

Alternaria-induced barrier dysfunction of nasal epithelial cells: role of serine protease and reactive oxygen species

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Background: Upper airway barrier dysfunction has been associated with chronic rhinosinusitis and allergic rhinitis. *Alternaria* is commonly found in nasal secretion and plays a role in the pathogenesis of airway diseases. The aim of this study was to investigate the effects of *Alternaria* on the junctional complex of nasal epithelial cells.

Methods: Air-liquid interface nasal epithelial cultures from the inferior turbinate of septal surgery patients were stimulated with *Alternaria alternata*. Production of intracellular reactive oxygen species (ROS) and transepithelial resistance (TER) was measured. The expression of tight junction (TJ) and adherens junction (AJ) molecules was determined using real-time reverse transcriptase-polymerase chain reaction, Western blot analysis, and confocal microscopy. Protease activity in *Alternaria* was determined using protease inhibitors and heat inactivation.

Results: *Alternaria* enhanced the production of ROS and reduced the TER. *Alternaria* decreased the messenger RNA and protein expression of TJs (zonula occludens-1,

occludin, and claudin-1), but did not influence the AJ molecule. When *Alternaria* was pretreated with serine protease inhibitor and heat inactivation, ROS, TER, and TJ molecule expression returned to their nonstimulated levels.

Conclusion: Serine protease in *Alternaria* altered nasal epithelial barrier function. Intracellular ROS induced by *Alternaria* may influence the barrier function of nasal epithelial cells and enhance the inflammatory process of nasal mucosa. © 2018 ARS-AAOA, LLC.

Key Words:

Alternaria; tight junctions; adherence junction; nasal epithelial cell; reactive oxygen species; protease

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Nasal epithelial cells play an important role in the innate immune system and the first exposure of mucosal cells to airborne pathogens is associated with mucosal inflammation. The physical barrier function of nasal mucosa is determined by the integrity of the apical junction complex. The apical junctional complex is composed of tight junctions (TJs) and adherens junctions (AJs).¹ TJs form the most apical intercellular junction between epithelial cells, responsible for the regulation of paracellular flux of ions and small molecules. TJs of nasal epithelial cells contain

proteins, such as the zonula occludens-1 (ZO-1), junctional adhesion molecule-1, and claudins.² AJs anchor epithelial cells to maintain cell-to-cell adhesion and transmit information from the environment to the interior of the cells and are comprised of the E-cadherin and nectin families.³ Apical junctional complex dysfunction leads to the abnormal entrance of external stimulants and initiates abnormal signal transduction and chemical mediator production.⁴ In chronic rhinosinusitis (CRS) and allergic rhinitis, impaired epithelial barrier function may lead to increased susceptibility to allergens, diminished transepithelial migration of leukocytes, and suppressed repair and proliferation of epithelial cells.^{5,6}

Fungi are ubiquitous in nature and some of them are associated with upper and lower airway diseases. Fungal components, like proteins and enzymes, induce the immune response and proteases facilitate pathogen access to target tissues and directly activate immune cells.^{7,8} Protease activity can induce chemical mediator production through cell membrane receptors, such as protease-activated receptor and Toll-like receptor, and can also generate

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hyperresponsiveness of airway mucosa.^{7,9} In addition, protease can directly influence the epithelial barrier functions.^{5,10} An imbalance of protease and protease inhibitors within the airway mucosa can cause the development of CRS and bronchial asthma.^{9,11} *Alternaria*, *Aspergillus*, and *Cladosporium* are commonly found in nasal secretion of normal and CRS patients.¹² These fungi can also develop and aggravate airway inflammatory diseases.^{8,9}

In CRS, the levels of ZO-1, occludin, and E-cadherin protein and mRNA were found to be decisively decreased for the barrier function of mucosal epithelium.^{5,13} *Alternaria*, a member of *Deuteromycetes*, is one of the most important allergenic fungi and contains potent airborne allergens that can induce allergic airway inflammation.^{7,14} *Alternaria* extract has increased the permeability of bronchial epithelial cells.¹⁵ However, the effect of airborne fungi on upper airway epithelial barrier function has not been commonly studied. In the present study, we have attempted to determine the effect of *Alternaria* exposure on the barrier function of human nasal epithelial cells and also tried to define the underlying mechanism for their action on epithelium.

