

Protease-Activated Receptor-2 Decreased Zonula Occludens-1 and Claudin-1 Expression and Induced Epithelial Barrier Dysfunction in Allergic Rhinitis

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Abstract

Background: Protease-activated receptor-2 (PAR-2)-modulated tight junctions (TJs) have been suggested to be involved in the pathogenesis of chronic inflammatory diseases. However, immunopathogenesis remains to be investigated among patients with allergic rhinitis (AR).

Objective: This study sought to investigate the role of PAR-2 in the modulation of epithelial barrier function and the expression of TJs in the nasal mucosa of AR patients.

Methods: The expression of TJs and PAR-2 of the nasal mucosa in AR patients and control subjects by immunohistochemistry, quantitative real-time polymerase chain reaction (qRT-PCR), and western blotting. *In vitro*, Primary human nasal epithelial cells (pHNECs) of AR patients were stimulated by Der p1 to analyze the correlation between PAR-2 and TJs expression. Der p1-induced pHNECs were treated with the PAR-2 agonist SLIGRL-NH₂ and antagonist FSLLRY-NH₂. Fluorescein isothiocyanate–dextran 4 kDa detection was employed as an indicator of epithelial permeability.

Results: Lower expression levels of TJs in the nasal epithelium of AR patients were observed in comparison with that in control subjects. The PAR-2 level was markedly increased following treatment with 1,000 ng/mL of Der p1 for 24 hours in a cellular model of AR. The expression of PAR-2 was increased in Der p1-induced pHNECs of AR patients and correlated inversely with zonula occludens (ZO)-1 and claudin-1. Treatment with Der p1 further downregulated TJs expression and promoted an increased epithelial permeability in Der p1-induced pHNECs.

Conclusions: PAR-2 could downregulate the expression of ZO-1 and claudin-1, which is involved in epithelial barrier dysfunction in AR.

Keywords

allergic rhinitis, tight junctions, primary human nasal epithelial cells, protease-activated receptor-2, ZO-1, Claudin-1

Abbreviations

PAR-2, Protease-activated receptor-2; TJs, Tight junctions; AR, Allergic rhinitis; ZO-1, zonula occludens-1; pHNECs, Primary human nasal epithelial cells; JAM-A, junction adhesion molecule-A; qRT-PCR, quantitative real-time polymerase chain reaction; FD4, Fluorescein isothiocyanate–dextran 4 kDa

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Introduction

Allergic rhinitis (AR), with an estimated prevalence of 30% in the Western population, is defined as an anaphylactic inflammation of the nasal mucosa resulting from an immunoglobulin E (IgE)-mediated allergy to inhaled allergens, pathogens, and other foreign substances.¹ Traditionally, AR is a chronic upper-airway inflammatory disease with symptoms of nasal obstruction, sneezing, nasal pruritis, and clear rhinorrhea,² which is influenced by multiple factors such as genes and the environment.³ Recently, dysfunctional epithelial integrity together with altered tight junctions (TJs) expression were found in patients with allergic inflammatory diseases.^{4,5}

The airway epithelial cells as a structural barrier play a crucial role against inhaled allergens and pathogens and are regulated greatly by TJs. TJs are intercellular junctional complexes located on the apical aspect of the lateral membranes of epithelial cells that consist of different transmembrane proteins.^{6,7} TJs are closely related to epithelial permeability and inflammatory changes, which play an important role in the maintenance of epithelial barrier function.⁸ Disruption of TJs function, expression, or both can lead to the entry of foreign pathogens, irritants, and allergens into the organism.⁸ Several previous studies have reported that in the epithelium of the human nasal mucosa *in vivo*, mRNAs of occludin, zonula occludens (ZO)-1, ZO-2, junction adhesion molecule (JAM)-A, and claudin 1, -2, -4, -7, -8, -12, -13, and -14 were detected.^{4,9} Some research on TJs has also shown that the exposure of Der p1 antigen increases epithelial permeability and decreases the expression of TJs in primary human nasal epithelial cells (pHNECs).^{5,10} Currently, knowledge about the underlying molecular mechanisms of epithelial barrier dysfunction in AR is lacking.

Protease-activated receptors (PARs) are transmembrane G-coupled receptors located on cell surfaces, which contribute to the chronic inflammatory diseases such as asthma, inflammatory bowel disease, and multiple sclerosis.^{11–13} It is known that the airway epithelium expresses PAR-1, PAR-2, and PAR-4, with PAR-2 having a much greater response to the stimulation of receptors than the others.¹⁴ PAR-2 presents in various kinds of cells including airway epithelial cells and participates in the regulation of the functions of these cells.¹⁵ It is of interest that these epithelial cells themselves are an important source of trypsin, an agonist of PAR-2.^{16,17} Some earlier studies had reported that PAR-2 signaling plays an important role in cockroach extract (CE)-induced airway hyper-responsiveness and allergic airway inflammation through the modulation of immune mediators.¹⁸ However, the precise mechanism

by which PAR-2 regulates the interactions of TJs remains unknown.

In this study, we proposed the hypothesis that PAR-2 expression is involved in epithelial barrier dysfunction and plays a vital role in the pathogenesis of AR. We aimed to investigate the role of PAR-2 in the modulation of epithelial barrier function and the expression of TJs in the nasal mucosa of patients with AR.

