

TRPV4-Mediated Epithelial Junction Disruption in Allergic Rhinitis Triggered by House Dust Mites

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Abstract

Background: Epithelial barrier disruption is a crucial feature of allergic rhinitis (AR). Previous reports have indicated the role of transient receptor potential vanilloid (TRPV) 4 in regulating the intercellular junctions in various cells. However, the role of TRPV4 and its regulation by T helper 2 cell cytokines in the epithelial cells of patients with AR remains unclear.

Objective: We aimed to elucidate the expression of TRPV4 in nasal epithelial cells and its cytokine-induced regulation, and to reveal its role in house dust mite-induced junction disruption in AR.

Methods: The expression of TRPV4 in nasal epithelial cells was measured using real-time polymerase chain reaction, western blot, and immunohistochemical assays, and the expression levels were compared between the patients with AR and healthy controls. Altered expression of TRPV4 was induced in cultured nasal epithelial cells by stimulation of interleukin (IL) 4, IL-13, and tumor necrosis factor alpha. In addition, expression of E-cadherin and zonula occludens 1 was induced in Der p 1-stimulated epithelial cells by treatment with either a TRPV4 agonist (GSK1016790A) or a TRPV4 antagonist (RN1734).

Results: TRPV4 expression was increased in epithelial cells harvested from the affected turbinates compared to those from the normal turbinates. The stimulation of cultured epithelial cells with IL-4 and IL-13 resulted in TRPV4 upregulation. Additionally, E-cadherin and zonula occludens 1 expression levels decreased in the cultured epithelial cells treated with GSK1016790A after stimulation with Der p 1, whereas Der p 1 stimulation alone showed no effect on junctional protein expression.

Conclusions: Increased TRPV4 expression occurred in epithelial cells harvested from patients with AR and epithelial cells stimulated by Th2 cytokines. Decreased junctional protein expression in epithelial cells after the stimulation by house dust mite allergen with TRPV4 agonist indicates a possible role of TRPV4 in the pathogenesis of allergen-induced epithelial barrier disruption in AR.

Keywords

allergic rhinitis, epithelial cells, epithelial barrier, transient receptor potential vanilloid channels, TRPV4, house dust mites, allergen, cytokine, E-cadherin, ZO-1

Introduction

Allergic rhinitis (AR) is an immunoglobulin E (IgE)-mediated disease in response to the inhalation of an allergen. Epithelial cells play a key protective role as they are the first physiological barrier against allergen infiltration and they also release epithelial-derived cytokines to promote T helper (Th) 2 immune responses.^{1,2} Barriers are established through cell-cell contact with apical junctional complexes consisting of tight junctions (TJ) and adherens junctions (AJ).³ Inhaled allergens,

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including house dust mites (HDMs) are known to disrupt epithelial junctional complexes with their proteolytic activities, inducing allergen sensitization.^{4,5} In addition, Th2 cytokines are also reported to induce barrier disruption in allergic diseases.⁶

Transient receptor potential vanilloid (TRPV) channels are one of the six subfamilies belonging to the transient receptor potential (TRP) superfamily, a group of non-selective cation channels associated with the transmission of sensory information.⁷ Among the six members of the TRPV family, TRPV1-TRPV4 have moderate permeabilities for calcium channels and are gated by various chemical or physical stimuli, including temperature, pH, and mechanical forces, such as shear stress and osmolarity.⁸ Although widely known as a type of receptor involved in nociception, various other functions of TRPV channels in human respiratory diseases have been investigated.⁹ In human airway epithelial cells, TRPV4 was reported to modulate epithelial calcium levels and regulate ciliary beat frequencies.^{10,11} In addition, previous studies revealed that TRPV4 inhibition reduced stretch-induced proinflammatory cytokine release, and TRPV4 activation induced by lower levels of shear stress, enhanced epithelial barriers.^{12,13}

Altered levels or function of TRPV4 expression might play a key role in the pathogenesis of AR, as TRPV4 is present in respiratory epithelial cells and involved in barrier function. It has recently been reported that the activation of TRPV1, stimulated by HDMs, induces allergic skin inflammation in animal models and interleukin (IL)-33 secretion in respiratory epithelial cells.^{14,15} However, the effect of allergens or allergic environment on TRPV4 in airway epithelial cells has not been evaluated precisely. Since the activation of protease-activated receptor is known to stimulate TRPV4, we hypothesized that junction disruption by protease-containing allergens, such as HDMs, might be associated with TRPV4.¹⁶ Therefore, the objectives of the present study are:

1. To determine the expression levels of TRPV4 in the nasal mucosa and nasal epithelial cells harvested from the inferior turbinates of patients with AR, and to identify the alteration of TRPV4 expression by cytokines in order to reveal its possible effects on the pathogenesis of AR.
2. To reveal whether activation or inhibition of TRPV4 affects HDM allergen-induced junction disruption in nasal epithelial cells.