Materials and methods

Primary nasal epithelial cell air-liquid interface culture

Human nasal epithelial cells were isolated from the inferior turbinate mucosa of 10 septal surgery patients. None of the subjects had any history of allergy, asthma, aspirin intolerance, systemic or local diseases, or corticosteroid or antibiotics use during the 4 weeks preceding the study. The study was approved by the institutional review board of the Daegu Catholic University Medical Center, and written informed consent that outlined the objectives of the research and experiments was obtained from each patient. Primary nasal epithelial cells were isolated according to previously described methods.¹⁶ The specimens were placed in Ham's F-12 medium supplemented with 100 IU penicillin, 100 μ g/mL streptomycin, and 2 μ g/mL amphotericin B. Nasal mucosa was rinsed with Ham's F-12 medium supplemented with antibiotics and incubated with 0.1% dispase (Roche Diagnostics, Mannheim, Germany) for 16 hours at 4°C. The epithelial cells were isolated by gentle agitation and cell suspensions were filtered through a No. 60 mesh cell dissociation sieve. The cells were suspended in Ham's F12 medium supplemented with antibiotics, 150 μ g/mL glutamine, 5 μ g/mL transferrin, 25 ng/mL epithelial growth factor, 15 μ g/mL endothelial cell growth supplement, 5 IU/mL insulin, 200 pmol/L triiodothyronin, 100 nmol/L hydrocortisone, and 15% fetal calf serum (FCS). Cell suspensions (10^6 cells/mL) were plated in 6-well culture plates and placed in a 5% CO₂-humidified incubator at 37°C. The culture medium was changed after 24 hours and every 2 days thereafter. When the epithelial cell cultures reached 80-90% confluence, the cells were seeded on 0.4-

μ m, 12-mm Transwell inserts (Corning Costar, Cambridge, MA) at a density of 2×10^5 cells/mL in medium. Once the epithelial cells grew to complete confluence, the medium was retrieved to adapt the air-liquid interface (ALI) culture in PneumaCult ALI basal medium (Stemcell, Vancouver, BC, Canada).

Treatment of ALI-cultured nasal epithelial cells

When the nasal epithelial cells reached confluence with mucociliary activity in the ALI system, 50 μ g/mL of *Alternaria alternata* (Greer Laboratories, Lenoir, NC) or heat-inactivated or protease inhibitor-treated *Alternaria* was applied to the upper chamber. *Alternaria* protease activity was blocked by heat inactivation at 70°C for 30 minutes. The *Alternaria* was pretreated with protease inhibitors (Roche, Indianapolis, IN) for 20 minutes with 500 μ g/mL of Pefabloc (*p*-aminoethylbenzenesulfonylfluoride) for serine protease inhibition, 10 μ mol/L of E-64 (trans-epoxysuccinyl-L-leucylamide-[4-guanidine] butane) for cysteine protease inhibition, and 1 μ mol/L of pepstatin for aspartic protease inhibition.

Measurement of TER

The capacity of *Alternaria* to disrupt epithelial junctions was determined by measuring TER, which provides a physical measure of the electrical resistance between cells. Nasal epithelial cells were apically stimulated with various concentrations of *Alternaria* and other stimulants for 24 hours and all 3 anion channel blockers were added to the apical solution. Voltage-clamp experiments were performed using EVOM-II (World Precision Instruments, New Haven, CT). The values for cell-covered filters were determined in standard units of ohms $\times$ square centimeter ($\Omega \cdot \text{cm}^2$) after subtracting the resistance of blank filters, and TER was expressed as a percentage compared with the nonstimulated condition at time zero. Cells treated with medium alone served as a negative control.

Measurement of intracellular ROS

Determination of intracellular ROS was done through the dichlorofluorescein method after stimulation of nasal epithelial cells for 1 hour with 50 μ g/mL of *Alternaria*, heat-inactivated *Alternaria*, protease inhibitor-treated *Alternaria*, or stimulation of epithelial cells after 5-mmol/L of glutathione treatment. We used 1 mmol/L of hydrogen peroxide (H₂O₂) in phosphate-buffered saline (PBS) as positive control. Glutathione was used to abolish intracellular ROS. The cells were incubated with 10 μ mol/L of a 5- (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate probe (Sigma-Aldrich, St. Louis, MO) in PBS for 35 minutes. Next, the cells were washed with PBS and incubated for 5 minutes in 90% dimethylsulfoxide (DMSO; Sigma-Aldrich). The cells were then transferred into black 96-well plates and the fluorescence of the samples was read (excitation = 488 nm, emission = 530 nm) using a spectrophotometer (FLUOstar Optima, BMG Labtech, Ortenberg, Germany). The values of each sample were expressed

TABLE 1. Sequencing data for primer for RT-PCR analysis of tight and adherens junction of nasal epithelial cells

Zonula occludens-1	Forward 5'-TGG TGT CCT ACC TAA TTC AAC TCA-3'	134 bp
	Reverse 5'-CGC CAG CTA CAA ATA TTC CAA CA-3'	
Occludin	Forward 5'-ACA GAG CAA GAT CAC TAT GAG ACA-3'	107 bp
	Reverse 5'-TGT TGA TCT GAA GTG ATA GGT GGA-3'	
Claudin-1	Forward 5'-CCA GTC AAT GCC AGG TAC GAA T-3'	130 bp
	Reverse 5'-TTG GTG TTG GGT AAG AGG TTG TT-3'	
E-cadherin	Forward 5'-ATA GAG AAC GCA TTG CCA CAT ACA-3'	106 bp
	Reverse 5'-TTC TGA TCG GTT ACC GTG ATC A-3'	
β -actin	Forward 5'-ACA GGA AGT CCC TTG CCA TC-3'	248 bp
	Reverse 5'-AGG GAG ACC AAA AGC CTT CA-3'	

bp = basepairs; RT-PCR = reverse transcript polymerase chain reaction.

in terms of the fluorescent intensity of intracellular ROS compared with their respective controls.

