Synergistic mucus secretion by histamine and IL-4 through TMEM16A in airway epithelium

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ABSTRACT

Synergistic mucus secretion by histamine and IL-4 through TMEM16A in airway epithelium

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Histamine is an important mediator of allergic reactions, and mucus hypersecretion is a major allergic symptom. However, the direct effect of histamine on mucus secretion from airway mucosal epithelia has not been clearly demonstrated. TMEM16A is a Ca^{2+}-activated chloride channel, and it is closely related to fluid secretion in airway mucosal epithelia. We investigated whether histamine directly induces fluid secretion from epithelial cells or submucosal glands (SMG) and mechanisms related therewith in allergic airway diseases.

In pig airway tissues from nose or trachea, histamine was a potent secretagogue that directly induced strong responses. However, gland secretion from human nasal tissue was not induced by histamine even in allergic rhinitis patients. Also, H1R and H2R were not noted in SMG by in situ hybridization.

Cultured primary human nasal epithelial (NHE) cells were used for the measurement of short circuit current changes with the Ussing chamber.

Histamine induced slight responses of anion secretions under normal conditions. The response was enhanced by IL-4 stimulation through

TMEM16A, which might be related to fluid hypersecretion in allergic rhinitis. Pretreatment with IL-4 augmented the histamine response that was suppressed by a TMEM16A inhibitor. TMEM16A expression was enhanced by 24 h treatment of IL-4 in HNE cells. The expression of TMEM16A was significantly elevated in an allergic rhinitis group, compared to a control group. We elucidated histamine-induced fluid secretions in synergy with IL-4 through TMEM16A in the human airway epithelium. In addition, we observed species differences between pig and human in terms of gland secretion to histamine.

Key words : histamine; mucus; airway; epithelium; submucosal gland; TMEM16A; IL-4

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I. INTRODUCTION

Allergic rhinitis or asthma is a chronic disease characterized by airway hyperreactivity, Th2-cytokine mediated inflammation, and mucus overproduction. Allergic airway inflammation is a multicellular process involving eosinophils, neutrophils, CD4+T lymphocytes, and mast cells.[1,2] Th2 inflammation plays a crucial role in allergic responses at multiple sites, and epithelial cells are closely involved therein, resulting in alteration of the airway microenvironment.[1] While epithelial cells function generally as a physical barrier, they also participate in maintaining mucociliary transport by regulating airway surface liquid.[3] The mucus covering the airway epithelium is commonly defined as a biphasic layer composed of a superficial gel layer and periciliary sol layer.[4] Fluid amounts in the mucus are regulated by a balance between Na^+ absorption and Cl^- secretion, which permits the flow of water through the epithelium as it passively follows the movement of ions.[5,6] Allergic airway inflammation shifts this balance generally towards fluid overproduction by secreting Cl^- and water through anion channels.[3,7,8] Among various apical ion channels, the cystic fibrosis

transmembrane conductance regulator (CFTR) has been well studied, because its mutation is the cause of cystic fibrosis, resulting in severe respiratory infection.[9] Besides CFTR, there is at least another ion channel through which Cl^- is secreted. Transmembrane member 16A (TMEM16A) is a protein, which is also known as anoctamin 1 (ANO1), that functions as a Ca^{2+}-activated chloride channel that is activated by $[Ca^{2+}]i$ mobilizing stimuli, such as ATP or UTP.[10-12]

Histamine, which is deposited in mast cells and basophils, is a major mediator of allergic diseases.[13] Histamine plays an essential role in the symptoms of allergic rhinitis, and the nasal provocation test using histamine has been used to induce allergic symptoms, such as itching, sneezing, rhinorrhea, and congestion.[14, 15] Nasal provocation tests with histamine have been shown to enhance nasal airway resistance and sneezing, and increase secretion in allergic patients.[16, 17] The histamine reaction mediated by histamine type 1 receptor (H1R) changes vascular permeability and promotes leukocyte infiltration, resulting in mucosal edema.[18] In addition, histamine increases the paracellular permeability of the epithelial barrier by interrupting E-cadherin adhesion.[19] Histamine, one of the neurotransmitters stored in the neurons of the central and peripheral nervous systems, has diverse biological roles, including in the innate immune response.[20] Therefore, various kinds of antihistamines against H1R have been applied to relieve allergic symptoms, and mucus hypersecretion can be reduced through H1R, which is present on central and peripheral histaminergic neurons. The presence of H1R on airway epithelial cells or submucosal glands and its association with mucin have been reported.[21-25] Furthermore, histamine causes a transient elevation of $[Ca^{2+}]i$ in the airway epithelium.[26]

However, a direct effect of histamine on mucus or fluid secretion from

airway epithelial cells and its mechanism have not been clearly demonstrated. Therefore, we investigated whether histamine directly induced mucus secretion from epithelial cells in human primary nasal epithelial cell and its mechanism.

II. MATERIALS AND METHODS

Human nasal epithelial cell cultures

Human nasal epithelial (HNE) cells were cultured as previously reported.[27, 28] A small portion of inferior turbinate mucosa was harvested from patients during nasal surgery. Passage 2 primary human nasal epithelial cells ($1x10^5$ cells/culture) were seeded in 0.5 ml of culture medium onto Transwell clear culture inserts (0.45mm pore size, Costar Co., Cambridge, MA). Cultured cells were grown submerged until they reached confluence. After the cells reached confluence, the media on the apical side was removed to create an air–liquid interface to allow for differentiation into ciliated columnar epithelial cells with polarity.

Nasal tissue harvest from patients

To determine submucosal gland secretion or expression of TMEM16A in human nasal epithelium, nasal tissues were obtained from 29 patients. Nasal tissues were harvested from a portion of the inferior turbinate during turbinoplasty. The subjects were categorized into allergic rhinitis (AR) (n=15, mean age 41.0 ± 4.7 yr) and non-allergic rhinitis (Non-AR) (n=14, mean age 36.5 ± 4.7 yr) groups. Informed consent was obtained from all patients, and this study was approved by the Institutional Review Board of Yonsei University College of Medicine. AR was diagnosed based on a

history of allergic symptoms, including nasal congestion, itchy sensation, rhinorrhea, or sneezing, and a positive multiple allergen simultaneous test (MAST) result (allergen-specific IgE level class ≥ 2).