Methods

Patients

Ten healthy control participants and ten patients with AR were enrolled in this study. Individuals with an

active smoking status, history of recent or recurrent exacerbation for upper and lower airway infection, or ongoing medical treatment for AR (nasal steroid, antihistamines) as well as any other medical condition within 8 weeks preceding the study were excluded. All participants in the AR group had persistent, moderate/severe symptoms, including rhinorrhea, sneezing, and nasal obstruction within at least the past year, and showed a positive result only for HDMs on a skin-prick test or a multiple-allergen simultaneous test (MAST) for inhalant allergens. The healthy control group consisted of participants who underwent rhinoplasty or closed reduction for nasal bone fracture without any symptoms for AR and showed negative results on a skin-prick test and MAST.

The protocol of this study was approved by the institutional review board for human studies at Korea University Medical Center, and all participants provided written informed consent. The clinical characteristics of the participants are summarized in Table 1.

Primary Human Nasal Epithelial Cell Culture

A nasal brush (Vansco Korea, Daecheon) was inserted between the inferior turbinate and nasal septum, and the brush was gently spun 20–30 times on the middle portion of the inferior turbinate. The brush tips were immersed and suspended gently, and then placed in a transfer media in 15 ml conical tubes. The sample tubes were centrifuged at 1,200 rpm/min for 3 minutes. The supernatant was carefully aspirated, and the cells were suspended in DMEM/F12 (Gibco, Dublin, Ireland) for washing. After pipetting several times, the tubes were centrifuged at 1,200 rpm/min for 3 minutes. The supernatant was aspirated, and the cells were suspended in bronchial epithelial cell growth supplement (BEpiCM) medium (Sciencell, Carlsbad, CA, USA) and placed in 6-well plates.

Real-Time Polymerase Chain Reaction

The epithelial cells were plated on 12-well plates and treated with 100 ng/ml of cytokines (IL-4, IL-13, tumor necrosis factor (TNF)- α ; Peprotech, Rocky Hill, USA) or 2 μ g/ml Der p 1 (Indoor biotechnology, Charlottesville, VA, USA) with or without 10 nM TRPV4 agonist (GSK1016790A) or 10 μ M antagonist (RN1734). After 24 h of incubation, RNA was extracted from the control and treated cells. To extract the total RNA from the epithelial cells, detached epithelial cells were lysed with QIAzol (Qiagen, Valencia, CA, USA), and the lysates were sequentially treated with chloroform, isopropanol, and ethanol to purify the mRNA; cDNA was synthesized from the RNA by using cDNA synthesis master mix (GenDEPOT, TX, USA).

Quantitative Real-Time Polymerase Chain Reaction

The gene expression in the epithelial cells of the inferior turbinate was measured by quantitative real-time polymerase chain reaction. The prepared cDNA was amplified and quantified using the SYBR Green master mix (Qiagen, Valencia, CA, USA). Polymerase chain reactions were performed using a real-time thermal cycler system (TP850) (Takara, Shiga, Japan) with 50 cycles of a two-step reaction that consisted of denaturation at 95°C for 15 s followed by annealing-extension at 60°C for 45 s. Data were analyzed using the ΔC_t method. The primer sequences are categorized in Table 2.

Immunohistochemistry

The inferior turbinate was immediately fixed after the surgery with 4% paraformaldehyde for paraffin embedding. The paraffin-embedded turbinate blocks were cut at 4- μ m thickness using a microtome and deparaffinized. For histologic analysis, turbinate sections were stained for TRPV4 (Abcam, Cambridge, MA, USA), E-cadherin (SantaCruz Biotechnology, Dallas, TX, USA), and zonula occludens (ZO)-1 (Abcam, Cambridge, MA, USA). The slides were examined with an Olympus BX51 microscope (Tokyo, Japan). Pictures were captured by using an Olympus DP72 microscope digital camera with DP2-BSW software.