ZO-1, occludin, claudin-1, and E-cadherin messenger RNA and protein expression

The messenger RNA (mRNA) expression of TJ and AJ molecules after treatment with *Alternaria* were determined through real-time reverse transcript polymerase chain reaction (RT-PCR). After 24-hour stimulation, total RNA from the nasal epithelial cells was extracted according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). One microliter of RNA was used for the reverse transcription into cDNA using a thermal cycler (DNAEngine Peltier, Bio-Rad, Berkeley, CA). Sequences of the primers used in this study are presented in Table 1. The reaction mixture consisted of 10 μ L of real-time PCR Master Mix (Toyobo Co, Osaka, Japan) and 0.8 μ L of primer in a final concentration of 0.4 μ mol/L in both cases, and the final volume of the mixture was adjusted to 20 μ L with the addition of diethylpyrocarbonate water. RT-PCR was performed using a MiniOpticon real-time PCR detection system (Bio-Rad): 1 cycle of 95°C for 1 minute, and then 40 cycles of 95°C for 15 seconds, 54°-60°C (according to the optimal annealing temperature) for 15 seconds, and 72°C for 45 seconds. Melting-curve analyses were performed by monitoring changes in SYBR Green fluorescence with increases in temperature (0.5°C increments at 10-second intervals) over a temperature range of 55°-95°C. All samples were amplified in triplicate. β -Actin was used as the internal control housekeeping gene. Relative gene expression levels were determined by normalizing expression relative to that of β -actin. The $2^{-\Delta\Delta CT}$ method was used for relative quantitation of mRNA levels.

For Western blot analysis, nasal epithelial cells were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% skim milk solution and incubated with antibodies against ZO-1,

occludin, claudin-1, E-cadherin, and 3-glyceraldehyde phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA). After incubation for 1 hour, the membranes were washed and then treated with peroxidase-conjugated anti-rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology). Bands were visualized using horseradish peroxidase conjugated secondary antibodies and an enhanced chemiluminescence system (Pierce, Rockford, IL). The band densities were measured using Multi Gauge version 2.02 software (Fujifilm, Tokyo, Japan). Protein expression levels were determined by normalization relative to GAPDH and then expressed as a percentage of treated cells vs untreated cells.

Confocal immunofluorescence microscopy

Confluent ALI nasal epithelial cells were fixed with 4% paraformaldehyde for 15 minutes. The inserts with cell monolayers were rinsed twice with 25 mmol/L glycine buffer, permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature, and then incubated with primary antibodies (Santa Cruz Biotechnology). ZO-1, occludin, claudin-1, and E-cadherin primary antibodies were diluted 1:100 in saline-sodium citrate antibody buffer at 4°C overnight. Nuclei were stained with 4',6'-diamidino-2-phenylindol (DAPI; Sigma-Aldrich, St. Louis, MO) diluted in PBS. After incubation with the primary antibody, the cells were incubated with the secondary antibody solution (anti-mouse IgG-fluorescein isothiocyanate [FITC] conjugate, 1:100 working dilution; or anti-rabbit IgG-FITC conjugate, 1:100 working dilution) for 2 hours at room temperature. The stain was then removed and the cells were washed 3 times with PBS. After the final washing step, the slides were coverslipped with fluorescent mounting medium (Dako, Glostrup, Denmark) and examined using a confocal microscope (Nikon, Tokyo, Japan). The median fluorescence intensities of FITC-labeled ZO-1, occludin, claudin-1, and E-cadherin were analyzed using NIS-elements AR software (Nikon).

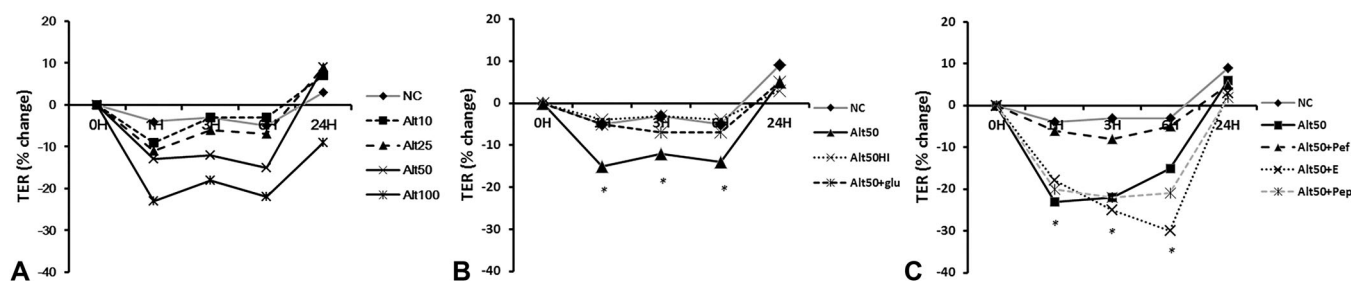


FIGURE 1. Effects of *Alternaria* on TER in air-liquid interface-cultured nasal epithelial cells. TER was measured at 0, 1, 3, 6, and 24 hours under various concentrations of *Alternaria* and stimulation conditions. Results are expressed as percent changes in TER relative to prestimulation conditions. TER levels tended to decrease in a concentration-dependent manner (A). Heat inactivation and glutathione treatment returned TER to baseline level (B). Pretreatment of nasal epithelial cells with serine protease inhibitor (Pefabloc) returned TER to baseline level (C). Alt 50 = *Alternaria* 50 $\mu\text{g}/\text{mL}$; E = E-64; glu = glutathione; HI = heat inactivation; NC = negative control; Pef = Pefabloc; Pep = pepstatin; TER = transepithelial resistance. * $p < 0.05$ vs baseline conditions; $n = 5$ for each experiment.