Materials and Methods

Subjects

The nasal mucosa of AR patients (n = 18) and control subjects (n = 26) were obtained from the Department of Otorhinolaryngology, Head, and Neck Surgery at the First Affiliated Hospital of NanChang University in China. The diagnosis of AR was made based on the combined evaluation of patients' clinical history, results of skin prick test, and allergen-specific IgE findings in the serum.¹⁹ Selected AR patients underwent endoscopic Vidian neurectomy. Those in the control group had no history of AR and no evidence of sinonasal mucosal inflammation under endoscopic examination but instead received endoscopic surgeries for other indications such as nasal septoplasty and optic nerve decompression. Biological specimens of inferior turbinate mucosa were obtained from AR patients and control subjects. The study protocols were approved by the local ethics committee of the First Affiliated Hospital of Nanchang University (Nanchang, China) [(2017) Medical Research Review no. (080)] and written informed consent was obtained during each preoperative clinic visit. Demographic data for AR patients and control subjects are listed in Table 1.

Cell Culture and Der p1-Induced Cellular Model of AR

Each specimen was washed with sterile saline after surgical removal and placed in a sterile centrifuge tube with 5 mL of phosphate-buffered saline containing 10% streptomycin. It was contraindicated to cut or separate specimens to refrain from mucosal damage. The submucosal tissue was degraded enzymatically to ensure purification. Each specimen was placed in a 15 mL centrifuge tube containing 10 mL of trypsin with a concentration of 1.4 mg/mL and incubated in 37° water for 1 hour before adding 1 mL of fetal bovine serum to stop the digestion. Each cell suspension was mixed and transferred to another centrifuge tube before adding 5 mL of pure minimal essential culture media. These tubes were inverted up and down to release more cells and these cells were transferred to centrifuge tubes containing cell suspensions. A total of 5 mL of Dulbecco's modified Eagle medium/F12 complete medium was added to the pellets

Table 1. Demographic and Clinical Characteristics of Subjects.

	AR	Control	P ^a
Simple size [No.]	18	26	N/A
Age(y), mean $\pm$ SD	36.63 $\pm$ 16.52	32.63 $\pm$ 20.93	N/A
Gender [M/F]	8/10	12/14	N/A
SPT(+) [No.]	18	0	P < 0.001
Asthma [No.%]	0	0	N/A
Atopy [No.%]	0	3 (11.5)	N/A

^aThese data were calculated by using Fisher's exact test, and $p < 0.05$ was considered as statistically significant. AR, Allergic rhinitis; SPT, skin prick test.

at the bottom of each centrifuge tube, and these cells were incubated for 1.5 hours at 37° and 5% CO₂. The cell suspension was centrifuged at 1,200 revolutions/min for 5 minutes, then cultured in a cell incubator with 5 mL of bronchial epithelial cell growth medium (BEGM) (Lonza Group, Basel, Switzerland) at 37° in 5% CO₂. BEGM media was changed every 48 hours.

The cells were incubated with different concentrations (0, 100, 500, and 1,000 ng/mL) of Der p1 (Prospec, Rehovot, Israel) for 24 hours until the cell layer reached 70% confluency. Cell lysates were collected at the time points of 0, 8, 16, and 24 hours, and quantitative real-time polymerase chain reaction (qRT-PCR) was performed to obtain the optimal time and optimal concentration of cellular model induced by Der p1 according to the epithelial permeability. Then, the cellular model was treated with deionized water, 50 μ mol/L of PAR-2-agonists (SLIGRL-NH₂; Toris, Cambridge, UK) and PAR-2-antagonists (FSLRY-NH₂; Toris, Cambridge, UK). This experiment involved four groups (i.e., control group of HCS, Der p1 group, Der p1 + SLIGRL-NH₂ group, and Der p1 + FSLRY-NH₂ group).

Immunohistochemistry

Standard immunohistochemical procedures were applied to the specimens. Details have been described previously.²⁰ Specimen slices were stained with secondary antibodies including mouse antibodies and rabbit antibodies as the following proteins: claudin-1(1:200; Lifespan Biosciences, Seattle, WA, USA), claudin-3 (1:100; Abcam, Cambridge, UK), claudin-7 (1:150; Invitrogen, Carlsbad, CA, USA), claudin-12 (1:100; Sigma-Aldrich, St. Louis, MO, USA), occludin (1:200; Abcam, Cambridge, UK), ZO-1 (1:100; Novus Biologicals, Centennial, CO, USA), JAM-A (1:250; Abcam, Cambridge, UK), and PAR-2 (1:200, Abcam, Cambridge, UK). Human nasal epithelial cells and submucosal gland tissue were analyzed using the ImageJ analysis software (National Institutes of Health,

Bethesda, MD, USA) and the results were expressed as average optical density values per unit area.