Western Blotting

Protein expression of TRPV4 and cell-to-cell adhesion molecules in the epithelial cells were detected by western blotting. Protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with the E-cadherin antibody (SantaCruz Biotechnology, Dallas, TX, USA), ZO-1 antibody (Abcam, Cambridge, MA, USA), and TRPV4 antibody (Abcam, Cambridge, MA, USA) for the target molecule, or the β -actin antibody (SantaCruz Biotechnology, Dallas, TX, USA) for reference. Proteins were visualized with a ChemiDoc imaging system (Bio-Rad, Hercules, CA, U.S.A.). The protein bands were analyzed using ImageJ.

Statistical Analysis

Statistical analysis was conducted using SPSS version 20.0 (IBM Corp., Armonk, NY). Comparisons of clinical characteristics or experimental data between the healthy controls and patients with AR were analyzed using the Student's t-test. One-way analysis of variance was used for determining the difference between cytokine- or TRPV4 agonist-/antagonist-treated samples. P-values less than 0.05 were considered to represent statistical significance.

Table 1. Patient Characteristics of the Study Groups.

Characteristics	Allergic Rhinitis	Healthy Controls	P-Value
No. of patients	10	10	
No. of male/female	7/3	8/2	0.906
Age(y), mean range	28.1 $\pm$ 11.7	33.8 $\pm$ 15.4	0.362
Serum total IgE (IU/mL)	342.2 $\pm$ 234.1	75.9 $\pm$ 23.8	0.002
Serum eosinophil (%)	4.60 $\pm$ 2.18	2.14 $\pm$ 1.05	0.005
SNOT-22	41.101 $\pm$ 11.21	11.2 $\pm$ 4.37	<.001
Endoscopic turbinate grade	5.40 $\pm$ 0.52	2.50 $\pm$ 0.71	<.001

Table 2. Forward and Reverse Primer Sequences Used in the Study.

Transcript	Orientation	Primers (5'-3')
TRPV1	Forward	AC ACA CCT GAT GGC AAG GAC
	Reverse	AG ACT GCC TAT CTC GAG CAC
TRPV3	Forward	TC CTC ACC TTT GTT CTC CTC CT
	Reverse	CG CAA ACA CAG TCG GAA ATC AT
TRPV4	Forward	CA ACA ACG ACG GCC TCT C
	Reverse	GG ATG ATG TGC TGA AAG ATC C
ZO-1	Forward	ACA GTG CCT AAA GCT ATT CCT GTG A
	Reverse	TCG GGA ATG GCT CCT TGA G
E-cadherin	Forward	TGC TCT TGC TGT TTC TTC GG
	Reverse	TGC CCC ATT CGT TCA AGT AG
GAPDH	Forward	GAG TCA ACG GAT TTG GTC GT
	Reverse	TTG ATT TTG GAG GGA TCT CG

Results

TRPV4 Expression Was Increased in the Epithelial Cells Harvested from Allergic Turbinates

To investigate the gene expression level of each TRPV channel in the nasal epithelial cells, mean FPKM values of TRPV1-6 obtained from RNA sequencing were evaluated (Supplementary Figure S1). Expression of the TRPV1, TRPV3, and TRPV4 genes in the nasal epithelial cells has been confirmed previously, whereas the expression of the TRPV2, TRPV5, and TRPV6 genes has been rarely observed. Therefore, we conducted an experimental study to verify the difference in the expression of TRPV1, TRPV3, and TRPV4 in the nasal epithelial cells derived from allergic and normal turbinates.

The mRNA levels of TRPV1 and TRPV4 were greater in the epithelial cells harvested from the patients with AR than in those from the healthy controls, while the mRNA expression of TRPV3 showed no definite difference between the two groups (Figure 1(A) to (C)). In the turbinate tissue homogenate, the transcriptional level of TRPV4 was slightly higher in the allergic turbinates than in the controls, although no definite statistical difference was identified (Figure 1(D) and (E)).