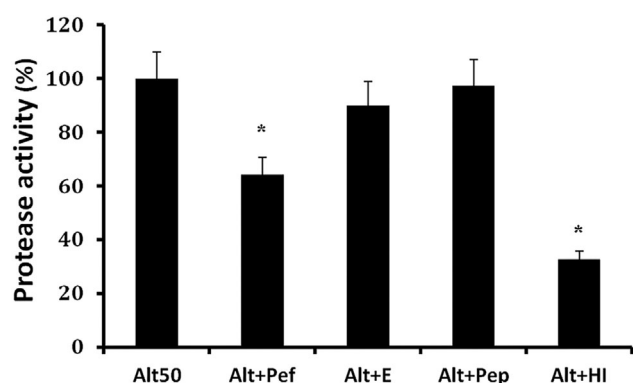


FIGURE 2. Protease activity of *Alternaria* in various conditions. Protease activity was significantly inhibited with serine protease inhibitor, Pef, and HI. Bars represent percent intensity compared with Alt 50. Alt 50 = 50 *Alternaria* $\mu\text{g}/\text{mL}$; E = E-64; HI = heat inactivation; Pef = Pefabloc; Pep = pepstatin. * $p < 0.05$ vs Alt 50; $n = 7$ for each experiment.

Determination of protease activity

The protease activity of *Alternaria* was determined a protease activity assay kit (Cayman, Ann Arbor, MI). Briefly, *Alternaria*, heat-inactivated *Alternaria*, and *Alternaria* pretreated with protease inhibitors were placed in 96-well black plates with protease substrate for 20 minutes at room temperature on an orbit shaker. The value of protease activity was determined with an excitation wavelength of 485 nm and an emission wavelength of 520 nm using FLUOstar Optima (BMG Labtech).

Statistical analysis

The results are presented as mean $\pm$ standard deviation (SD). The statistical significance of differences between control and experimental data was determined using a Wilcoxon signed rank test for single comparisons, Kruskal-Wallis test for multiple comparisons of mRNA and protein expression, and a one-way analysis of variance followed by Dunnett's test for fluorescence intensity (SPSS version 25.0, IBM, Armonk, NY). $p < 0.05$ was considered statistically significant. All results were obtained from at

least 5 independent individuals and every experiment was performed in duplicate.

Results

Effects of *Alternaria* on TER of nasal epithelial cells

Nasal epithelial cell integrity was determined through TER. The mean TER of the nontreated nasal epithelial cells was $497.9 \pm 176.7 \Omega \cdot \text{cm}^2$, and, when nasal epithelial cells were treated with 50 $\mu\text{g}/\text{mL}$ of *Alternaria*, TER decreased to $425.7 \pm 152.4 \Omega \cdot \text{cm}^2$ after 1 hour, $440.9 \pm 214.3 \Omega \cdot \text{cm}^2$ after 3 hours, and $429.7 \pm 185.5 \Omega \cdot \text{cm}^2$ after 6 hours of treatment. In Figure 1 TER is expressed as percent change compared with nonstimulated conditions at time zero. When the nasal epithelial cells were cultured with various concentrations of *Alternaria*, TER tended to decrease in a concentration-dependent manner. After 24 hours of treatment, the TER levels of the nasal epithelial cells were returned to baseline level. However, at 100 $\mu\text{g}/\text{mL}$ of *Alternaria*, TER level did not return to baseline level. The TER of the nasal epithelial cells exposed to heat-inactivated *Alternaria* was almost the same as that of the nontreated group. When the nasal epithelial cells were pretreated with glutathione or stimulated with *Alternaria*, which was pretreated with serine protease inhibitor (Pefabloc), TER level returned to that seen at baseline (463.4 - $475.6 \Omega \cdot \text{cm}^2$ with glutathione and 458.1 - $473.1 \Omega \cdot \text{cm}^2$ with Pefabloc). However, cysteine and aspartic protease inhibitors did not influence the *Alternaria*-induced TER suppression (Fig. 1).

Protease activity of *Alternaria*

The protease using activity of *Alternaria* extract with or without pretreatment with a variety of protease inhibitors or heat inactivation was determined. The serine protease inhibitor, Pefabloc significantly reduced the protease activity of *Alternaria*, whereas the cysteine and aspartate protease inhibitors did not significantly influence the protease activity of *Alternaria* (36%, 10%, and 7%, respectively). Heat inactivation more strongly suppressed the protease activity of *Alternaria* (67%) ($p < 0.05$; Fig. 2).