Optical measurement of mucus secretion rates (mucus bubble method)

Individual gland secretion was measured as previously described.[29, 30] A piece of nasal turbinate mucosa was dissected from the turbinate bone from patients or pigs and mounted in a Sylgard-filled chamber with serosa in the bath (pH 7.4 and 290 mOsm Krebs-Ringer bicarbonate buffer containing glucose and 1 μM indomethacin to minimize prostaglandin release). The tissue surface was cleaned and dried, and about 10 μl of water saturated mineral oil was layered onto its surface. Experiments were performed at 37℃; the experimental chamber was filled with warmed, humidified 95% O_2-5% CO_2. Pharmacological agents were diluted to their final concentrations with warmed, gassed bath solution and were added to the basolateral side of the tissue through complete bath replacement. Secreted fluid from individual glands was visualized within the oil layer under oblique illumination and was digitally imaged with the macro lens of a camera. Images were analyzed using Image J software (http://rsb.info.nih.gov/ij/), and mucous volumes were determined from the size of the spherical bubbles. Secretion rates were determined at 5-min intervals.

Pig nasal and tracheal tissues were also harvested from adult pigs (n=4) that were euthanized after they were used for surgical training, and the mucosal tissues were prepared as previously reported.[31] This experiment was also approved by the Institutional Animal Care and Use Committee. Individual gland secretion was measured as previously described.[29,30]

Measurement of short circuit current (Isc) change with the Ussing chamber

The cultured HNE cells were mounted in Ussing chambers (4-channel system EVC4000, World Precision Instruments, Sarasota, FL). The mounted cells were clamped with an automatic voltage clamp, and the short-circuit current (*Isc*) was measured. The epithelium was bathed on both sides with 5 ml of warmed Krebs-Ringer bicarbonate buffer (pH 7.4, 290 mOsm) containing glucose and 1 μM indomethacin to minimize prostaglandin release, which was circulated in gas (95% O2 to 5% CO2). The mounted cells were clamped with an automatic voltage clamp, and the short-circuit current (*Isc*) was measured. Data were analyzed with LabChart 7 (v7.2.4) software (ADInstruments).

Measurement of intracellular calcium

To determine the change in intracellular Ca^{2+} level caused by histamine, HNE cells were loaded with 5 μM Fluo-3 AM (Invitrogen) for 30 min at 37℃. The cells were illuminated with a 488-nm light, and the emitted fluorescence was measured using a 525-nm emission filter. The fluorescence intensity was analyzed using MetaMorph software (Version 7.1, Universal Imaging Corp., USA).

In situ hybridization

Human nasal turbinate tissues were fixed and reacted using RNA probes from human H1R, H2R, and H3R cDNA. The specimens were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight at 4℃, dehydrated in 30% sucrose in PBS overnight at 4℃, embedded in OCT compound (Sakura, Tokyo, Japan), and stored at 80℃ until use. Tissues

were sectioned at 5-μm thickness for in situ hybridization. RNA probes for H1R were generated from a 411-base pair (bp) human H1R cDNA containing the +1082 to +1464 coding region and the 28 bp 3' untranslated region (Z34897.1); for H2R, from a 473-bp human H2R cDNA containing the +490 to +962 coding region (AY136744.1); for H3R, from a 372-bp human H3R cDNA containing the +1015 to +1338 coding region and the 48 bp 3' untranslated region (NM_007232.2).

Western blotting

HNE cells were lysed in lysis buffer (20 mM Tris-HCl [pH 7.5], 150mM NaCl, 1mM Na2EDTA, 1mM EGTA, 1% Triton, 2.5mM Sodium pyrophosphate, 1mM Na3VO4, 1ug/ml Leupeptin, 1mM β-glycerophosphate. The concentrations of the lysates were quantified using BCA Reagent (Thermo Scientific), and equal amounts of each lysate were separated onto an SDS-polyacrylamide gel (30% acrylamide in stock solution) by electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore; Bedford, MA, USA). The transfer buffer was composed of Tris (25mM), glycine (192mM), and methanol (20% (v/v) in DW. The PVDF membranes were blocked with 5% BSA (Sigma Aldrich) in Tris-buffered saline Tween 20 for 1h at room temperature. The blot was incubated overnight with primary antibodies in 0.5% Tween 20 in Tris-buffered saline (TTBS). After washing with TTBS, the blot was further incubated for 1h at room temperature with secondary antibody in TTBS and visualized using an ECL kit (Amersham; Little Chalfont, Buckinghamshire, UK).

Immunofluorescence

HNE cells on Transwell inserts were rinsed with PBS and fixed by adding 500 μl of methanol (Millipore) to the apical side for 20 min at room temperature (RT). After washing, cells were blocked with 1% BSA for 1 h and then incubated overnight at 4℃ with 300 μl (1:200) of primary antibodies diluted in DAKO antibody diluent including 1% background reducing component. The next day, secondary antibody was added at RT for 30 min. After washing with TTBS, DAPI was added and incubation was continued at RT for 2 min.

Measurement of airway surface liquid thickness

The thickness of the airway surface liquid (ASL) thickness was measured in HNE cells according to the method described by Tarran et al.[32] The HNE cells were washed with PBS and loaded with 20 μL of PBS containing 0.2% Texas Red-dextran (Invitrogen). A per-fluorocarbon (Fluorinert FC-770; 3M, St. Paul, MN) was then added to the apical surface to prevent evaporation of the ASL. After 12 h of incubation, cells were observed using confocal microscope (LSM 700; Carl Zeiss MicroImaging Inc., Thornwood, NY) and the ASL thickness was measured at three predetermined points in the cultures (one central, two circumferential) via XZ scans and analyzed using Image J software 7 (http://rsb.info.nih.gov/ij/).

Materials

Histamine (H1R agonist, H7125), thioperamide (H3R antagonist), amiloride (A7410), carbachol, ATP (A9187) were purchased from Sigma Aldrich (St. Louis, MO, USA). Amthamine dehydrobromide (H2R agonist) and (R)-α-methylhistamine dihydrobromide (H3R agonist) were

purchased from Tocris (Bristol, UK). IL-4 (204-IL) was purchased from R&D Systems. Primary antibodies against TMEM16A (14476S) and LC3B (3868S) were purchased from Cell Signaling and the p62 antibody (H00008878-MO1) was purchased from Abnova. Anti-rabbit and anti-mouse secondary antibodies were purchased from Jackson Immune Research.