Western Blotting

Western blotting was performed in a manner consistent with the method described by Yan et al.²¹ The human nasal mucosa was ground to a powder with liquid nitrogen, added with 300 μ L of lysate phenylmethylsulfonyl fluoride (PMSF), and lysed on ice for 30 minutes. The supernatant was clarified by centrifugation at 12,000 rpm for 5 minutes and the protein concentration was measured using the bicinchoninic acid protein assay kit. The obtained samples were heated at 95°C for 15 minutes, naturally cooled on ice, and then resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% polyacrylamide gels. After electrotransference of the proteins to nitrocellulose membranes, the membranes were blocked for 1 hour by 5% bovine serum albumin and incubated first with primary antibodies for 12 hours at 4°C with diluted rabbit anti-human claudin-1 (1:200; Lifespan Biosciences, Seattle, WA, USA), rabbit anti-human claudin-3 (1:1,000; Abcam, Cambridge, UK), rabbit anti-human claudin-7 (1:500; Invitrogen, Carlsbad, CA, USA), rabbit anti-human claudin-12 (1:1,000; Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-human claudin (1:1,500; Abcam, Cambridge, UK), rabbit anti-human ZO-1 (1:1,000; Novus Biologicals, Centennial, CO, USA) rabbit anti-human JAM-A (1:1,000; Abcam, Cambridge, UK), and mouse anti-PAR-2 (1:1,000; Abcam, Cambridge, UK). Membranes were then incubated with anti-mouse or anti-rabbit antibody as the secondary antibody for 2 hours. Finally, the protein bands were visualized by incubating the membranes with SuperSignal West Pico maximum sensitivity substrate (Thermo Fisher Scientific, Waltham, MA, USA) and processed using the Bio-Rad Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA).

RNA Isolation and qRT-PCR Analysis

The methodologies of mRNA isolation and qRT-PCR used have been described elsewhere.²² Total RNA was extracted from nasal biopsy specimens with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in a manner consistent with the manufacturer's directions. Complementary DNA was synthesized by 4 μ g of total RNA with an oligo (dT) 18 primer, while M-MLV reverse transcriptase (TaKaRa Bio, Kusatsu, Japan) was used for reverse transcription. qRT-PCR was conducted using the ABI PRISM 7600 detection system (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Taq (TaKaRa Bio, Kusatsu, Japan). The primer sequences of claudin-1, -3, -4, -7, and -12;

Table 2. Primer Sequences Used for RT-qPCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
β -Actin	GGACATCCGCAAAGACCTGT	CTCAGGAGGAGCAATGATCTTGA
Claudin-1	GGATTTACTCCTATGCCGGCGACAACA	CTCTGCGACACGCAGGACATCCA
Claudin-3	CATCACGTCGCAGAACATCTG	TCGTACACCTTGCACTGCATCT
Claudin-7	AGGCATAATTTTCATCGTGG	GAGTTGGACTTAGGGTAAGAGCG
Claudin-12	AGTACTCCACAAGCTTGCCT	TCTCCAGTTGGGAAGCAGAG
ZO-1	CGGTCCTCTGAGCCTGTAAG	GGATCTACATGCGACGACAA
JAMA-A	GCGGCTGAAATGGTTGTTTG	TCCACAACAAGAGCTCCCAT
Occludin	CGAGGAGTGGGTAAAAATGTGT	ACTGTCAACTCTTTCCACATAGTCAGA
PAR-2	CTGCTGATTCTGTGGTCAC	CCAATGGCCAGAGAGAGGAA

RT-qPCR, reverse transcription quantitative polymerase chain reaction; ZO-1: zonul occludens-1; JAM-A: junctional adhesion molecule-A; PAR-2: protease activated receptor-2.

ZO-1; JAM-A; occludin; PAR-2; and the housekeeping genes encoding β -actin (ACT2B) are listed in Table 2.

Paracellular Flux Measurements

Fluorescein isothiocyanate–dextran 4 kDa (FD4) (Sigma-Aldrich, St. Louis, IL, USA) was used as a surrogate marker of layer integrity. The nasal epithelial permeability was determined at 0, 8, 16, and 24 hours in basolateral medium with a fluorescence reader. FD4 concentration was calculated and expressed in picomoles (pmols).

Statistical Analysis

Data statistical analyses were performed with the Graph Pad Prism 6 statistical package (GraphPad Software, La Jolla, CA, USA). Count data were analyzed by nonparametric testing. Different groups were compared by one-way analysis of variance followed by Tukey's multiple comparison testing. Correlation analysis was performed with the Spearman test. Values were considered statistically significant at $p < 0.05$.

Results

TJs and PAR-2 Expression at Levels of Protein and mRNA in the Nasal Epithelium of AR Patients and Control Subjects

Epithelial barrier integrity was evaluated in AR patients and control subjects. Immunohistochemistry, western blotting, and qRT-PCR were used to investigate the expression levels of TJs and PAR-2 in the nasal epithelium. We found that the expression levels of claudin-1, -3, -7, and -12; JAM-A; ZO-1; and occludin were significantly decreased in the nasal epithelium of AR patients as compared with in control by immunohistochemistry and western blotting (Figures 1(A) and (B) and 2(A) and (B)). Conversely, the expression of PAR-2 was

increased in the nasal epithelium of AR patients as compared with in control group (Figures 1(A) and (B) and 2(A) and (B)).