Immunohistochemistry results showed increased expression of TRPV4 in the allergic nasal epithelium (Figure 2(B)) compared to the normal mucosa (Figure 2(A)). The prominent expression of TRPV4 was observed in ciliary cells and basal cells but not in

goblet cells. TRPV4 was also expressed in the submucosal glands and infiltrating cells in the nasal mucosa.

Increased TRPV4 Expression by Th2 Cytokine Stimulation

To evaluate whether the expression of TRPV1, TRPV3, and TRPV4 is regulated by cytokines, the nasal epithelial cells were treated with Th1 cytokines (TNF- α) as well as Th2 cytokines (IL-4 and IL-13). The transcriptional expression of TRPV1 and TRPV4 in the nasal epithelial cells derived from the patients with AR was higher than that in the normal epithelial cells before cytokine treatment (Figure 3(A) and (C)). In the normal epithelial cells, the expression levels of TRPV1 and TRPV4 were higher after IL-4 and IL-13 treatment. In contrast, in the allergic nasal epithelium, no definite increase in TRPV4 mRNA expression was identified after either Th1 or Th2 cytokine stimulation. In contrast, a change in mRNA expression level of TRPV3 occurred only after TNF- α treatment in the normal epithelial cells (Figure 3(B)).

Since numerous experimental studies on the role of TRPV1 in AR have been published previously, we focused on the regulation of TRPV4 by Th2 cytokines. Immunoblotting analysis indicated increased expression of TRPV4 in the normal epithelial cells treated with IL-4 and IL-13 (Figure 3(D) and (E)). Despite the higher protein expression of TRPV4 in the allergic nasal epithelial cells before cytokine treatment than in the healthy nasal epithelial cells, it was observed that Th1 or Th2 cytokine

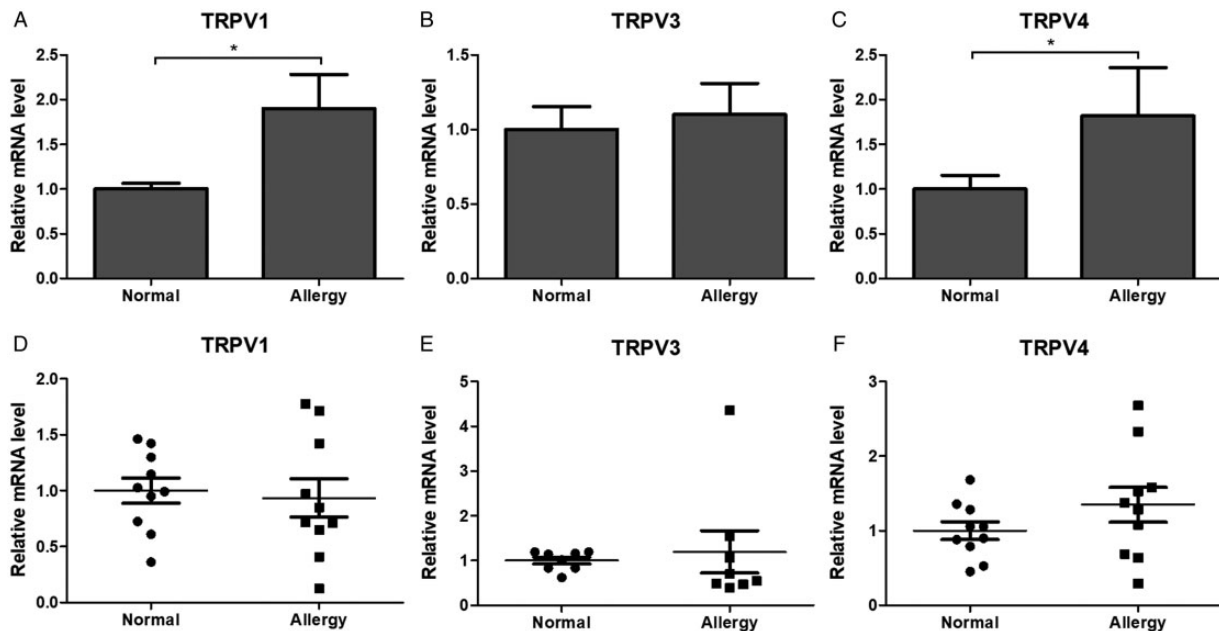


Figure 1. Transcriptional analysis of TRPV1, TRPV3, and TRPV4 in epithelial cells and mucosal tissue harvested from normal ($n = 10$) and allergic turbinates ($n = 10$). A–C, mRNA expression of TRPV1, TRPV3, and TRPV4 in primary nasal epithelial cells obtained from normal controls and patients with allergic rhinitis. D–F, TRPV1, TRPV3, and TRPV4 mRNA levels in inferior turbinate mucosa harvested from normal controls and patients with allergic rhinitis * $p < .05$.

stimulation did not regulate TRPV4 expression in the allergic epithelial cells.

