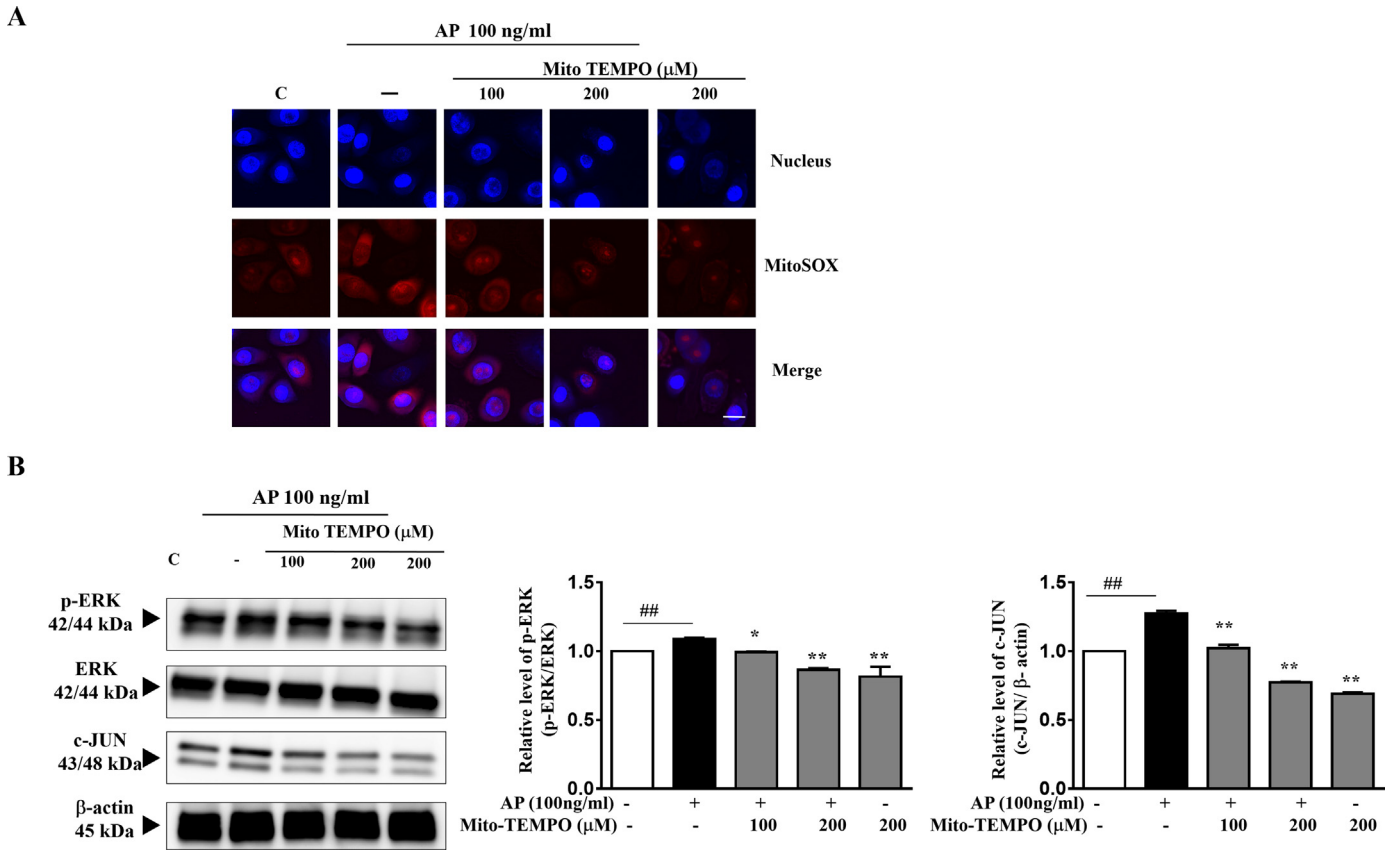

Fig. 5. *Effects of Aspergillus protease-induced mitochondrial ROS on MAPK activation and AP−1 expression.* (A) *Aspergillus* protease (AP)-induced mitochondrial ROS production was evaluated in primary epithelial bronchial cells by confocal microscopy with or without Mito-TEMPO treatment (magnification: 130 ×). (B) JNK and ERK1/2 phosphorylation and c-JUN expression (B) were analyzed by western blotting. Images were acquired with LAS image analysis software and quantification was performed with ImageJ software. β-Actin was used as a loading control. *P < 0.05, **P < 0.01 vs. *Aspergillus* protease stimulation without Mito-TEMPO; #P < 0.05, ##P < 0.01 vs. control group (no *Aspergillus* protease stimulation or Mito-TEMPO pretreatment).