Next, qRT-PCR revealed decreased mRNA levels of claudin-1, -3, -7, and -12; JAM-A; occludin; and ZO-1 in the nasal epithelium of AR patients when compared with that in control subjects (Figure 3(A) to (G)). Inversely, the expression of PAR-2 was markedly increased in the nasal epithelium of AR patients as compared with among control group ($p < 0.0001$; Figure 3(H)). Also, after exploring the relationship between PAR-2 and TJs in the nasal epithelium of AR patients, it was found that PAR-2 was negatively correlated with claudin-1 ($r = -0.8641$; $p < 0.001$; Figure 4(A)) and ZO-1 ($r = -0.714$; $p = 0.009$; Figure 4(G)) of AR patients. There was no difference noted between the mRNA expression levels of claudin-3, -7, and -12; JAM-A; and occludin correlated with PAR-2 (Figure 4(B) to (F)).

Overall, these results indicate that decreased expression levels of claudin-1 and ZO-1 in the nasal epithelium of AR patients are correlated inversely with PAR-2.

TJs and PAR-2 Expression mRNA in Der p1-Induced AR Cellular Model

The pHNEC culture system induced by Der p1 was used to explore the effects of PAR-2 on epithelial barrier function. Cultured pHNECs in vitro were cocultured with different concentrations of Der p1 for 24 hours. The expression levels of claudin-1 and ZO-1 were markedly decreased after treatment with 500 ng/mL or 1,000 ng/mL of Der p1 in a dose-dependent manner. The optimal concentration of Der p1 for in vitro testing was determined (Figure 5(A)). Then, cultured pHNECs in vitro were treated with 1,000 ng/mL of Der p1 at different time points. It was found that claudin-1 and ZO-1 were significantly decreased at different time points after being treated with 1,000 ng/mL of Der p1 in a

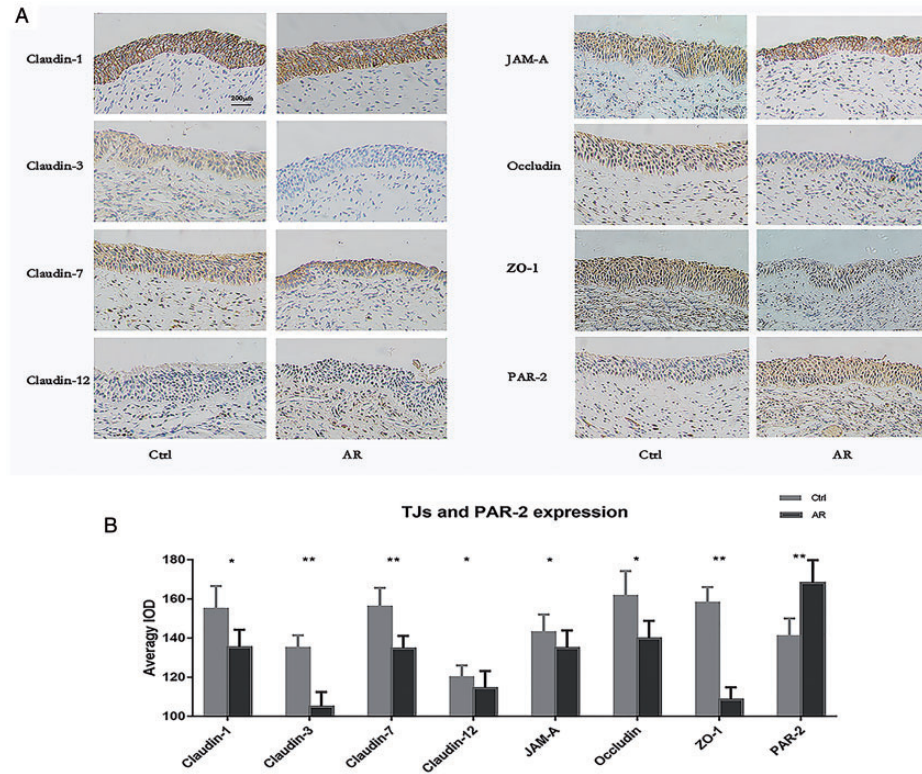


Figure 1. TJ and PAR-2 expression levels in the nasal mucosa epithelium of AR patients and control were analyzed by immunohistochemistry. Immunohistochemical staining images of claudin-1, -3, -7, and -12; JAM-A; occludin; ZO-1; and PAR-2 in the nasal mucosa epithelium of AR patients and HCS are shown in A and B ($\times 400$). A, The expressions of claudin-1, -3, -7, and -12; JAM-A; occludin; and ZO-1 in the nasal mucosa epithelium of AR patients were lower than in control. Meanwhile, the expression of PAR-2 was significantly increased in the nasal mucosa epithelium of AR patients than in control. B, The corresponding expression levels are shown as bar graphs. These data are expressed as the mean $\pm$ standard error of the mean (SEM). * $p < 0.05$ and ** $p < 0.01$.