TRPV4 Agonist- and Antagonist-Induced Regulation of Nasal Epithelial Cell-Adherent Molecules

To determine the effect of TRPV4 in regulating cell-to-cell junctions after allergen stimulation in the nasal epithelial cells, we evaluated the expression of the TJ marker (ZO-1) and AJ marker (E-cadherin) in the

epithelial cells exposed to the TRPV4 agonist (GSK1016790A) and TRPV4 antagonist (RN1734) after stimulation with HDM extracts.

When the primary nasal epithelial cells harvested from normal turbinates were treated solely with HDM extract for 24 h, no definite change was identified in the mRNA expression of ZO-1 (Figure 4(A)) or E-cadherin (Figure 4(B)). However, ZO-1 and E-cadherin mRNA expression decreased in the epithelial cells treated with GSK1016790A after HDM stimulation for 24 h. In the

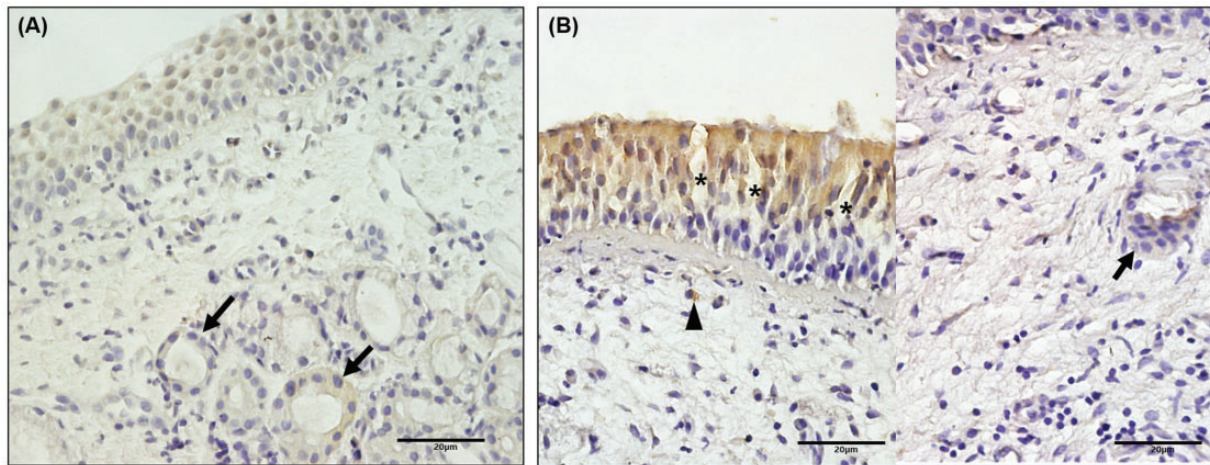


Figure 2. Immunohistochemical localization of TRPV4 in normal and allergic turbinates. In the normal turbinates (A) and allergic turbinates (B), TRPV4 was detected on the epithelium surface, submucosal glands (arrow), and inflammatory cells (arrowhead). The intensity of the staining was stronger in the nasal epithelium of the patients with allergic rhinitis than in that of the controls. *Goblet cells. Bar = 20 μ m.