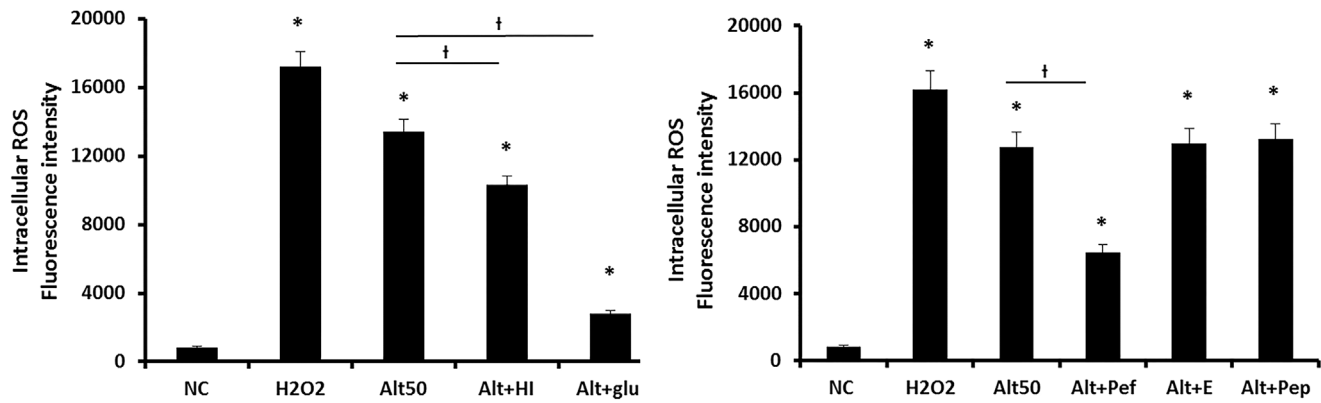


FIGURE 3. Effects of *Alternaria* on production of intracellular reactive oxygen species (ROS) in nasal epithelial cells. Intracellular ROS was measured after 1 hour of stimulation and hydrogen peroxide (H₂O₂) was used as a positive control. Alt 50 = *Alternaria* 50 μ g/mL; E = E-64; HI = heat inactivation; glu = glutathione; NC, negative control; Pef = Pefabloc; Pep = pepstatin; ROS = reactive oxygen species. * p < 0.05 vs baseline; n = 5 for each experiment.

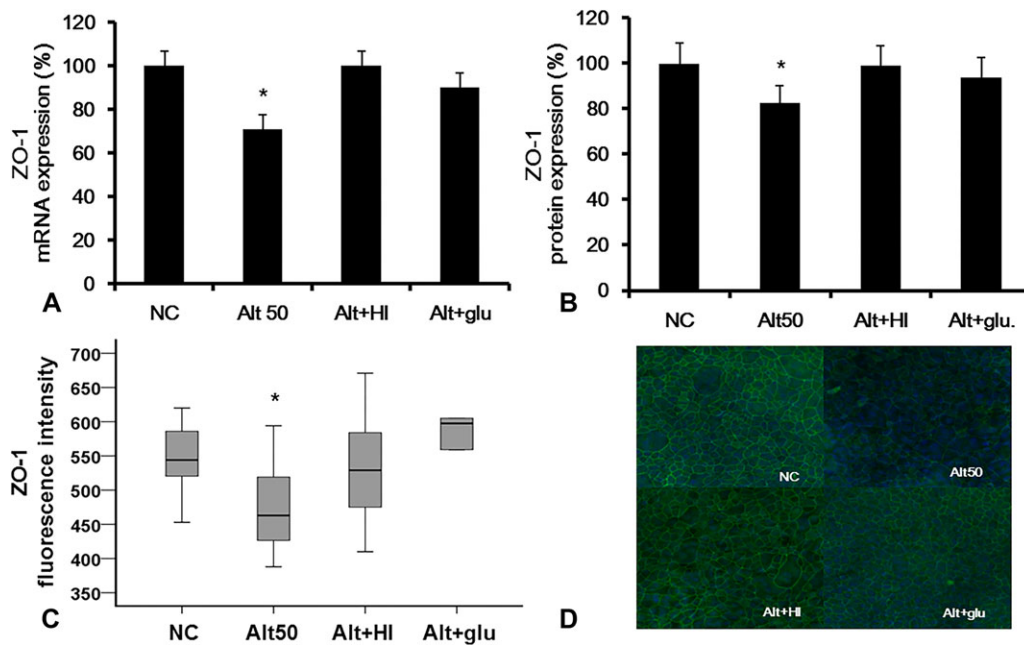


FIGURE 4. Effects of *Alternaria* on ZO-1 messenger RNA and protein expression in air-liquid interface-cultured nasal epithelial cells. After 24-hour stimulation, *Alternaria* significantly inhibited ZO-1 mRNA (A) and protein expression (B). The mean fluorescence intensity of ZO-1 was significantly decreased with *Alternaria* treatment of the epithelial cells (C). Representative confocal microscopy finding of immunofluorescence staining for ZO-1 merged with 4',6'-diamidino-2-phenylindol ($\times 400$) (D). Alt 50 = *Alternaria* 50 μ g/mL; glu = glutathione; HI = heat inactivation; NC = negative control; ZO-1 = zonula occludens. * p < 0.05 vs NC; n = 5 for each experiment.