Statistics

Data are presented as mean ± SE, unless otherwise indicated. IBM SPSS Statistics software (Ver. 23) was used for Mann-Whitney or t-test. A difference was considered significant at a P value less than 0.05.

III. RESULTS

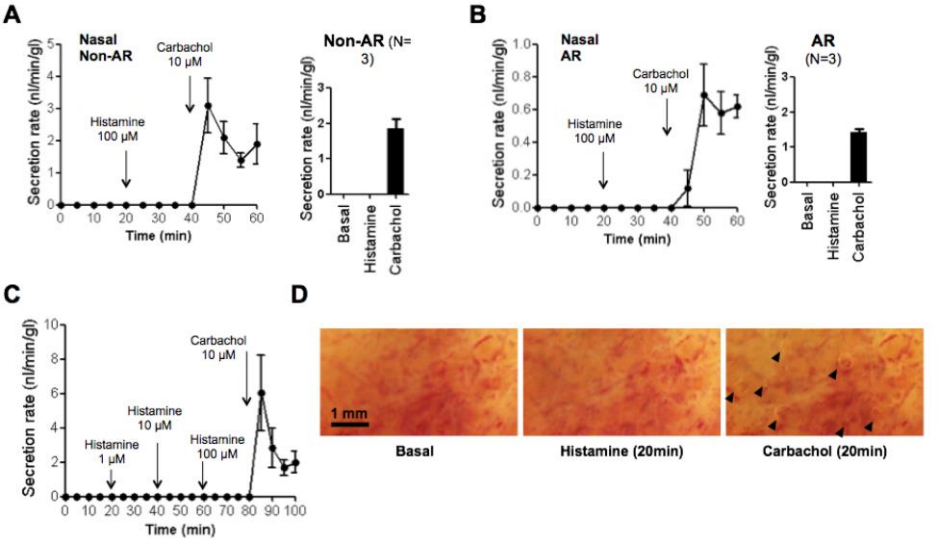
Histamine did not induce gland secretion in freshly excised human nasal tissue

Mucus secretion from submucosal glands was determined using freshly excised human nasal turbinate mucosae, as described in the Methods section. We used 100 μM histamine to determine the responses in other experiments, as used in a study by Lee RJ et al.[33] No notable histamine-induced change in bubble size was observed in nasal tissue from non-allergic rhinitis patients and, according to our optical measurement, the secretion rate was not detectable (n=3, 31 glands). However, use of 10 μM carbachol as a positive control induced robust mucus secretion (1.86 ± 0.2 nl/min/gl, n=3, 19 glands, Fig. 1A). To our surprise, gland secretion in response to histamine in allergic patients was also not observed, whereas

carbachol induced a strong response (1.4 ± 0.1 nl/min/gl, n=3, 20 glands, Fig. 1B and 1D). The nasal mucosae were treated with 1, 10, or 100 μM histamine to observe secretion from submucosal gland. Gland secretion was not induced by any concentration of histamine (Fig. 1C).

The expression of histamine receptor 1, 2, and 3 mRNA in human nasal tissue

The expression of H1R, H2R and H3R mRNA was investigated in the epithelium of human nasal turbinate using *in situ* hybridization (Fig. 1E). H1R mRNA was found in epithelial cells, but was not found in submucosal glands. H2R mRNA was found to be expressed similarly to H1R, as it was found in epithelial cells, but it was barely seen in submucosal glands. Interestingly, H3R mRNA was identified in both epithelial cells and submucosal glands.



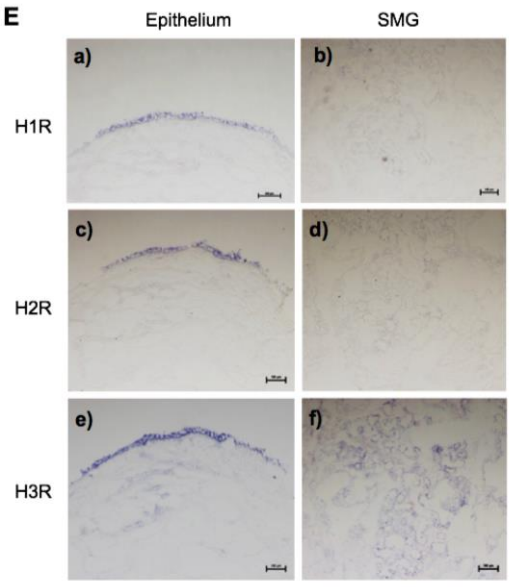


Fig. 1. Submucosal gland secretion in response to histamine in human nasal turbinate tissue and the expression of histamine receptor subtypes determined using *in situ* hybridization in human nasal turbinate tissue. A. A representative plot of the secretion rate and a summary of the averaged secretion rates in non-allergic patients. B. A representative plot of the secretion rate and a summary of the averaged secretion rates in allergic patients. C. A representative plot of the secretion rate in response to various concentrations (1 μM, 10 μM, and 100 μM) of histamine. Histamine failed to cause a glandular response in human nasal tissue. D. Examples of mucus bubbles formed under oil on the surface of nasal turbinates before and after stimulation with 100 μM histamine followed by 10 μM carbachol. E. a and b) H1R. c and d) H2R. e and f) H3R. H1R mRNA was found in epithelial cells, but it was not found in submucosal glands. H1R and H2R mRNA were found in epithelial cells, but barely seen in submucosal glands. H3R mRNA was identified in both epithelial cells and submucosal glands.

