

Nuclear erythroid 2-related factor 2 activation inhibits house dust mite-induced sinonasal epithelial cell barrier dysfunction

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Background: Dysregulated sinonasal epithelial cell (SNEC) barrier function has been proposed to contribute to the pathogenesis of sinonasal inflammatory conditions such as allergic rhinitis (AR) and chronic rhinosinusitis (CRS). Allergens such as house dust mite (HDM) have been reported to disrupt SNEC barrier integrity. We have recently identified nuclear erythroid 2-related factor 2 (Nrf2) activation via sulforaphane (SFN) stimulation to stabilize SNEC barrier function. The purpose of this study was to explore whether Nrf2 activation could ameliorate HDM-induced SNEC barrier dysfunction.

Methods: Human SNECs (HSNECs) were grown from patients at the air-liquid interface (ALI). HSNECs were stimulated with HDM with or without pharmacologic activation of Nrf2 with SFN. HSNECs were then stained for the epithelial cell junction protein zonula occludens-1 (ZO-1) and cell surface localization was evaluated by confocal microscopy. Transepithelial electrical resistance (TER) and paracellular fluorescein isothiocyanate (FITC)-dextran permeability was measured in response to stimulation with HDM and SFN.

Results: HDM stimulation caused a global disruption of the protein ZO-1 along with an associated decrease in TER

($p < 0.001$) and increased FITC-dextran paracellular permeability ($p < 0.0001$). Enhancing Nrf2 activation through treatment with SFN prior to stimulation with HDM was associated with increased localization of ZO-1 at the cell surface and statistically significant increases in TER ($p < 0.05$) and decrease in paracellular FITC-dextran permeability ($p < 0.001$).

Conclusion: This is the first study to demonstrate that HDM-induced SNEC barrier dysfunction may be preventable by Nrf2 activation. The Nrf2 antioxidant pathway may represent a potential therapeutic target for allergen-induced sinonasal inflammation. © 2017 ARS-AAOA, LLC.

Key Words:

house dust mite; Nrf2; sinonasal epithelial barrier dysfunction; permeability; allergy

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Allergic rhinitis (AR) affects nearly 1 in 6 Americans and causes symptoms including nasal congestion,

rhinorrhea, and sneezing.¹ AR is characterized by immunoglobulin E (IgE)-mediated sinonasal inflammation in response to allergen exposure.^{1,2} Common allergens include seasonal sources such as pollen and grass, episodic such as animal dander, and perennial sources such as house dust mite (HDM). The sinonasal cavity possesses mechanisms to counteract exposure to allergens including mucociliary clearance and the sinonasal epithelial cell (SNEC) layer acting as a physical barrier. Indeed, SNEC barrier dysfunction has been proposed to be a contributing factor in disorders of sinonasal inflammation such as AR or chronic rhinosinusitis (CRS).^{3–5}

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The SNEC barrier is formed by intercellular tight and adherens junction proteins that are scaffolded to the cytoplasmic actin skeleton by proteins such as zonula occludens-1 (ZO-1).³ Disruption of these protein-protein interactions results in increased passage of fluid and antigenic stimuli. When these antigens reach the underlying interstitial tissue they cause sensitization through interaction with antigen-presenting cells, as well as a dysregulated inflammatory response through activation of inflammatory cells and a resultant release in inflammatory cytokines. Interestingly, exposure to HDM antigens has previously been shown to destabilize barrier function *in vitro* and *in vivo*.^{6–8} Identification of pathways that improve barrier stability may therefore be beneficial in treating sinonasal inflammatory disease.

Recent studies have demonstrated the barrier stabilizing effects of activation of the nuclear erythroid 2-related factor 2 (Nrf2) pathway.^{9–11} Under normal conditions, Nrf2 is sequestered in the cytoplasm by Kelch erythroid cell-derived protein with CNC homology (ECH)-associating protein 1 (Keap1). Upon pathway activation, Nrf2 is released and translocates to the nucleus where it acts as a transcription factor, binding to antioxidant response elements (AREs), and acting as a master regulator of the cytoprotective response.⁹ We have recently found that activation of the Nrf2 pathway using the small-molecule activator sulforaphane (SFN) restored SNEC barrier function *in vitro* in response to the oxidative stress particulate matter and cigarette-smoke extract.^{10,11} Furthermore, pharmacologic activation of the Nrf2 pathway has been demonstrated to reduce allergic asthma in mice through enhancement of airway epithelial cytoprotective function.⁹ We therefore hypothesized that Nrf2 activation using the small molecule activator SFN may block allergen-induced SNEC barrier disruption *in vitro*.