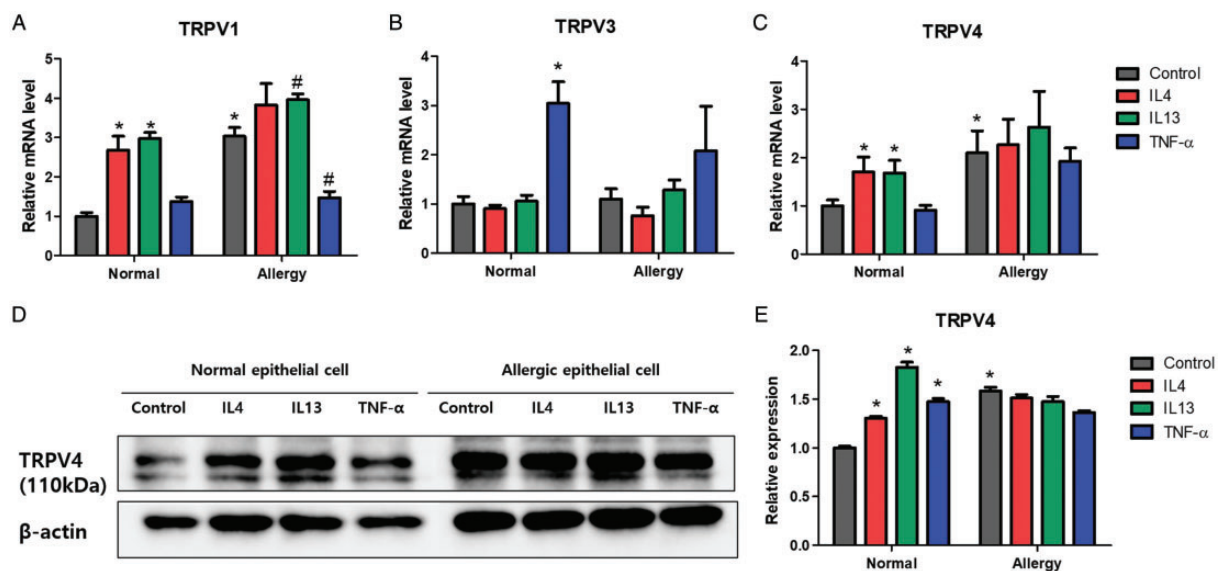


Figure 3. Cytokine regulation of TRPV1, TRPV3, and TRPV4 expression levels in primary nasal epithelial cells ($n = 5$). Transcription level alterations of (A) TRPV1, (B) TRPV3, and (C) TRPV4 after stimulation with T-helper (Th) cell type 1 (TNF- α) and Th2 (IL-4, IL-13) cytokines in normal and allergic nasal epithelial cells. (D and E) Representative immunoblot image for regulation of protein level expression of TRPV4 by cytokine stimulation with graphic and statistical analysis. Statistical significance is indicated by * $p < .05$ compared to normal control and # $p < .05$ compared to allergic nasal epithelial cells.