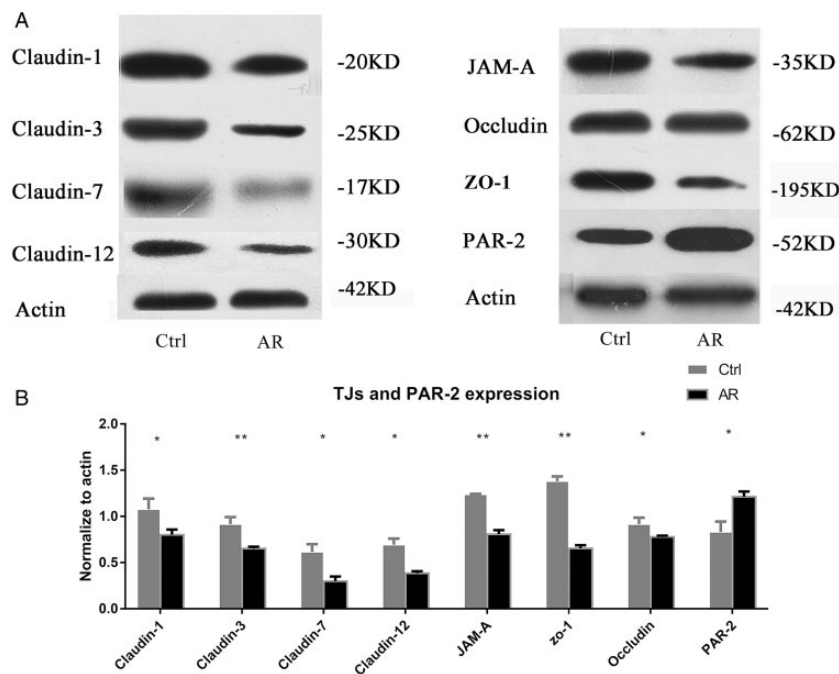


Figure 2. TJ and PAR-2 expression levels in the nasal mucosa epithelium of AR patients and control were analyzed by western blotting. A, Western blotting for the expressions of claudin-1, -3, -7, and -12; JAM-A; occludin; ZO-1; and PAR-2 in the nasal mucosa epithelium of AR patients ($n = 18$) and control subjects ($n = 26$). β -actin was used as a loading control. B, The corresponding expression levels are shown as bar graphs. These data are expressed as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$.

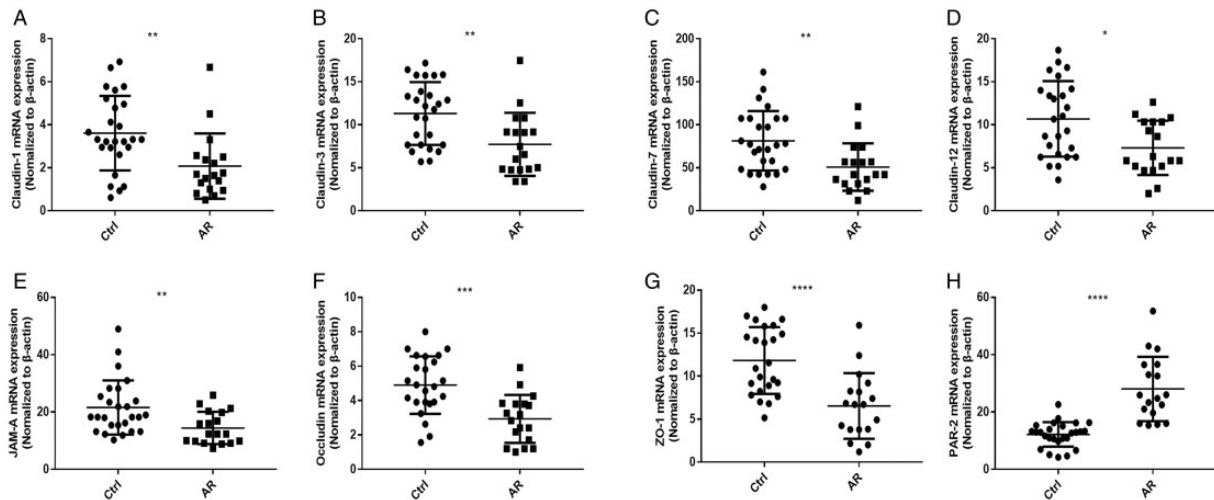


Figure 3. The mRNA expression levels of TJs and PAR-2 in the nasal mucosa epithelium of AR patients and control were analyzed by qRT-PCR. A–H, qRT-PCR for the mRNA expressions of claudin-1, -3, -7, and -12; JAM-A; occludin; ZO-1; and PAR-2 in the nasal mucosa epithelium of AR patients as compared with in control. The corresponding expression levels are shown as dot plots with medians and interquartile ranges. These data are expressed as the mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$.