Subjects and methods

Human subjects

Eight human subjects (4 controls, 2 CRS without nasal polyps, 2 CRS with nasal polyps) were enrolled in this study. The research protocol was approved through our Institutional Review process, and all subjects gave signed informed consent. Specimens were obtained from non-immunocompromised patients greater than 18 years of age undergoing elective endoscopic sinus surgery for conditions including CRS. Allergy testing is not routinely performed prior to inclusion in the study; therefore, subject sensitivity to HDM is unknown.

SNEC culture at the air-liquid interface

SNECs were cultured at the air-liquid interface (ALI) as described by our group.^{11–14} Epithelial cells were isolated from tissue samples by enzymatic digestion and grown in culture on 10-mm culture dishes. Once the cells reached confluence, they were dissociated, resuspended in bronchial

epithelial cell growth medium (BEGM), and plated onto Falcon filter inserts (0.4 μ m pore size; Becton Dickinson, Franklin Lakes, NJ) coated with human type IV placental collagen (Sigma, St. Louis, MO; Type VI). When confluent, BEGM was removed and the cells grown at an ALI using LHC Basal Medium:DMEM-H (Gibco, Halethorpe, MD) (50:50). Each set of cultures came from a separate patient source and was maintained at the ALI with the apical surfaces remaining free of medium for at least 3 weeks prior to study.

Treatment of SNECs with HDM antigens

HDM antigens were obtained from Greer Laboratories (Lenoir, NC). Cells were stimulated apically with 50 μ g/mL of Der p 1 and Der f 1 antigens diluted into ALI medium. SNEC cultures were pretreated for 72 hours with 10 μ M sulforaphane (Toronto Research Chemicals, Toronto, ON, Canada) or with vehicle (0.1% dimethylsulfoxide [DMSO]).

In vitro permeability: transepithelial electrical resistance measurements

Transepithelial electrical resistance (TER) was monitored using the EVOM2 Voltohmmeter with the STX2 chopstick probe (World Precision Instruments, Sarasota, FL). To measure TER, 500 μ L of prewarmed ALI media was incubated in the apical compartment of the transwell for 30 minutes at 37°C. This media was replaced with the treatment medium and TER was recorded as time zero. Additional measurements at both 1 hour and 4 hours poststimulation were then performed. TER from each well was normalized to its prestimulation (time zero) TER.

In vitro permeability: paracellular fluorescein isothiocyanate–dextran flux

In vitro permeability was evaluated by quantifying paracellular flux of 4-kDa fluorescein isothiocyanate (FITC)–dextran beads as described.⁹ Briefly, SFN or vehicle control was added to the basolateral media 72 hours prior to HDM stimulation. HDM antigens were applied to the apical surface following SFN pretreatment. Two milligrams per milliliter (2 mg/mL) FITC–dextran was incubated at the apical surface of the SNEC culture for the duration of HDM treatment. Four hours poststimulation, the basolateral medium was collected and read for fluorescence in triplicate on a plate reader at an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

Immunocytochemistry: ZO-1 and junctional adhesion molecule-A localization

ALI cultures were washed and fixed with ice-cold 4% paraformaldehyde for 15 minutes at room temperature. The filter membranes were cut from the inserts, permeabilized with 0.3% Triton-X (EMD Chemicals, Gibbstown, NJ), blocked with 10% normal donkey serum,