Histamine induced modest Isc change in HNE cells

Because the effect of histamine on anion secretion has not yet been directly proven in the human nasal epithelium, we first investigated the change in *Isc* caused by histamine in cultured human nasal epithelial (HNE) cells. Interestingly, histamine induced an *Isc* change, but the response was modest (Fig. 2A). The changes in *Isc* caused by the various treatments were as follows (Fig. 2C): 100 μM amiloride, -23.7 ± 10.9 μA/cm^2 (n=12); 100 μM histamine, 2.7 ± 1.7 μA/cm^2 (n=12); 100 μM ATP, 12.0 ± 7.7 μA/cm^2 (n=5). ATP, used as a positive control, induced a strong change in *Isc*, indicating anion secretion. Treatment with 10 μM T16Ainh-A01, a TMEM16A inhibitor [34], significantly suppressed the histamine- or ATP induced responses (Fig. 2B and 2C, n=5). Because histamine weakly changed *Isc* in HNE cells, alteration of the [Ca^{2+}]i response in HNE cells was measured. Following the application of 100 μM histamine after the basal period, and 100 μM ATP, a strong increase in [Ca^{2+}]i was noted (Fig. 2D and 2E).

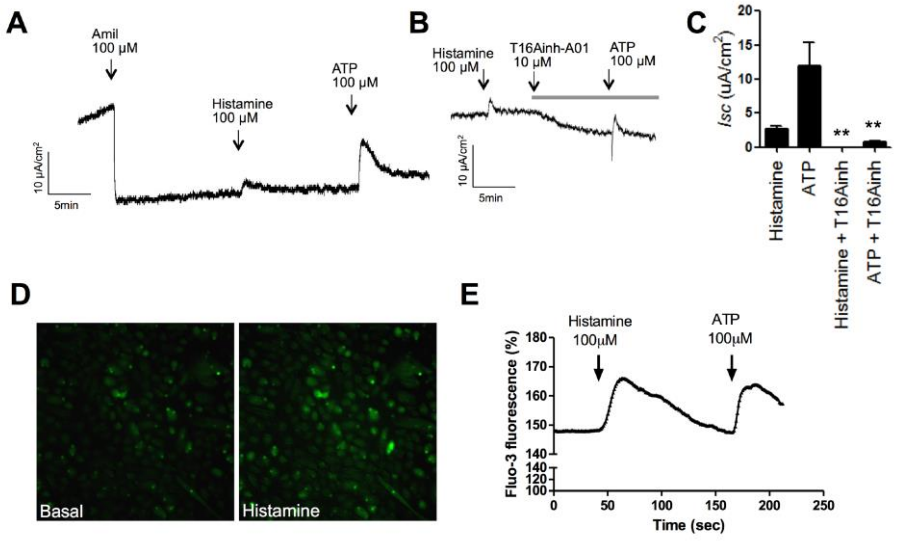


Fig. 2. Short circuit current (*Isc*) changes caused by histamine and ATP (a, b, and c) in cultured human nasal epithelial (HNE) cells and the measurement of intracellular calcium in Fluo-3-loaded HNE cells (d and e). A. A typical *Isc* recording in HNE cells. The histamine induced *Isc* change was modest compared to the ATP-induced current change. B. The histamine-induced response was suppressed by 10 μM T16Ainh-A01. C. Summary of *Isc* change in HNE cells. The response caused by histamine was 1/5 that of the response caused by ATP. Histamine- and ATP-induced *Isc* changes were significantly suppressed by T16Ainh-A01 (**; $p<0.005$). D. Cells were treated with 100 μM histamine after the basal period, and an increase in $[Ca^{2+}]i$ was noted. E. Histamine (100 μM), followed by ATP (100 μM), stimulated an increase in $[Ca^{2+}]i$ in HNE cells.

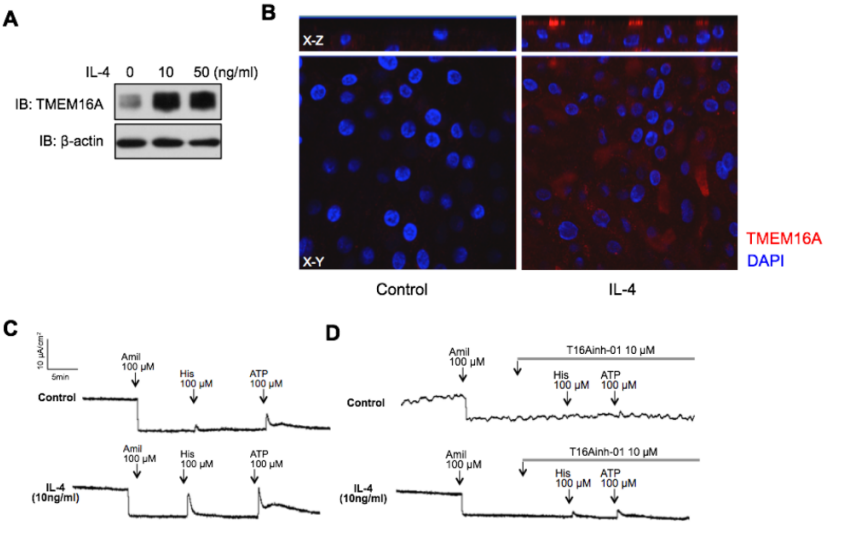
Histamine enhanced Isc change under IL-4 via TMEM16A

Contrary to our expectations, the response to histamine was modest in HNE cells. We assumed that this response might be augmented by stimulation with IL-4, one of the major cytokines associated with the allergic mechanism. First, because TMEM16A is a calcium activated chloride channel and histamine is an agonist which in our studies elevated $[Ca^{2+}]i$, we evaluated whether IL-4 could increase the expression of TMEM16A in HNE cells. The level of TMEM16A protein was strongly increased by 10 ng/ml or 50 ng/ml of IL-4 during a 24 h treatment (Fig. 3A, Fig. 3B). Next, we determined the *Isc* change in response to histamine or ATP after pretreatment with 10 ng IL-4 for 24 h. The histamine-sensitive *Isc* change in IL-4 pretreated cells was 9.6 ± 3.3 $\mu A/cm^2$ (n=9) and it was significantly increased (Fig. 3C and 3E, *; $p<0.05$, **; $p<0.005$). The ATP-induced *Isc* in IL-4 pretreated cells was 14.0 ± 3.2 $\mu A/cm^2$ (n=9),

which also represents a significant enhancement compared to control cells without IL-4 (Fig. 3D and 3F). Use of 10μM TA16inh, a TMEM16A inhibitor, significantly suppressed the histamine (0.3 ± 0.2 μA/cm^2, n=6) and ATP responses (4.0 ± 0.8 μA/cm^2, n=7) in IL-4 treated NHE cells (Fig. 3D, 3E, and 3F).