Aspergillus protease. Compared to cells treated with *Aspergillus* protease, migration was markedly reduced in the Mito-TEMPO-pretreated group (Fig. 6C), possibly due to the decrease in IL-8 level resulting from mitochondrial ROS scavenging. Taken together, these data suggest that *Aspergillus* protease-induced mitochondrial ROS activate MAPK and AP-1, thereby enhancing the inflammatory response in human primary airway epithelial cells.

4. Discussion

Allergenic proteases can cause inflammatory response through cellular damage, including epithelial cell shrinkage [2,5], which can in turn induce damage-associated molecular patterns such as ROS and uric acid production [30–32]. Previous studies have reported increased ROS levels in asthma patients [33] and mitochondrial dysfunction in allergic airway inflammation [27]. However, the mechanism underlying allergenic-protease-induced increase in ROS level remains poorly understood. Here, using *Aspergillus* proteases, we investigated the mechanistic basis for protease-mediated inflammation associated with ROS production in the allergenicity of the airway epithelium, as epithelial cells are known to play a crucial role in the initiation and orchestration of allergic immune responses [6,21].

Our main finding was that *Aspergillus* proteases enhanced mitochondrial ROS generation, which is associated with down regulation of UCP2, a mitochondrial inner membrane protein. Down regulation of UCP2 is possibly involved in TGF-β-SMAD4 signaling. Consequently, *Aspergillus* protease-induced mitochondrial ROS induced early immune response such as the expression of inflammatory mediators, resulting in tissue damage via recruitment of leukocytes and induction of other cytokines. We found here that *Aspergillus* proteases regulate the expression of inflammatory cytokines including IL-1β, −6, and −8, TGF-β, and MCP-1 at the transcriptional level as well as the secretion of IL-1β, −6 and −8, and TGF-β. MCP-1 is known to stimulate macrophage and monocyte migration [34] and functions along with IL-8 as a chemoattractant that activates neutrophils [35]. IL-1β and IL-6 induces an acute immune response and lymphocyte activation. Moreover, IL-6 is a pleiotropic cytokine that is present at a high level in the bronchoalveolar lavage fluid of asthmatic patients [36,37]. TGF-β level is also elevated in severe asthma patients, and this cytokine is related to airway remodeling and its expression is accelerated in autocrine manner [38]. Collectively, our data suggest that *Aspergillus* proteases induce proinflammatory cytokines such as IL-1β and −6 which stimulate the production of other cytokines and of chemokines, such as IL-8 that promotes leukocyte migration, thereby activating a cascade that leads to persistent inflammation.

In this study, inhibition of mitochondrial ROS by treatment with Mito-TEMPO reduced IL-6 and −8 secretion and inhibited IL-1β, −6, and −8, and MCP-1 mRNA expression in an AP-1-dependent manner. This results show that mitochondrial ROS is an important role in *Aspergillus* protease-induced inflammation. In addition, elevated generation of mitochondrial ROS via siRNA-mediated UCP2 gene silencing suggests a crucial role of UCP2 in *Aspergillus* protease-mediated inflammation. These results suggest that *Aspergillus* proteases exacerbate or induce airway inflammation via a mitochondrial ROS/AP-1 signaling and that UCP2 may be related with the cellular defense against oxidative stress by controlling mitochondrial ROS generation during fungal protease infection.