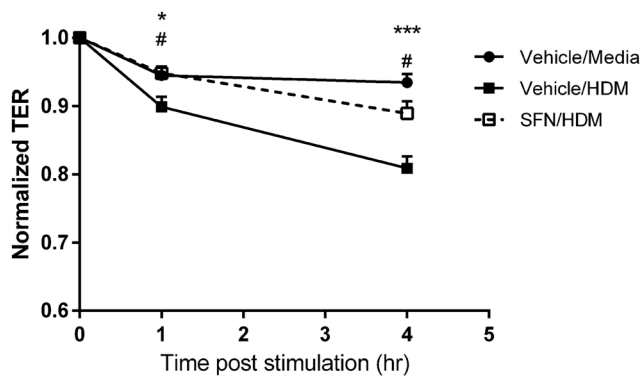


FIGURE 1. TER measurements following exposure to HDM antigens (* $p < 0.05$ DMSO vs HDM, *** $p < 0.001$ DMSO vs HDM, # $p < 0.05$ HDM vs SFN/HDM). Data are representative of mean $\pm$ SEM, $n = 8$. DMSO = dimethylsulfoxide; HDM = house dust mite; SEM = standard error of the mean; SFN = sulforaphane; TER = transepithelial electrical resistance.

and incubated (4°C, overnight) with AlexaFluor 594 mouse anti-ZO-1 and goat anti-junctional adhesion molecule-A (JAM-A) (LifeSpan Biosciences, Seattle, WA). After washing, the filter membranes were mounted on glass slides with 95% glycerol. Confocal images were collected with a LSM700 confocal laser-scanning microscope (Zeiss, Oberkochen, Germany). Fluorescence area was quantified from maximum intensity projections using ImageJ (NIH, Bethesda, MD; <http://imagej.nih.gov/ij/>).

RNA extraction and reverse transcription

Total RNA was isolated with the RNeasy Mini kit (Qiagen, Valencia, CA) using the manufacturer's protocol. RNA was quantified spectrophotometrically and absorbance ratios at 260/280 nm were > 1.80 for all samples studied. Five hundred nanograms of total RNA was reverse transcribed in a 20- μ L volume with random hexamer primers (Invitrogen), 20 U of RNase inhibitor (Applied Biosystems, Foster City, CA), and the Omniscript RT kit (Qiagen, Valencia, CA) under conditions provided by the manufacturer.

Real-time polymerase chain reaction: interleukin-1 β expression

Real-time polymerase chain reaction (PCR) was performed in a StepOnePlus (Applied Biosystems, Foster City, CA) using the TaqMan Gene Expression system (Thermo Fisher Scientific, Cockeysville, MD). The cycle parameters used were 95°C for 20 seconds, then 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. This was followed by 95°C for 15 seconds, 60°C for 60 seconds, and 95°C for 15 seconds. Amplicon expression in each sample was normalized to its 18S RNA content. The level of expression of interleukin 1 β (IL-1 β) messenger RNA (mRNA) was determined as the delta cycle threshold (Δ Ct), as described. Fold-change was calculated as $2^{\Delta\Delta C_t}$. Negative controls, consisting of reaction mixtures containing all components except target RNA, were included with each PCR run.

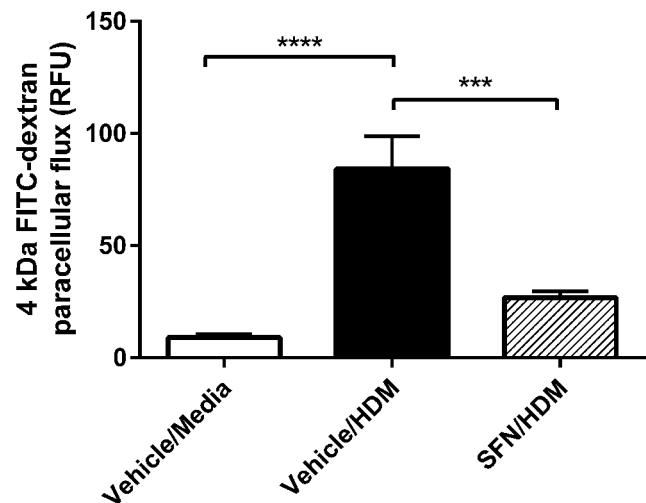


FIGURE 2. Transepithelial permeability as assessed by FITC-dextran flux following exposure to HDM antigens (* $p < 0.05$ DMSO vs HDM, *** $p < 0.001$ HDM vs SFN/HDM). Data are representative of mean $\pm$ SEM, $n = 8$. DMSO = dimethylsulfoxide; HDM = house dust mite; FITC = fluorescein isothiocyanate; RFU = relative fluorescence unit; SEM = standard error of the mean; SFN = sulforaphane.