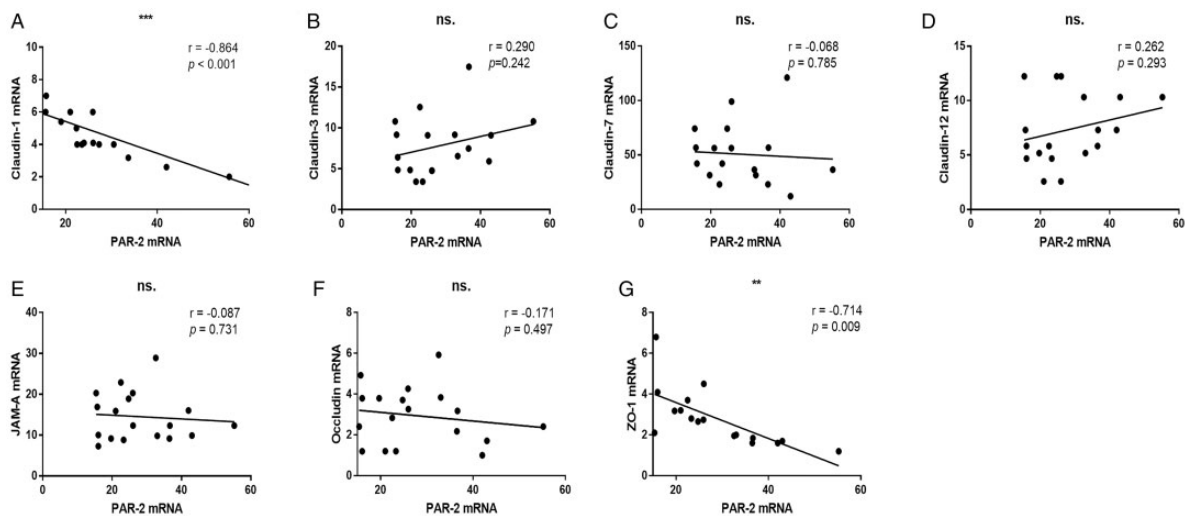


Figure 4. Correlation analysis among the expression levels of TJ and PAR-2 mRNAs in the nasal mucosa epithelium of AR patients. A and G, The expression of PAR-2 mRNA was negatively correlated with claudin-1 ($r = -0.864$; $p < 0.001$) and ZO-1 ($r = -0.714$; $p = 0.009$) in AR patients. B–F, There was no significant correlation of mRNA expression among PAR-2 and claudin-3, -7, and -12; JAM-A; and occludin. These data are expressed as the mean $\pm$ SEM. ** $p < 0.01$; *** $p < 0.001$. n.s., not significant.

time-dependent manner (Figure 5(B)). Meanwhile, the expression of PAR-2 appeared markedly increased after treatment with different concentrations of Der p1 at different time points in a dose- and time-dependent manner (Figure 5(A) and (B)).

FD4 epithelial permeability, a surrogate marker for the integrity of the epithelial barrier, was significantly increased after treatment with 1,000 ng/mL of Der p1 in the pHNECs of AR patients. Different concentrations of Der p1 all had significant effects on FD4 permeability at 24 hours (Figure 5(C)). Based on these data, treatment

with 1,000 ng/mL of Der p1 for 24 hours was ideal to evaluate the potential mechanisms involved in PAR-2-induced epithelial barrier dysfunction.

PAR-2 Activation Decreased TJs Expression and Increased Epithelial Barrier Permeability in Der p1-Induced pHNECs

To further explore the role of PAR-2 on the expression of TJs in Der p1-induced pHNECs, cultured pHNECs were incubated with PAR-2 agonist (SLIGRL-NH2) or

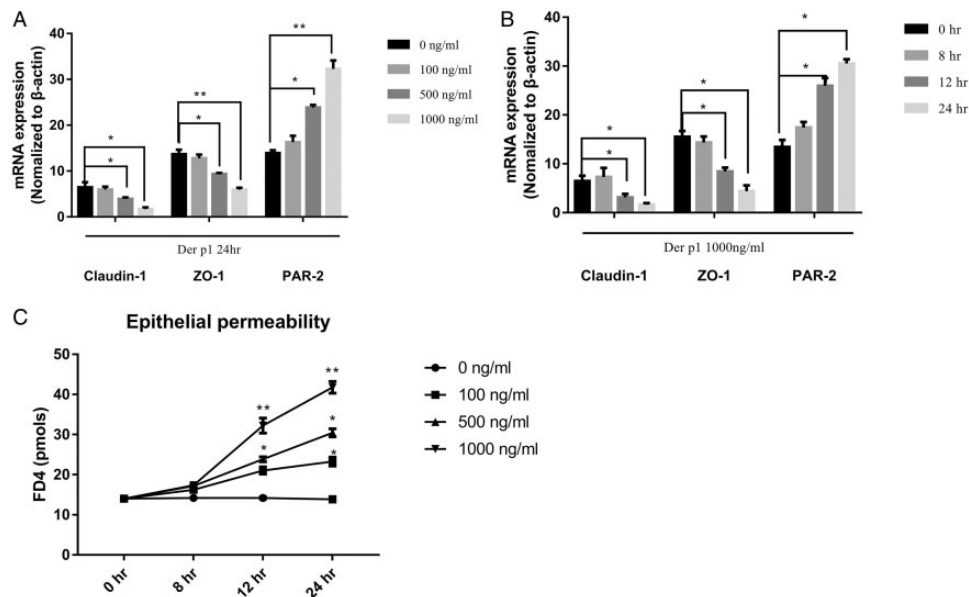


Figure 5. Stimulation of Der p1 resulted in the downregulation of mRNA expression of TJs and the upregulation of mRNA expression of PAR-2 in a dose- and time-dependent manner. A, Effects of different concentrations of Der p1 on TJs and PAR-2 mRNA expression in the nasal epithelial cells of AR patients. B, Effects of different time points of 1,000 ng/mL of Der p1 on TJs and PAR-2 mRNA expression in pHNECs. C, Treatment with different concentrations of Der p1 on FD4 permeability at different time points. These data are expressed as the mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. n.s., not significant.

antagonist (FSLRY-NH2) at a concentration of 1,000 ng/mL of Der p1 for 24 hours. As a control, the pHNECs of AR patients were treated with the deionized water. The Der p1 group showed significantly increased PAR-2 mRNA expression as compared with control group ($p < 0.05$), while, at the same time, the PAR-2 mRNA level was increased significantly in Der p1 in the SLIGRL-NH2 group as compared with in control group ($p < 0.05$; Figure 6(A)). Meanwhile, no difference between the Der p1 + FSLRY-NH2 group and Der p1 group was found (Figure 6(A)).