ASL thickness measurement

We measured the ASL thickness in HNE cells after Texas Red-dextran loading under confocal microscope (Fig. 3G). The ASL thickness in histamine (100 μM)-treated cell (6.7 ± 0.6 μm) was thicker than that in the control (4.4 ± 0.2 μm). Co-stimulation of IL-4 with histamine (8.5 ± 0.6 μm) induced more prominent ASL thickness than histamine alone (Fig. 3H, *; $p<0.05$).



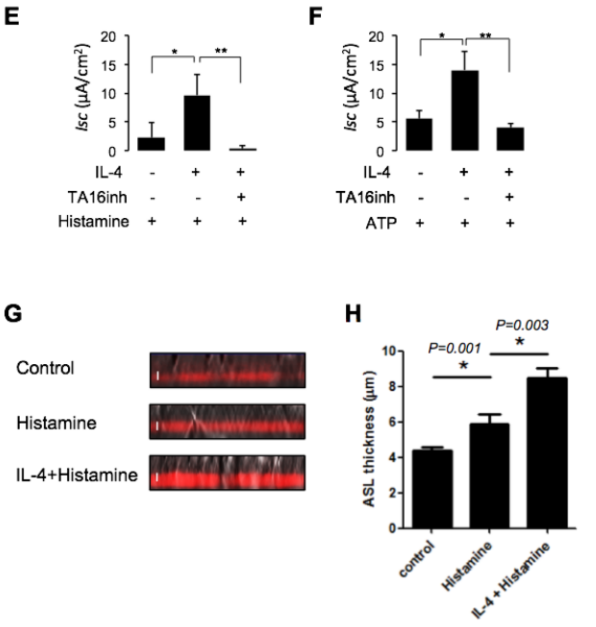


Fig. 3. The effect of IL-4 on TMEM16A and the histamine or ATP response in HNE cells. A. Western blot showing increased expression of TMEM16A caused by IL-4 treatment. B. Representative images taken with a confocal microscope showing the expression of TMEM16A (red) in HNE cells. Nuclei were also stained with DAPI (blue). IL-4 induced TMEM16A expression. C. A typical *Isc* recording showing augmented histamine- or ATP sensitive *Isc* resulting from pretreatment with IL-4 in HNE cells. D. A typical *Isc* recording showing the inhibitory effect of TA16inh-A01 on histamine- or ATP-sensitive *Isc*. E. Summary of *Isc* changes caused by histamine in HNE cells. Histamine-sensitive *Isc* was significantly increased by IL-4 and suppressed by TA16inh-A01. F. Summary of *Isc* changes caused by ATP in HNE cells. ATP-sensitive *Isc* was significantly increased by IL-4 and suppressed by TA16inh-A01. G. Representative confocal images of ASL loaded with Texas Red-dextran (White bar scale = 5 μm). H. Summary of ASL thickness measurements.

TMEM16A expression in allergic rhinitis patients

We compared the protein expression of TMEM16A in nasal turbinate tissues from normal control or allergic rhinitis patients (Fig. 4A and 4B). β-actin was used as a loading control. When we compare the relative increased fold (TMEM16A/β-actin ratio), it was significantly increased in allergic rhinitis (7.7 ± 1.8) than in normal control (2.0 ± 0.7, ***; $p<0.01$*).

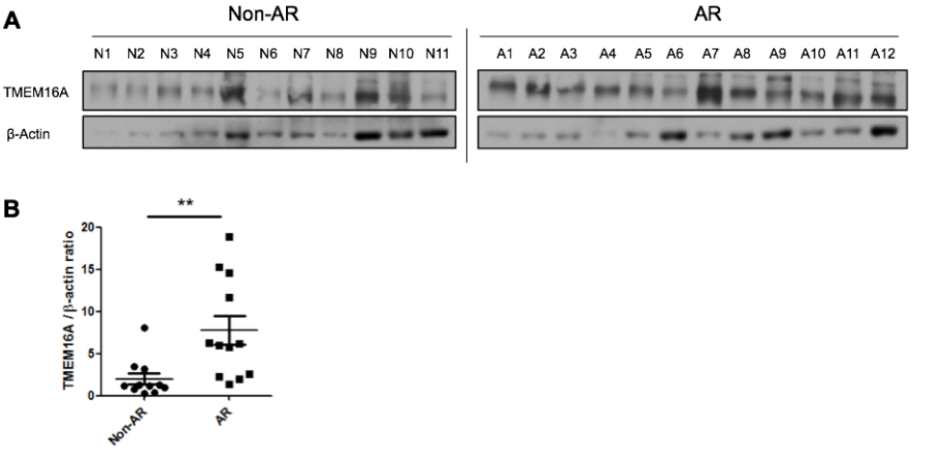


Fig. 4. Expression of TMEM16A analyzed by Western blot from patients. a) TMEM16A expression of crude extracts prepared from inferior turbinate mucosa of normal (non-AR; n=11) and allergic rhinitis (AR; n=12) patients. β-actin was used as a loading control. b) The comparison of normalized TMEM16A expression in between non-AR and AR patients.

Histamine induced a strong response in pig nasal and tracheal epithelia

Mouse nasal serous cells were previously shown to undergo *Isc* change in response to histamine.[33] As we hypothesized that a species difference may exist, we evaluated the histamine response in pig nasal and tracheal mucosal tissues. The histamine-induced *Isc* change in freshly excised mucosal epithelium derived from either the nose or trachea of a pig was measured (Fig. 5). In contrast to human nasal tissue, histamine induced a notable change in *Isc* in both nasal and tracheal tissues from pig (n=4). The changes in *Isc* due to treatment were as follows. In pig nasal tissue (Fig. 5A), 100 μM amiloride, -4.625 ± 5.1 μA/cm^2 ; 100 μM histamine, 1.75 ± 1.3 μA/cm^2 ; 100 μM carbachol, 2.7 ± 1.6 μA/cm^2. In pig tracheal tissue (Fig. 5B), 100 μM amiloride, -3.3 ± 4.0 μA/cm^2; 100 μM histamine, 2.5 ± 1.4 μA/cm^2; 100 μM carbachol, 0.7 ± 0.7 μA/cm^2. The H2R agonist, H3R agonist, and H3R antagonist did not result in changes in *Isc* in pig nasal or tracheal tissues (Fig. 5A and 5B). The response of submucosal glands in pig airway tissues was also determined. Treatment with 100 μM histamine also induced notable fluid secretion from pig nasal submucosal glands (n=3, 28 glands, Fig. 5C), resulting in a secretion rate of 0.14 ± 0.05 nl/min/gl. The response to 10 μM carbachol was 0.55 ± 0.07 nl/min/gl. Surprisingly, in pig tracheal tissue (n=3, 34 glands, Fig. 5D), robust gland secretion (2.6 ± 0.8 nl/min/gl) was noted for only 1 μM histamine, and this response was similar to the response from 10 μM carbachol (3.0 ± 0.6 nl/min/gl). The H3R antagonist failed to cause gland secretion in pig nasal or tracheal tissues (Fig. 5F).