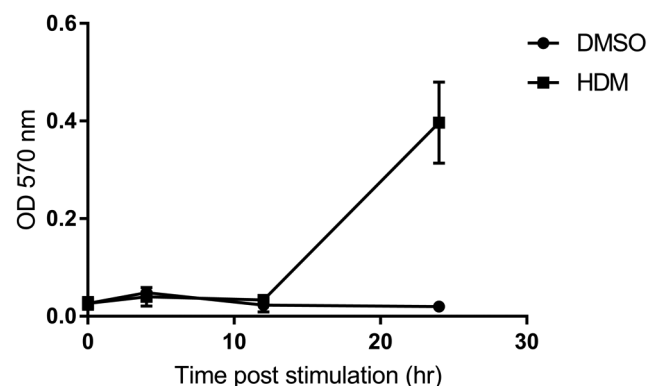


FIGURE 3. SNEC death assessed by LDH release after HDM antigen stimulation. * $p < 0.05$. Data are representative of mean $\pm$ SEM, $n = 3$. HDM = house dust mite; LDH = lactate dehydrogenase; OD = optical density; SEM = standard error of the mean; SNEC = sinonasal epithelial cell.

Lactate dehydrogenase cytotoxicity assay

Following HDM or mock treatment at the indicated time points, HSNECs were apically incubated with $1 \times$ phosphate buffered saline (PBS) for 30 minutes at 37°C. PBS was collected, centrifuged at 10,000g for 2 minutes, and assayed for lactate dehydrogenase (LDH) concentration (Pierce, Cockeysville, MD).

Statistical analysis

Raw data were entered into a spreadsheet and statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA). Data are expressed as mean $\pm$ the standard error of mean (SEM). Statistical significance was determined by a nonparametric Kruskal-Wallis test and Dunn's post hoc testing. Differences were considered statistically significant at $p < 0.05$.

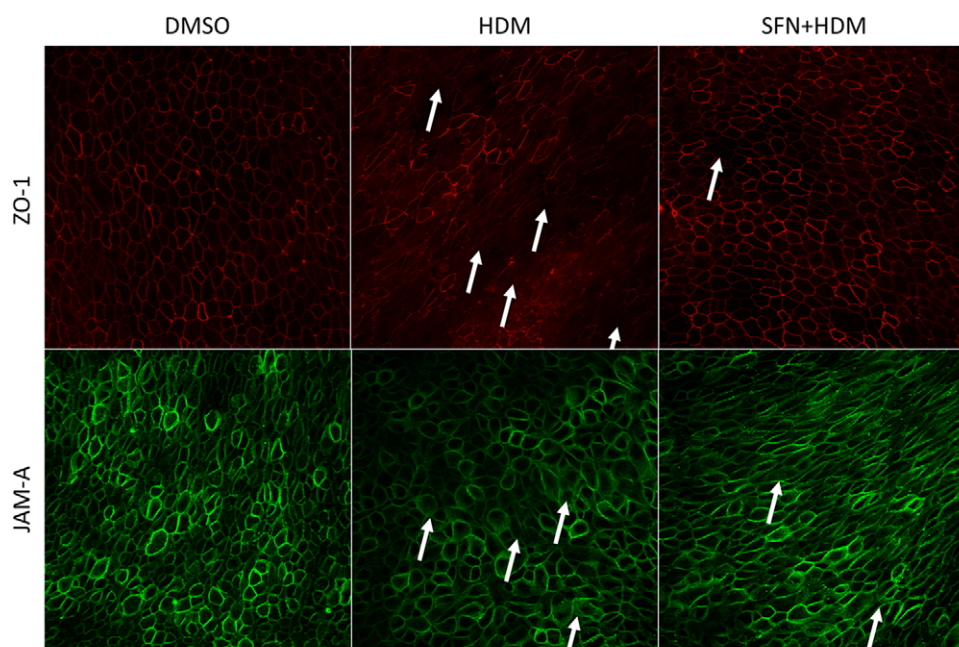


FIGURE 4. Immunofluorescence staining of ZO-1 (red) and JAM-A (green). Images are representative of 4 independent experiments for ZO-1 and 2 for JAM-A. DMSO = dimethylsulfoxide; HDM = house dust mite; JAM-A = junctional adhesion molecule-A; SFN = sulforaphane; ZO-1 = zonula occludens-1.