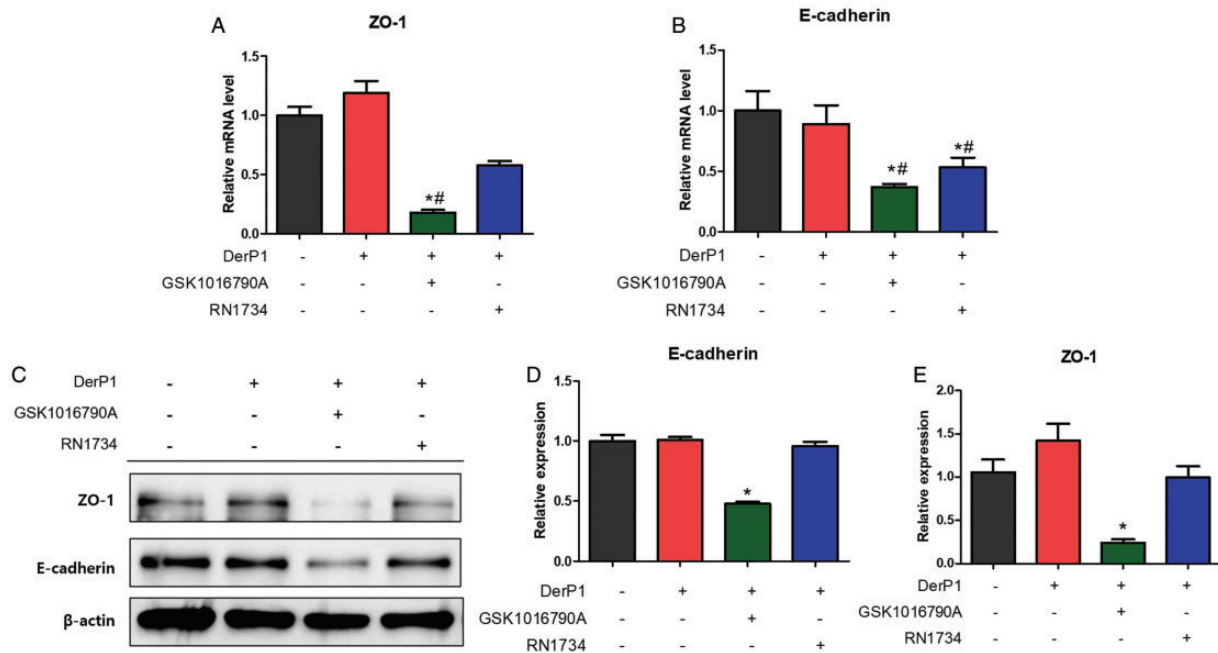


Figure 4. Regulation of adherent molecule expression (ZO-1, E-cadherin) by a TRPV-4 agonist and antagonist on primary nasal epithelial cells after allergen stimulation. Regulation in mRNA level of (A) ZO-1 and (B) E-cadherin by GSK1016790A and RN1734 after Der p I stimulation. Representative immunoblot image for protein level alteration of ZO-1 and E-cadherin with (C) graphic and (D and E) statistical analysis. Statistical significance is indicated by * $p < .05$ compared to non-treated epithelial cells and # $p < .05$ compared to Der p I treated epithelial cells.

epithelial cells treated with RN1734, diminished mRNA expression of ZO-1 was less evident, and no definite decrease in transcriptional expression was observed for E-cadherin.

In immunoblotting analysis, the normal epithelial cells solely stimulated by HDM showed no definite change in protein expression of ZO-1 and E-cadherin compared to the controls. However, after treatment with GSK1016790A, ZO-1 and E-cadherin expression decreased, while RN1734 treatment elicited no definite change in the protein expression level of adherent molecules (Figure 4(D) and (E)).

Discussion

Epithelial cell barrier disruption is an important mechanism in the pathogenesis of AR. Preserving epithelial cell barrier function can reduce the type 2 immune response induced by the influx of harmful environmental molecules, including allergens, thus is regarded as a promising therapeutic strategy for AR treatment in the future.¹⁷ Previous studies have reported enhanced permeability due to junction disruption with decreased expression of ZO-1 and E-cadherin in the nasal epithelial cells of patients with AR.^{18,19} The precise mechanism of barrier deficiency in AR is still being established, and the results from this study suggest that increased expression of

TRPV4 in the nasal epithelial cells of patients with AR might contribute to the disruption of the epithelial barrier induced by HDMs.

The mRNA expression of TRPV1 and TRPV4 from whole nasal mucosa showed no difference between controls and patients with AR. However, when the turbinate epithelial cells were evaluated separately, the expression of TRPV1 and TRPV4 was higher in patients with AR than in the control group. To date, the most comprehensively studied TRPV channel in AR is TRPV1.²⁰ Despite the known involvement of TRPV4 in asthmatic patients, the role of TRPV4 in AR has not yet been identified. Regarding nasal epithelial cells, the presence of TRPV4 and TRPV4-mediated calcium response using fluorometric calcium imaging in ciliated cells has been previously reported.¹⁰ The localization of TRPV4 in nasal ciliary cells was also reported, suggesting that TRPV4-mediated ciliary dysfunction could be a possible disease mechanism.²¹ Consistent with these results, immunohistochemistry from the present study showed the most prominent expression of TRPV4 in the ciliary epithelium, particularly in the ciliary cells of the inferior turbinate. In addition, TRPV4 was also identified in submucosal glands and inflammatory cells.

Increased TRPV4 expression by stimulation of Th2 cytokines, as seen in normal epithelial cells, was not observed in allergic epithelial cells. An explanation for

this could be that the expression of TRPV4 had already increased because of the stimulation by Th2 cytokines for a prolonged period and, therefore, it did not respond to additional *in vitro* stimulation. In a recent study of the choroid plexus epithelial cells, there was no change in TRPV4 after stimulation with anti-inflammatory cytokines, such as IL-4 and IL-10.²² It has been reported that the activation of TRPV4 in the lung epithelial cells increased the secretion of IL-6 and IL-8. However, to our knowledge, there have been no studies on the relationship between Th2 cytokines and TRPV4 in airway epithelial cells.¹³ Our findings showed increased expression of TRPV4 because of Th2 stimulation, which supports the role of TRPV4 in allergic airway diseases.