UCP2 is known to be regulated at multiple levels such as the genetic

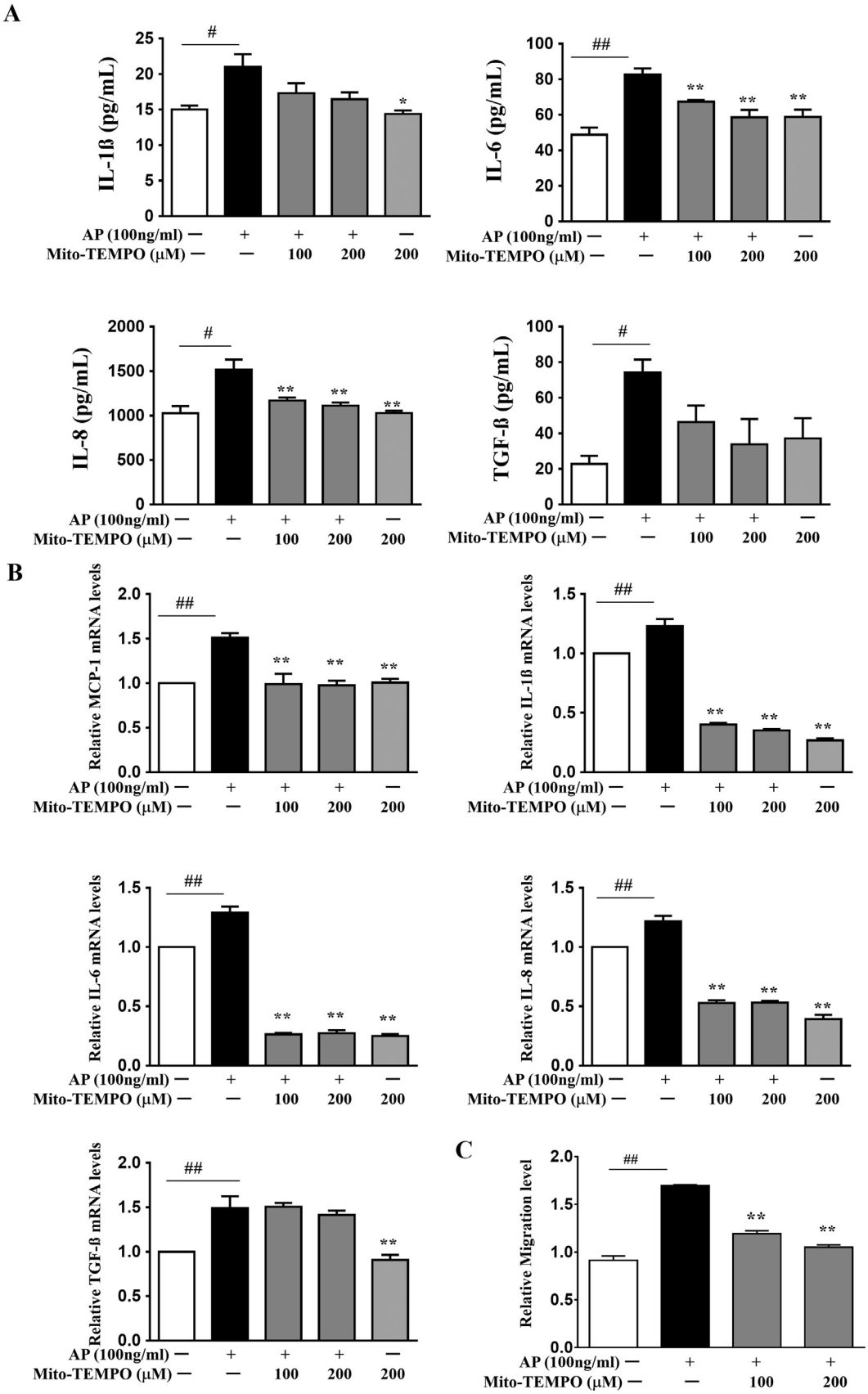

Fig. 6. *Aspergillus protease-induced mitochondrial ROS induces inflammatory cytokine production and gene expression and stimulates neutrophil migration*. (A, B) IL− 1β, − 6, − 8, and TGF-β levels in primary bronchial epithelial cells (A) and gene expression (B) were evaluated following *Aspergillus* protease (AP) treatment. (C) Migration of human primary neutrophils induced by mitochondrial ROS produced by primary bronchial epithelial cells stimulated with *Aspergillus* protease (AP). *P < 0.05, * *P < 0.01 vs. *Aspergillus* protease stimulation for 50 min without Mito-TEMPO; #P < 0.05, ##P < 0.01 vs. control group (no *Aspergillus* protease stimulation and no Mito-TEMPO pretreatment).

and protein level, and during UCP2 proton conductance. In the transcriptional regulation, several transcription factors and regulatory proteins, such as peroxisomal proliferator-activated receptors and peroxisome proliferator-activated receptor gamma coactivator1-alpha regulate UCP2 transcription level by binding to the UCP2 promoter region [39]. SMAD is involved in the primary pathway of TGF-β, and it has been reported that SMAD4 binds to the UCP2 promoter region in poorly differentiated breast cancer cells [10,39]. We investigated the effects of TGF-β-SMAD4 signaling on UCP2 expression using siRNA-mediated SMAD4 knockdown and TGF-β receptor inhibitor in *Aspergillus* protease-treated primary epithelial cells. The results showed that *Aspergillus* proteases induced SMAD4 expression and SMAD4 knockdown recovered UCP2 levels. Moreover, in the case of treatment of TGF-*β* receptor inhibitor, SB-431542 reduced the SMAD4 level and enhanced UCP2 expression. These results suggest that suppression of UCP2 expression following *Aspergillus* protease infection is associated with upregulation of SMAD4 by TGF-β signaling in primary bronchial epithelial cells.

In summary, our results demonstrate that mitochondria are important sources of ROS for inflammatory stimulation of airway epithelial cells at early time points. Modulation of mitochondrial ROS could influence early immune response, such as the production of proinflammatory mediators by blocking AP-1 activation. In addition, this study has shed light on a novel pathway involving TGF-β, SMAD4, UCP2, and mitochondrial ROS in fungal protease-induced inflammatory response. Finally, our improved understanding of the allergenic fungal protease-induced inflammatory mechanism in the bronchial epithelium will help in developing intervention strategies for the regulation of inflammatory response in allergic airway diseases.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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