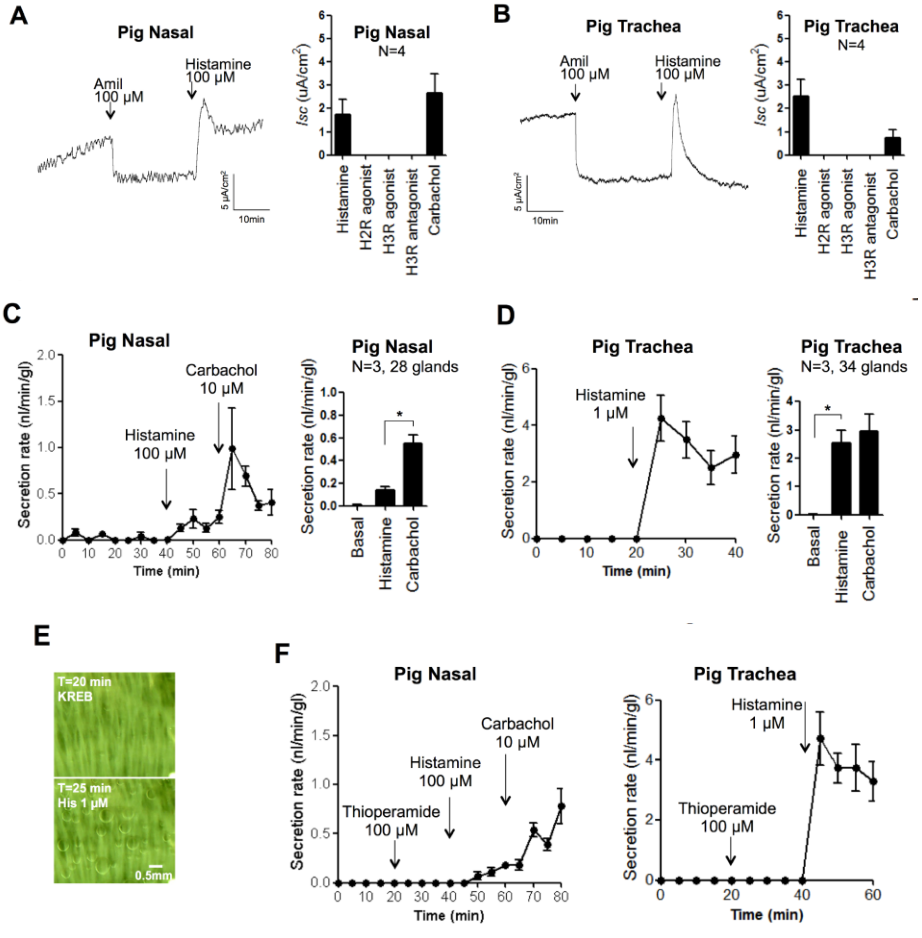


Fig. 5. Short circuit current (*Isc*) changes and submucosal gland secretion in pig nasal and tracheal tissues. A. A typical *Isc* recording and a summary of *Isc* change in pig nasal tissue. A strong response was observed upon addition of histamine, but not upon use of an H2R agonist, H3R agonist or H3R antagonist. B. A typical *Isc* recording and a summary of *Isc* change in pig tracheal tissue. A robust response was observed when histamine was used, but not when an H2R agonist, H3R agonist or H3R antagonist were

used. C. A representative plot and a summary of the secretion rate of a submucosal gland in pig nasal tissue. A strong response to histamine was observed. D. A representative plot and a summary of the secretion rate of a submucosal gland in pig tracheal tissue. A robust response was observed upon addition of 1 μM histamine. E. Representative photos showing a mucus bubble formed in the oil on the surface of the pig trachea when KRB solution was used in the bath (upper panel) and after 5 min of 1 μM histamine treatment (lower panel). F. Representative plots of the secretion rate in response to thioperamide (H3R antagonist) in pig nasal and tracheal tissue. Thioperamide failed to induce any gland secretion.

IV. DISCUSSION

The role of histamine in mucus secretion in the airway epithelium

Mucus hypersecretion is one of the major features of airway diseases, such as allergic rhinitis, allergic asthma, sinusitis, cystic fibrosis, and chronic obstructive pulmonary disease.[35] Therefore, understanding the mechanisms of mucus hypersecretion is important for the development of disease-specific treatments. Histamine is mostly released from mast cells stimulated by an IgE-dependent allergic reaction [36], and antihistamine reduce acute allergen challenge-induced mucus secretion and plasma exudation in allergic patients.[37] In rat tracheal epithelium, the depletion of goblet cell mucin granules has been observed after histamine application, which indicates that mucus, including mucin secretion, is induced by histamine.[22] MUC2 and MUC5AC, secreted gel-forming mucins, are upregulated by histamine in NCI-H292 cells.[23, 24] However, the influence of

histamine on anion secretion via ion channels in human nasal epithelium is still not clear. Fluid secretion from the submucosal glands in response to histamine has not been elucidated because of the limitations of experimental methods. The bubble method that was applied in this study is a well established method, and can optically measure fluid secretion from individual submucosal glands and detect a change in the secretion rate at a picoliter level. Histamine is known to stimulate robust airway surface liquid secretion, which is mediated by calcium-activated chloride channels, at a 100 μM concentration, a saturation concentration that induces maximal intracellular Ca^{2+} in human nasal epithelial cells.[33] In murine serous acinar cells of nasal mucosa, 100 μM histamine was shown to increase $[Ca^{2+}]i$ along with cell shrinkage.[38] Taken together, these results suggest that histamine is a secretagogue that stimulates fluid secretion in the airway epithelium.