Next, it was observed that the mRNA expression levels of claudin-1 and ZO-1 were markedly decreased in Der p1-induced pHNECs as compared with among control ($p < 0.01$). The combination of Der p1 with SLIGRL-NH2 in Der p1-induced pHNECs resulted in the decreased mRNA expression of claudin-1 and ZO-1 as compared with the Der p1-only treated group ($p < 0.05$) and control group ($p < 0.001$; Figure 6(B) and (C)). In addition, the mRNA expression levels of claudin-1 and ZO-1 were not statistically significant among AR patients treated with Der p1+FSLRY-NH2 as compared with the Der p1-only treated group (Figure 6(B) and (C)). Lastly, we employed a Der p1-induced AR cellular model to study epithelial permeability after Der p1 challenge and further analyzed the effects of SLIGRL-NH2 and FSLRY-NH2 on nasal mucosal permeability (Figure 7). It was found that the Der p1+SLIGRL-NH2 group showed a significant

impact regarding FD4 passage in comparison with the other groups (Figure 7). Furthermore, it was observed that both the Der p1+FSLRY-NH2 group and Der p1 group nearly had the same effect in altering the FD4 passage as compared with among control group (Figure 7).

Taken together, these findings suggest that Der p1 increased the expression of PAR-2 in pHNECs while PAR-2 activation decreased the expression levels of ZO-1 and claudin-1 as well as further destroyed the epithelial barrier function.

Discussion

AR is a chronic, prevalent inflammatory disorder of the nasal mucosa, with the pathophysiologic mechanisms responsible for the severity of disease being only partly understood. A dysfunctional nasal epithelial barrier of AR patients has been hypothesized to result in excessive allergen exposure and stimulation of inflammatory cells.²³ We confirmed that PAR-2 decreased ZO-1 and Claudin-1 expression together with epithelial barrier dysfunction was found in pHNECs with Der p1-induced AR. Our study reveals for the first time at the cellular level that Der p1-induced pHNECs increased the mRNA expression of PAR-2, which activation decreases ZO-1 and Claudin-1 expression and further disrupts the barrier function of AR patients.

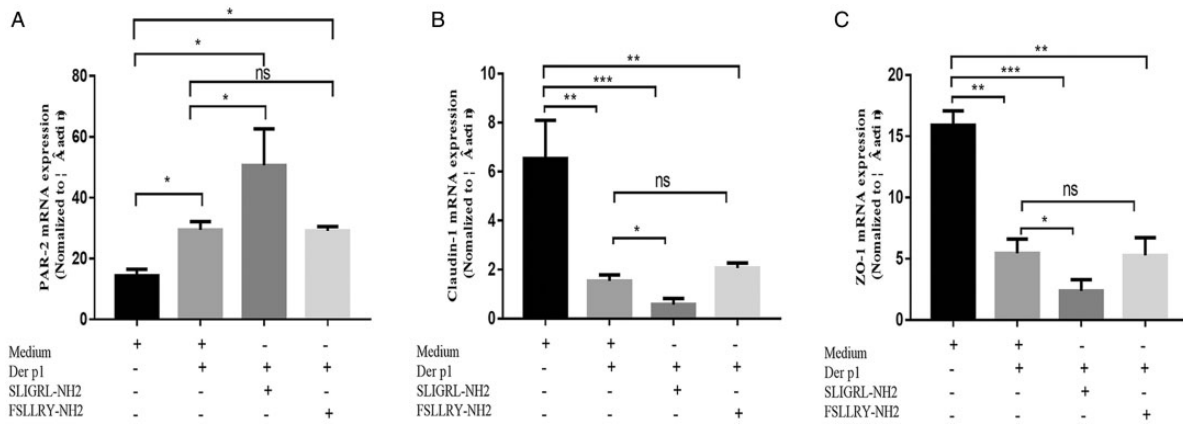


Figure 6. PAR-2 downregulated the expression of TJs in Der p1-induced pHNECs. A, Effects of Der p1 on the expression of PAR-2 mRNA in pHNECs. B and C, Effects of PAR-2 agonist and antagonist on mRNA expression of TJs in pHNECs of AR patients. These data are expressed as the mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ n.s., not significant.

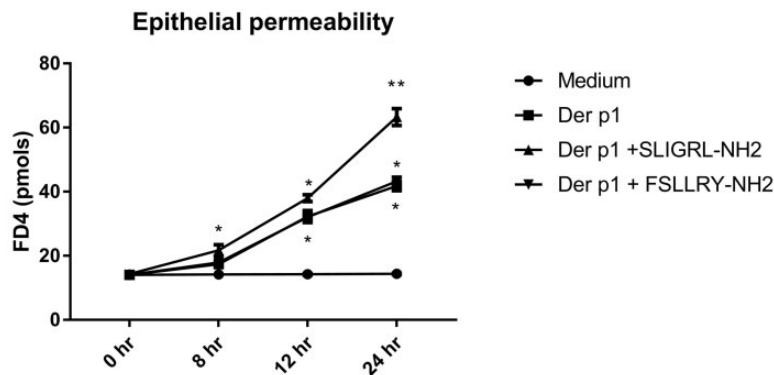


Figure 7. Der p1-induced pHNECs model of AR patients designed to study the epithelial permeability *in vitro*. FD4 passage in Der p1-induced pHNECs at different time points. These data are expressed as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$.