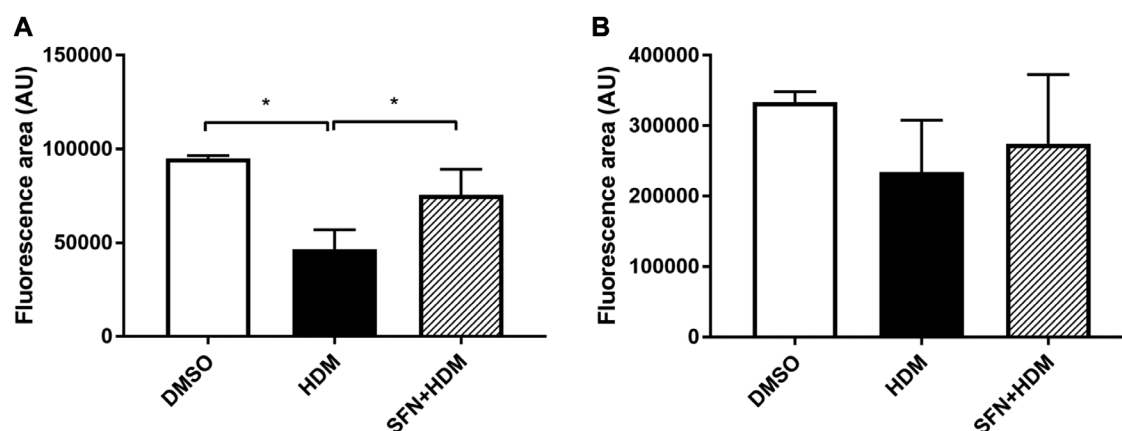


FIGURE 5. (A) Quantification of area of ZO-1 immunofluorescence. Data are representative of mean $\pm$ SEM, * $p < 0.05$. (B) JAM-A immunofluorescence was not statistically significant. AU = arbitrary units; DMSO = dimethylsulfoxide; HDM = house dust mite; JAM-A = junctional adhesion molecule-A; SEM = standard error of the mean; SFN = sulforaphane; ZO-1 = zonula occludens-1.

Results

HDM antigen-induced SNEC permeability is inhibited by activation of the Nrf2 pathway

To determine the effect of HDM antigens on the SNEC barrier, we exposed HSNEC cultures grown at ALI to HDM antigens Der p 1 and Der f 1 together with and without the Nrf2 activator SFN. The resulting changes in epithelial barrier function were assessed first via TER, where decreased electrical resistance indicates increased permeability. Analysis of barrier function via TER demonstrated that HDM-induced disruption of the SNEC barrier was reduced by

Nrf2 activation at both 1 hour and 4 hours after HDM antigen exposure (Fig. 1). We next confirmed the results using a second manner of assessment of epithelial permeability, namely paracellular flux of FITC-conjugated beads. Again, we found that HDM antigens induced barrier permeability as indicated by increased flux of FITC-dextran across the apical to the basolateral surface and that this effect was significantly reduced by pretreatment with SFN (Fig. 2). We confirmed that the barrier destabilizing effect of HDM within 4 hours was not due to cell death as assessed by LDH release (Fig. 3).

HDM antigen-induced disruption of ZO-1 cell surface localization is inhibited by activation of the Nrf2 pathway

Next we tested whether SFN could restore SNEC barrier function as assessed by cell surface localization of ZO-1, a scaffold protein linking the cytoplasmic actin skeleton to tight and adherens junction proteins³ and the JAM-A. Immunofluorescence staining of ZO-1 demonstrated marked disruption of ZO-1 and JAM-A cell surface/plasma membrane localization following HDM antigen exposure (Fig. 4). Nrf2 activation via SFN pretreatment demonstrated a reduction in this disruption, indicating a preservation of epithelial barrier integrity and increased plasma membrane localization of ZO-1 (Fig. 4). This finding was confirmed with objective quantification of area of ZO-1 staining (Fig. 5). Although Nrf2 activation did appear to be associated with a reduction in JAM-A disruption, objective quantification was not statistically significant.