H1R is associated with the major allergic mechanism in response to histamine [39], and H1R antagonists are used to abolish histamine-induced nasal symptoms.[40] The IgE dependent mechanism is the major pathophysiology of the allergic reaction. However, the upregulation of H1R is also noted in patients with allergic airway disease,[21,25] and the IL- 4 mediated mechanism is a possible signaling pathway.[41] These results suggest that the features of hyperresponsiveness, including mucus hypersecretion, might stem from upregulation of H1R in allergic conditions. Therefore, we hypothesized that mucus secretion from nasal epithelium in response to histamine would be increased in allergic rhinitis compared to non-allergic rhinitis. Interestingly, we failed to observe any submucosal gland secretion in response to histamine in both allergic and non-allergic rhinitis; this result may be related to the lack of detection of H1R in

submucosal glands by *in situ* hybridization (Fig. 1E). However, Dinh QT et al.[21] reported expression of H1R in submucosal gland mucus cells of human nasal turbinate by immunohistochemistry; the discrepancy between this study and our data may be the result of different experimental methods. Human nasal epithelial cells, contrary to our expectation, displayed only a modest change in *Isc* upon stimulation with 100 μM histamine, which was much lower than the response caused by ATP. This was unanticipated, as we had observed the expression of H1R in the epithelial layer, as shown in Fig. 1E. Although we did not quantify or compare the expression level of H1R between allergic and non-allergic rhinitis, the role of histamine in fluid secretion from human nasal epithelial cells seems to be minimal in the normal state.

Species difference between human and pig

The experimental goal of this study was to identify mucus or fluid secretion; we did not quantify the secretion of mucin granules stored in goblet cells. The degranulation of goblet cells by histamine may develop through histamine receptors, as previously reported. While we found that histamine was not a potent secretagogue in human nasal epithelium under normal conditions, we did observe strong fluid secretion induced by histamine in the pig airway epithelium. A difference between humans and pigs in response to substance P has also been reported. Pig tracheal glands are more sensitive to substance P than are human tracheal glands [42, 43], but pig nasal glands are unresponsive to substance P.[31] We did not determine the response to histamine in human tracheal glands in this study, and organ differences may be present in the pig. These results indicate that histamine is a weak mediator of fluid secretion in the human nasal epithelium

compared to pig airway epithelium under normal conditions.

Is H2R or H3R related to mucus secretion in the airway epithelium?

As described above, H1R is closely associated with allergic reactions, but H2R and H3R have also been suggested to have roles in mucus secretion. We observed the expression of H3R in a human nasal submucosal gland by *in situ* hybridization, as has been previously reported in rats by immunohistochemistry.[44] H3R was reported to be associated with postsynaptic regulation of H1R [45,46] and inhibition of H3R with thioperamide stimulated degranulation in mouse nasal gland acinar cells when investigated using histology.[44] However, thioperamide failed to induce gland secretion in either human or pig airway tissues in our functional assay of gland secretion or the Ussing experiment. The H3R agonist also failed to induce a response in pig or human epithelium. In a previous study, guinea pig trachea responded to an H2R agonist and goblet cell secretion was induced.[47] However, our functional assay failed to observe any response due to the H2R agonist. Taken together, H2R and H3R were not involved in mucus secretion in our experiments; further investigation will be required to elucidate their role in allergic responses.

Emerging role of TMEM16A in allergic airway diseases

TMEM16A, as described in the Introduction, mediates Cl^- secretion, resulting in fluid hypersecretion. We previously reported that house dust mite extract activates protease activated receptor (PAR)-2 and Cl^- secretion via Ca^{2+}-activated chloride channel in human airway epithelial cells.[8] Rievaj J *et al*. demonstrated that allergic sensitization with ovalbumin enhances ATP-sensitive *Isc* responsiveness of murine trachea to PAR-2

activation.[3] These results imply that ATP-sensitive anion secretion may be highly involved in the pathophysiology of allergic airway diseases in terms of fluid hypersecretion. To the best of our knowledge, we have, for the first time, shown that TMEM16A expression is enhanced in the nasal tissue from allergic rhinitis patients. Furthermore, we provided results that indicate that IL-4 increases protein levels and the functional activity of TMEM16A to histamine or ATP in human nasal epithelium. Th2 cells release IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13.[48] Among these cytokines, IL-4 plays a particularly important regulatory role in allergic inflammatory mechanisms, including IgE production and in the activation of eosinophils, basophils and mast cells.[48] In our data, histamine caused a modest change in *Isc* in normal HNE cells, but the change was increased under 24-h incubation with IL-4. The histamine response was suppressed by the TMEM16A inhibitor, implying that the IL-4 augmentation of fluid secretion by histamine likely occurs through the TMEM16A channel.Zhang Y et al. reported on the overexpression of TMEM16A and co-localization of MUC5AC in the nasal polyp from chronic rhinosinusitis patients.[49] They also found that IL-13 increased percentages of TMEM16A- and MUC5AC-positive cells in nasal epithelial cells.[49] In bronchial epithelial cells, IL-4 treatment also increased TMEM16A expression, mucous cell metaplasia, and the percentage of cells expression MUC5AC.[50] Therefore, TMEM16A, besides the role of anion / fluid secretion, could be involved in goblet cell metaplasia, and targeting TMEM16A could be a novel treatment strategy to alleviate fluid overproduction and disease severity in airway inflammatory diseases, such as allergic rhinitis, asthma, or sinusitis. Furthermore, one study has reported that IL-4 is a potent modulator of CFTR or ENaC in human bronchial

epithelium.[51] The authors thereof found that IL-4 evokes a two-fold increase in cAMP analog-sensitive *Isc* and marked inhibition of amiloride sensitive *Isc*. Gianottia A. et al showed that CaCCinh-A01 inhibited cAMP and calcium activated chloride secretion in mouse trachea and found unexpected CFTR inhibitory activity by CaCCinh-A01.[52] Therefore, all of these changes, including TMEM16A, can affect ion compositions and the amount of airway surface fluid under allergic conditions in airway epithelium. An investigation of cytokine-dependent ion transport alterations may provide novel strategies for the treatment of allergic airway diseases.