Effects of *Alternaria* on intracellular ROS of nasal epithelial cells

Hydrogen peroxide and *Alternaria* strongly induced intracellular ROS production from nasal epithelial cells. *Alternaria*-induced intracellular ROS production was significantly inhibited when the nasal epithelial cells were pretreated with glutathione or when they were treated with heat-inactivated *Alternaria* (p < 0.05). When the *Alternaria* was pretreated with protease inhibitors, the serine protease inhibitor (Pefabloc) significantly inhibited intracellular ROS production from nasal epithelial cells (p < 0.05).

However, cysteine and aspartic protease inhibitors did not influence *Alternaria*-induced intracellular ROS production (Fig. 3).

Effects of *Alternaria* on expression TJ and AJ molecules

Real-time RT-PCR and Western blot analysis were performed to determine whether *Alternaria* treatment could influence the expression of TJ and AJ mRNA and protein. In Figures 4–7, mRNA and protein expressions are described as percent change compared with the nontreated

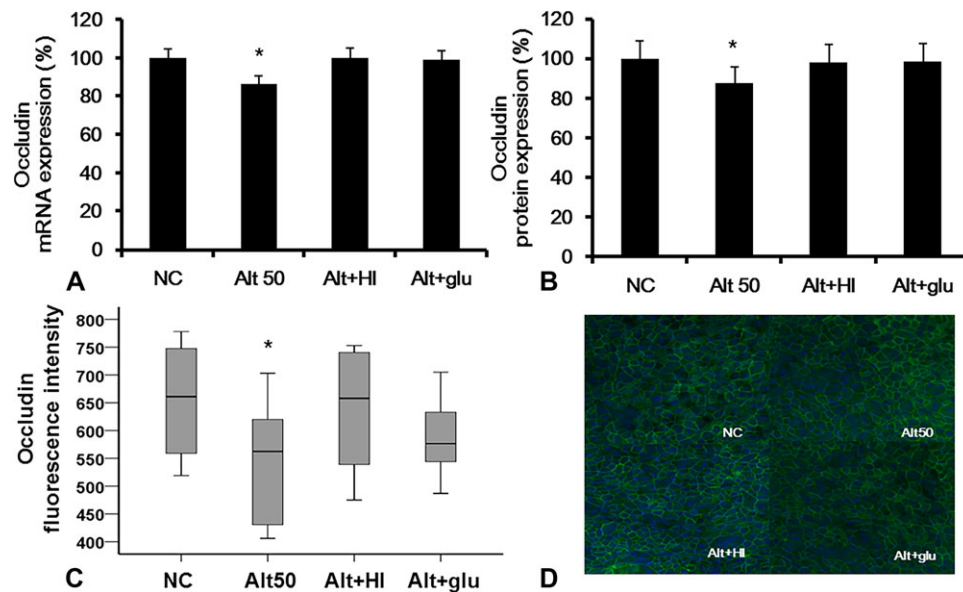


FIGURE 5. Effects of *Alternaria* on occludin mRNA and protein expression in air-liquid interface-cultured nasal epithelial cells. After 24-hour stimulation, *Alternaria* significantly inhibited occludin messenger RNA (A) and protein expression (B). The mean fluorescence intensity of occludin was significantly decreased with *Alternaria* treatment of the epithelial cells (C). Representative confocal microscopy finding of immunofluorescence staining for occludin merged with 4',6'-diamidino-2-phenylindol (×400) (D). Alt 50 = *Alternaria* 50 μ g/mL; glu = glutathione; HI = heat inactivation; NC = negative control. * p < 0.05 vs NC; n = 5 for each experiment.