The airway epithelium, as the first line of defense as a physical barrier against exotic allergens, has recently been shown to play an immunomodulatory role in allergic asthma.²⁴ A previous study found that the exposure of human bronchial epithelial cells to cigarette smoke extract significantly prompted the downregulation of ZO-1 and ZO-2 proteins.^{25,26} Meanwhile, it was reported that the activation of PAR-2 led to the disruption of TJs and increased the barrier permeability through the activation of p38. MAPK, which may lead to the initiation and exacerbation of respiratory inflammation.²⁷ In agreement with these data,^{4,28} our study examined that lower expression levels of claudin-1, -3, -7, and -12; JAM-A; ZO-1; and occludin were found in patients with AR at both the tissue and cellular levels. Here, we also demonstrated that the expression of PAR-2 was significantly upregulated in patients with AR, which negatively correlated with the claudin-1 and ZO-1 levels of AR patients. These findings showed that PAR-2 can

have a vital role in AR, which points to the existence of an important mechanism in the pathology of AR.

House dust mite extracts induce increased secretion from submucosal glands in the respiratory tract and increase calcium ion influx within pulmonary sensory neurons through the activation of PAR-2.²⁹ Der p1 antigen exposure has previously been reported to increase epithelial permeability and decrease the TJ proteins JAM-A and claudin-1 in samples of cultured primary sinonasal epithelial cells in the context of chronic rhinosinusitis.³⁰ We used a Der p1-induced AR cellular model to study the underlying connections between PAR-2 and TJs. In our study, we evaluated *in vitro* whether pHNECs showed increased nasal epithelial permeability after treatment with different concentrations of Der p1. Importantly, we demonstrated for the first time at the cellular level that TJs and PAR-2 mRNA expression levels were significantly changed at 24 hours following a challenge with 1,000 ng/mL Der p1, suggesting that

the barrier was affected in a dose- and time-dependent manner. Therefore, we believe that the Der p1-induced AR cellular model is a suitable model by which to explore the effects of PAR-2 on nasal epithelial function.

To further understand the suppression of TJs in the airway epithelia of patients with allergic disease, we evaluated the effects of SLIGRL-NH2 and FSLLRY-NH2 on the expression of TJs in Der p1-induced pHNECs. Interestingly, we found that, as compared with in control and Der p1 group, the expression of PAR-2 mRNA in pHNECs was significantly increased after treatment with SLIGRL-NH2. Several previous reports have shown that the activation of PAR-2 may play a pivotal role in the pathogenesis of allergic respiratory diseases.^{31–33} Our findings are consistent with the results of these studies and indicate that Der p1, an exogenous allergenic peptidase derived from the house dust mite, can cause PAR-2 activation directly in airway epithelial cells.³⁴ We have also observed that SLIGRL-NH2 decreased the expression of claudin-1 and ZO-1 in Der p1-induced pHNECs, which was expected because of the increasing PAR-2 expression. Our observations on pHNECs indicate that PAR-2 agonists can regulate paracellular permeability, and our observations here similarly support the outcomes of previous studies. However, the exact mechanism of increased PAR-2 expression in AR patients is not fully known. FSLLRY-NH2, an antagonist of PAR-2, has previously been reported to act as an anti-inflammatory agent in hydrogen peroxide-induced HepG2.³⁵ Intriguingly, beyond our expectations, FSLLRY-NH2 had no effect on decreasing the mRNA expression of claudin-1 and ZO-1 in Der p1-induced pHNECs and did not result in decreased epithelial permeability. These data clearly showed that the signaling for Der p1-induced pHNECs regulation of these TJs genes may be different from that for PAR-2 induced gene regulation. This discrepancy might be explained by that the increased inflammatory signals and blocking action by FSLLRY-NH2 could not represent all possible mechanisms of AR in our cellular model.

There are some limitations to our study, which should be discussed. First, we studied the effects on isolated pHNECs; whether other cells such as dendritic cells experience similar effects is still unknown. Second, the molecular mechanisms by which PAR-2 regulates interactions of TJs are not clear. Further study is required to clarify the effects of PAR-2 on TJ expression *in vivo*. Third, we found that FSLLRY-NH2 had no effect on decreasing the expression of claudin-1 and ZO-1 in Der p1-induced pHNECs within 24 hours. Further research is warranted to determine the optimal dosage and timing to limit the off-target effects of PAR-2 antagonists. In a nutshell, this study provides new insights into the epithelial barrier defects of AR patients. These results reveal that PAR-2 activation can destroy the epithelial barrier

by promoting the expression of PAR-2 and enrich the pool of evidence regarding our understanding of AR. More research is still needed to reveal the precise mechanisms by which PAR-2 regulates the interactions of TJs, which may ultimately help in developing new preventive and therapeutic targets in relevant allergic diseases in the future.

Declaration of Conflicting Interests

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