IV. CONCLUSION

In summary, we found that histamine induced modest anion secretion under normal conditions, but this response was enhanced under IL-4 stimulation, which may be related to mucus hypersecretion in allergic airway diseases. IL-4 increased the expression of TMEM16A, one of the major anion channels for fluid secretion, and the histamine or ATP response was mediated through TMEM16A. Taken together, histamine seems to play a minor role in fluid secretion in human nasal epithelium under normal conditions, but IL-4, which can be released from Th2 cells during allergic inflammation, might elevate TMEM16A expression through which fluid hypersecretion occurs as a result of stimulation by histamine (Fig.6).

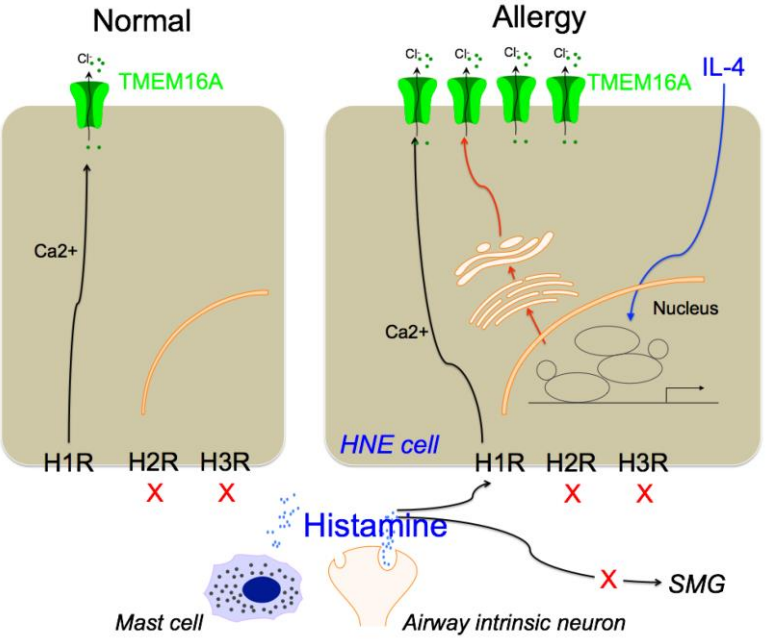


Fig. 6. Schematic drawing of the histamine response in the human nasal epithelium. Histamine induces modest anion secretion under normal conditions, but the response is enhanced upon IL-4 stimulation, which might be related to mucus hypersecretion in allergic rhinitis.

Therefore, we suggest that a therapeutic agent targeting IL-4 or TMEM16A would be effective in alleviating fluid hypersecretion in allergic rhinitis. In addition, we observed no response to an H2R agonist, H3R agonist or H3R antagonist in the airway epithelium. Interestingly, pig nasal and tracheal mucosa showed good responses to histamine, indicating a species difference in between human and pig. Consequently, this aspect of our work should be given careful consideration when studying histamine-related mucus secretion with pig airway epithelium.

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ABSTRACT(IN KOREAN)

기도상피에서 TEME16A 이온 통로를 경유하는 히스타민과 IL-4의 점액분비 상승작용 기전

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강 주 완

점액 과분비는 알레르기 반응의 주요 증상으로, 알레르기 반응의 매개 물질 중 하나인 히스타민은 점액 분비를 유도한다고 알려져 있다. 하지만, 아직까지 히스타민이 기도점막의 상피세포나 점막하 분비샘에서 점액 분비를 직접적으로 유도할 수 있는지에 대한 연구는 미흡하다. TMEM16A은 칼슘에 의해 활성화되는 염소이온 통로로 기도점막상피에서 점액 분비를 조절하는 중요한 역할을 하는 것으로 알려져 있다. 이에, 저자들은 히스타민이 기도점막상피와 점막하 분비샘에서 직접적으로 분비에 영향을 미치는지, 또한 이 과정에 TMEM16A가 관여하는지 그리고 알레르기 염증과의 상관관계 여부에 대하여 알아보고자 하였다.

연구 결과 히스타민은 돼지의 비점막과 기관지점막의 점막하 분비샘에서 점액 분비를 강하게 유도하였으나, 사람의 비점막 점막하 분비샘과 알레르기 비염 환자 비점막에서는 히스타민은 점액 분비를 유도하지 않았다. 또한 in situ hybridization를 이용하여 사람의 비점막에서 히스타민 수용제 1,2 발현을 조사하였을 때, 히스타민 수용체 1,2가 모두 발현한 상피세포와는 달리 점막하 분비샘에서 두 수용체 모두 발현되지 않았다.

한편, 사람의 비점막 상피세포를 배양한 후 Ussing

chamber를 이용하여 단락전류의 흐름을 측정한 결과, 히스타민은 약하게 음이온 분비를 유도하였고, 알레르기 염증 반응이 미치는 효과를 알아보기 위하여 알레르기 관련 사이토카인인 인터루킨4를 처리하였을 때 음이온 분비 효과가 더욱 강하게 유도되었다. TMEM16A의 길항제를 처리하였을 때는 이러한 반응이 억제되어, 히스타민에 의한 점액 분비 유도 과정에 TMEM16A이 관여함을 확인할 수 있었다. 마지막으로 사람 비점막 상피세포를 배양하여 인터루킨4를 처치한 결과 TMEM16A의 발현이 증가하였으며, 알레르기 비염 환자에서도 정상인에 비해 TMEM16A의 발현이 증가되어 있는 것을 확인할 수 있었다.

이를 종합하여 볼 때, 히스타민은 사람의 비점막에서 점액분비를 유도할 수 있으며, 알레르기 반응 (인터루킨4)에 의한 TMEM16A의 과발현과 상호작용하여 점액 분비를 강하게 유도하고, 이러한 히스타민에 의한 점액 분비는 종에 따라 다르게 나타날 수 있다고 생각된다.

핵심되는 말 : histamine; mucus; airway; epithelium; submucosal gland; TMEM16A; IL-4