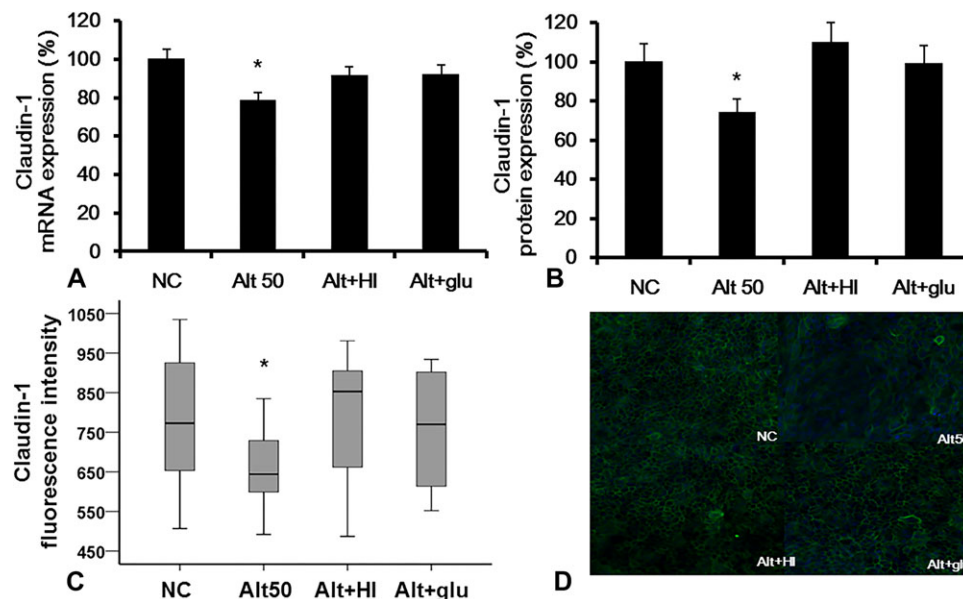


FIGURE 6. Effects of *Alternaria* on claudin-1 messenger RNA and protein expression in air-liquid interface-cultured nasal epithelial cells. After 24-hour stimulation, *Alternaria* significantly inhibited claudin-1 messenger RNA (A) and protein expression (B). The mean fluorescence intensity of claudin-1 was significantly decreased with *Alternaria* treatment of epithelial cells (C). Representative confocal microscopy finding of immunofluorescence staining for claudin-1 merged with 4',6'-diamidino-2-phenylindol (×400) (D). Alt 50 = *Alternaria* 50 μ g/mL; glu = glutathione; HI = heat inactivation; NC = negative control; treatment. * p < 0.05 vs NC; n = 5 for each experiment.

control group. The results indicate that *Alternaria* significantly suppressed mRNA expression for ZO-1, occludin, and claudin-1 in nasal epithelial cells to $29.2 \pm 17.6\%$, $15.5 \pm 13.5\%$, and $21.4 \pm 18.7\%$, respectively, compared with the nontreated group. *Alternaria* also reduced TJ protein expressions (ZO-1, $27.3 \pm 15.5\%$; oc-

cludin, $22.2 \pm 16.4\%$; and claudin-1, $25.8 \pm 18.7\%$) and fluorescence intensities in nasal epithelial cells (p < 0.05; Figs. 4–6). However, *Alternaria* did not influence the AJ molecule, E-cadherin mRNA, and protein expression (Fig. 7). When the nasal epithelial cells were pretreated with glutathione or treated with heat-inactivated *Alternaria*,

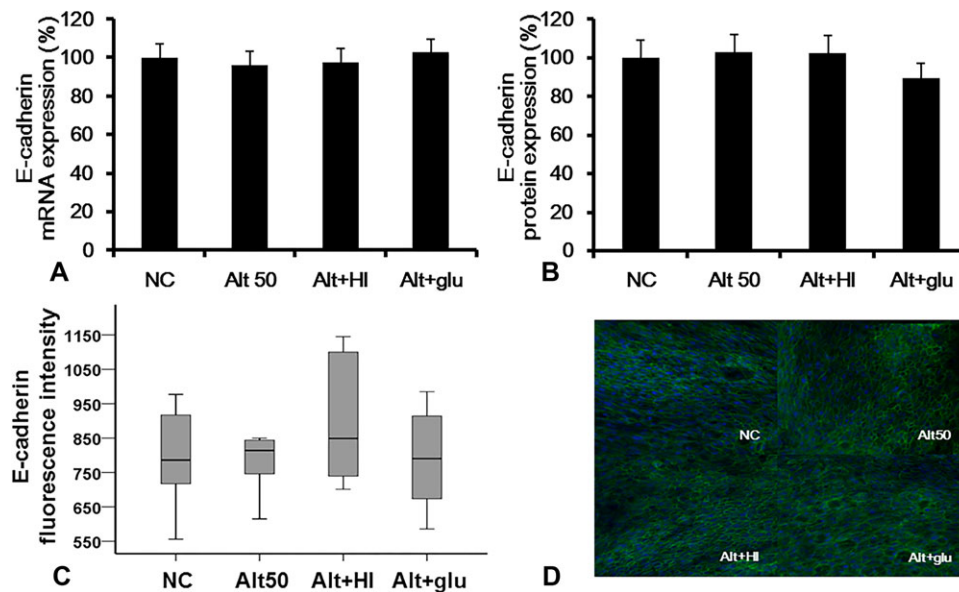


FIGURE 7. Effects of *Alternaria* on E-cadherin mRNA and protein expression in air-liquid interface-cultured nasal epithelial cells. After 24-hour stimulation, *Alternaria* did not influence E-cadherin messenger RNA (A) and protein expression (B). The mean fluorescence intensity of E-cadherin was not changed with *Alternaria* treatment of epithelial cells (C). Representative confocal microscopy finding of immunofluorescence staining for E-cadherin merged with 4',6'-diamidino-2-phenylindol ($\times 400$) (D). Alt 50 = *Alternaria* 50 $\mu\text{g/mL}$; glu = glutathione; HI = heat inactivation; NC = negative control. * $p < 0.05$ vs NC; $n = 5$ for each experiment.

TJ and AJ molecule expression levels were unchanged (Figs. 4–7).