HDM antigen-induced activation of inflammatory cytokine expression is not reduced by SFN pretreatment

The SNEC response to allergens and inflammatory stimuli is often characterized by increased expression of inflammatory cytokines such as IL-1 β . We exposed HSNECs to HDM antigen and as expected observed an increase in expression of IL-1 β as determined by quantitative PCR (qPCR) (Fig. 6). Interestingly, Nrf2 activation via SFN pretreatment did not alter the induction of IL-1 β expression (Fig. 6).

Discussion

SNEC barrier dysfunction has been proposed to be a contributing factor in disorders of sinonasal inflammation such as AR or CRS.^{3–5} Indeed, barrier dysfunction has been reported in these conditions as assessed by immunofluorescence staining of tissue, stimulation of HSNECs with barrier destabilizing agents, and TER of tissue samples.^{3,4,7,8} However, to date, few pathways have been identified that can stabilize SNEC barrier function and reduce barrier destabilization.

One pathway that has recently been identified to inhibit barrier destabilization is Nrf2 through reducing oxidative stress.^{10,11} HDM, however, has previously been implicated to destabilize the SNEC barrier through protease activity and degradation of barrier proteins.^{6,7} The data in our studies suggest that HDM antigen may also act to destabilize the SNEC barrier through oxidative stress. Alternatively, activation of the Nrf2 pathway may stabilize the barrier at baseline. Indeed, 1 study reported increased airway epithelial barrier stability in murine airway epithelial cells isolated from Keap1 knockout mice compared to controls.⁹

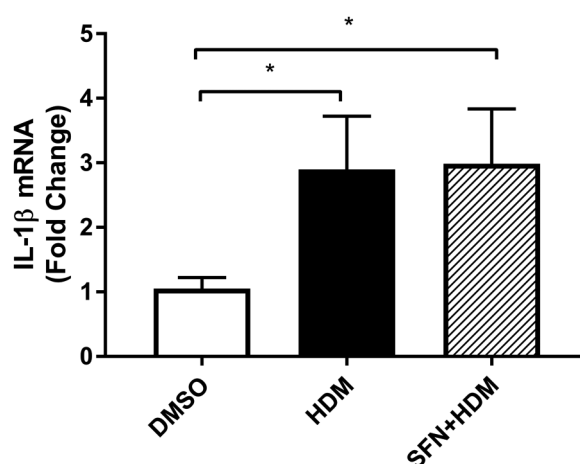


FIGURE 6. qPCR for mRNA transcripts of IL-1 β . * $p < 0.05$, $n = 5$. Data are representative of mean $\pm$ SEM. DMSO = dimethylsulfoxide; HDM = house dust mite; IL-1 β = interleukin 1 β mRNA = messenger RNA; qPCR = quantitative polymerase chain reaction; SEM = standard error of the mean; SFN = sulforaphane.

It is interesting that Nrf2 activation did not reduce HDM-induced inflammatory cytokine activation as assessed by IL-1 β gene expression (Fig. 6). This is in contrast to previous studies in which SFN pretreatment reduced cigarette-smoke extract-induced inflammatory cytokine expression.¹¹ These data may suggest a different mechanism of action for HDM and cigarette-smoke extract. One of the limitations of our study, however, was a low sample size. This may therefore make a small change in gene expression difficult to detect. Another limitation was the constrained number of inflammatory cytokines and chemokines we investigated. A screen involving many inflammatory cytokines and chemokines may potentially uncover genes whose expression may be affected by SFN pretreatment.

Conclusion

This preliminary study is the first to demonstrate that HDM-induced SNEC barrier dysfunction is inhibited by Nrf2 pathway activation via SFN pretreatment. These data also demonstrate the SNEC barrier stabilizing effects of SFN to multiple barrier destabilizing stimuli.^{10,11} In our current study, we noted a trend toward increased barrier disruption in SNECs from CRS patients exposed to HDM antigen compared to controls; however, this difference was not statistically significant. Given our limited sample size and combination of control and CRS patient phenotype, future studies dedicated to examining the effects of HDM antigen-induced barrier disruption on disease status with increased sample size are indicated. In conclusion, Nrf2-mediated stabilization of the SNEC barrier may represent a therapeutic target in AR and CRS. Further research regarding the Nrf2 pathway's ability to stabilize allergen-induced epithelial barrier dysfunction is warranted. 

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