The role of TRPV4 in barrier function has been reported in various cells. The TRPV4-mediated epithelial barrier disruption with decreased E-cadherin expression was identified in a murine acute lung injury model and breast cancer cells.^{10,23} In addition, TRPV4 inhibition attenuated the mechanical stretch-induced barrier dysfunction in a murine ventilation model and human lung epithelial cells.¹³ Recent reports have demonstrated the contribution of TRPV4 in epithelial cell-to-cell disruption with decreased E-cadherin levels as well as attenuated Th2 response via TRPV4 inhibition in asthmatic murine models, supporting our results.²⁴

In this study, Der p 1 was used as an allergen to validate the role of TRPV4 in allergen-induced barrier dysfunction. HDMs are important antigens that are the leading cause of perennial AR, and all participants with AR in this study were sensitized to *D. pteronyssinus* and *D. farina*.²⁵ Der p 1, a major component of HDMs, has cysteine protease activity.²⁶ Studies of the protease activity of Der p 1 on A549 cells reported that stimulation of Der p 1 induced Ca^{2+} influx through the activation of the PAR-2 receptors, thus promoting the release of proinflammatory cytokines.^{27,28} Coupling of PAR-2 and TRPV4 has been observed; consequently, activation of PAR-2 induces sustained activation of TRPV4. Therefore, we hypothesized that TRPV4 might be involved in junction disruption because of protease activities in HDM-induced AR.^{29,30}

Previous studies on epithelial barrier function in HDM-induced AR have reported increased epithelial permeability with reduced expression of TJ proteins compared to that in healthy controls.^{31,32} Nevertheless, several *in vitro* studies on the impact of allergens on the airway epithelial barrier have reported that the junctional proteins could not be decreased by HDM extract alone, but also depended on ATP, Ca^{2+} influx, and EGFR.^{33,34} Consistent with these findings, the results from our study showed that Der p 1 alone did not decrease the expression of ZO-1 and E-cadherin after the 24 h stimulation. However, in the presence of the

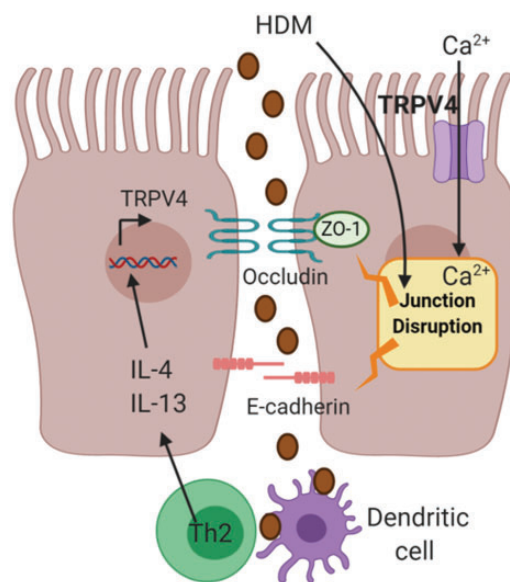


Figure 5. Hypothetical graphic depicting TRPV4 in the regulation of adherent molecule expression on epithelial cells stimulated by Th2 cytokine and house dust mite allergen (Figure created with Biorender.com).

TRPV4 agonist GSK1016790A, decreased expression of both junctional proteins was identified, indicating that TRPV4 mediates HDM-induced junctional disruption.

Conclusion

To our knowledge, this is the first study that evaluated the function of TRPV4 in the nasal epithelial cells of patients with AR. Our results indicate that the expression of TRPV4 in nasal epithelial cells could be upregulated by Th2 cytokines, and the destruction of the epithelial barrier because of HDMs mediated by TRPV4 activation may be aggravated by increased TRPV4 expression in patients with AR (Figure 5). Further studies are needed to evaluate the role of TRPV4 in epithelial junction disruption by stimulation of other allergens, such as pollens.

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Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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Supplementary figure 1. The mean FPKM values of TRPV channels obtained from RNA sequencing data of primary nasal epithelial cells (n=6)