Discussion

Respiratory epithelial cells are the first mucosal cells exposed to pathogenic stimuli and serve as an innate defense system through the production of physical barriers and chemical mediators and molecules that are known to kill or neutralize microorganisms.^{1,17} Fungi are ubiquitous in the airway and their spores enter the airway through inhalation, but they are rarely pathogenic in healthy individuals. *Alternaria* is a common pathogen found in airway secretion and is associated with the pathogenesis of CRS and asthma.^{9,12,18} We used 50 $\mu\text{g/mL}$ of *Alternaria*, due to the higher concentration of *Alternaria*-induced morphologic changes, which had a cytotoxic effect on nasal epithelial cells.^{7,19} At higher concentrations of *Alternaria*, the morphologic change may not return to normal physiologic structure, and TER level of nasal epithelial cells did not return to baseline with 100 $\mu\text{g/mL}$ of *Alternaria*. *Alternaria* exposure to nasal epithelial cells decreased the expression of TJ components ZO-1, claudin-1, and occludin, but not of AJ components like E-cadherin. Serine protease of *Alternaria* perturbs nasal epithelial barrier function with the overproduction of ROS.

ROS are highly reactive oxygen molecules that act as a part of balanced intracellular homeostasis and facilitate cell-to-cell communication.²⁰ The effect of ROS on cellular functions depends on the amount of ROS as well as the exposure time to ROS. ROS is required for the production of proinflammatory cytokines from immune cells. Innate im-

mune responses against microorganisms require ROS signaling through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) or nucleotide oligomerization domain-like receptors (NLRs), and promote the secretion of chemical mediators.^{7,18,21} Exposure of airway epithelial cells to *Alternaria* components (such as the mycotoxin, alt-toxin II) induces oxidative stress through nicotinamide adenine dinucleotide phosphate (NADPH) dual-oxidase 1 located within the apical membrane of epithelium.^{22,23} Protease of fungi can facilitate pathogen access to target tissue and cause the direct activation of cells. Protease-activated receptors (PARs) in respiratory epithelial cells are associated with activation and morphologic change by fungi. *Alternaria* has been found to enhance PAR2 and PAR3 mRNA expression in nasal epithelial cells.^{18,24} In this study, *Alternaria* strongly enhanced the production of intracellular ROS in nasal epithelial cells. *Alternaria* may enhance the production of intracellular ROS through PAR-associated pathways in nasal epithelial cells, or nonenzymatic components of *Alternaria* could influence the production of ROS through PRRs.

The junctional complex of nasal epithelial cells acts as a physical barrier to prevent pathogens from entering into the nasal mucosa.²⁵ We used normal nasal epithelial cells to determine the effect of *Alternaria* on the innate physiologic barrier function of nasal epithelial cells. TER may represent the permeability and molecular structure of the epithelial junctional complex.²⁶ *Alternaria* significantly decreased TER within 1 hour after exposure and this reduction was maintained for at least 6 hours. After 24 hours, *Alternaria*-induced TER change returned to non-stimulated levels. TJ-associated molecules, ZO-1, occludin,

and claudin-1 mRNA and protein expressions were significantly downregulated with *Alternaria* treatment. This indicates that proteases in *Alternaria*, particularly serine protease, play a major role in impeding the barrier function of nasal epithelial cells. Protease activity in an in-vitro culture system or in intracellular ROS production may not be maintained for 24 hours. Treatment of nasal epithelial cells with the ROS scavenger glutathione and serine protease-treated *Alternaria* blocked the influence of *Alternaria* on epithelial barrier dysfunction and TJ molecule expression. Serine protease in *Alternaria* and intracellular ROS played a major role in *Alternaria*-induced nasal epithelial barrier dysfunction. However, *Alternaria* did not influence AJ molecules in nasal epithelial cells. This result is consistent with that of a previous report, which indicated that, in CRS epithelial cells, ZO-1 was rarely expressed, whereas E-cadherin was even more strongly expressed compared with normal ethmoid mucosa.²⁷ *Alternaria* can induce the production of various chemical mediators from nasal epithelial cells.^{21,24,28} T-helper type 2 cytokines and proinflammatory cytokines alter intercellular junctional protein expression and epithelial permeability of nasal epithelial cells.^{13,29,30} Although we do not know the exact roles of the chemical mediators produced from the nasal

epithelial cells or which mediator influences the epithelial barrier function, the chemical mediators produced from nasal epithelial cells by airborne fungi can induce barrier dysfunction in nasal epithelial cells. Fungal protease and chemical mediators could synergistically perturb the barrier function of nasal epithelial cells and promote the penetration of pathogens with aggravation of tissue inflammation.

In this study, we have shown that *Alternaria* affected TJ molecule expression but not AJ molecule expression. *Alternaria*-induced nasal epithelial barrier dysfunctions were associated with the serine protease and intracellular production of ROS. However, the exact mechanism of nasal epithelial barrier dysfunction and oxidative stress of epithelial cells could not be explained by our study findings. *Alternaria* can directly affect the expression of TJ molecules through the interaction of serine protease with epithelial receptors or indirectly affect the expression of TJ molecules through oxidative stress-induced intracellular ROS production in nasal mucosa. Barrier dysfunction is an important mechanism in the pathogenesis of nasal mucosal inflammation. *Alternaria* in nasal secretion can enhance the inflammatory process through alternation of TJ structure and function. These pathologic conditions can develop or aggravate allergic rhinitis or CRS.

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