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Influence of Orai1 intervention on mouse airway epithelium reactions in vivo and in vitro

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ARTICLE INFO

Article history:

Received for publication August 23, 2011.

Received in revised form September 14, 2011.

Accepted for publication September 19, 2011.

ABSTRACT

Background: Orai1 is crucial for store-operated Ca^{2+} entry and Ca^{2+} release-activated Ca^{2+} channel activities. However, little is known about its function in allergic diseases.

Objective: To assess the influence of Orai1 intervention on mouse airway epithelium reactions in vivo and in vitro.

Methods: We used immunohistochemical staining, enzyme-linked immunosorbent assay, and real-time reverse transcription–polymerase chain reaction to evaluate Orai1 expression in nasal and tracheal mucosa epithelium of nonsensitized, control, and 2-aminoethoxydiphenyl borate (2-APB)–treated groups in vivo and in vitro. In addition, we analyzed concentrations of interleukin 1 β , interleukin 6, macrophage inflammatory protein 2, and tumor necrosis factor α in nasal lavage fluid, bronchoalveolar lavage fluid, and culture supernatant and their messenger RNAs in nasal and tracheal mucosa and cultured nasal and tracheal epithelium.

Results: Administration of 2-APB into the nostrils suppressed Orai1 expression in nasal and tracheal mucosa of treated mice compared with that in control mice and restrained the mediators in nasal lavage fluid, bronchoalveolar lavage fluid, and airway mucosa of treated groups compared with those in control groups. Similarly, the 2-APB intervention also alleviated Orai1 and the production of the mediators in culture supernatant and cultured airway epithelium under allergic conditions.

Conclusions: Our results indicate that 2-APB could effectively ameliorate reactions of upper and lower airway epithelial cells in mice in allergic states in vivo and in vitro.

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Introduction

For many years, nasal epithelial cells were considered to play a simple role as a mucosal barrier while being involved in the secretion of mucus and the removal of foreign agents by their cilia. However, recent studies have shown that airway epithelium has a broad range of activities. These cells in allergic individuals (patients with rhinitis and asthma) are in an activated state, with increased production of inflammatory mediators, such as interleukin (IL) 6, IL-8, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor α (TNF- α),^{1,2} which contributes to enhancement of allergic reactions.

Evidence is increasing that intracellular Ca^{2+} signals regulate a diverse array of physiologic processes, including secretion, cell growth, and gene expression.³ Changes in cytoplasmic Ca^{2+} levels are initiated by altered flux across plasma membrane and release from endoplasmic reticulum (ER). Store-operated Ca^{2+} entry (SOCE) is activated by depletion of ER Ca^{2+} stores and is a widely observed process of plasma membrane Ca^{2+} influx.⁴ Ca^{2+} release-

activated Ca^{2+} (CRAC) channels are the most extensively characterized SOCE pathway,⁵ and Orai1 has been identified as an essential component of CRAC channels.⁶ Mammalian cells have 2 additional proteins, Orai2 and Orai3, but their initiating Ca^{2+} currents are somewhat smaller.^{7,8}

Orai1 protein has been proven to be crucial for SOCE and CRAC channels,⁹ which are the principal manner of Ca^{2+} influx in mammalian immune system cells, such as mast cells, B cells, and T cells.^{10–12} Our previous study has provided evidence that Orai1 also exists in mice airway epithelial cells and is upregulated under allergic rhinitis (AR) conditions.¹³ Therefore, it is rational to assume that Orai1 protein may play a role in the pathogenesis of allergic airway diseases. It is well known that 2-aminoethoxydiphenyl borate (2-APB) has emerged as a useful pharmacologic tool in the study of SOCE. This drug affects SOCE by modulating the function of CRAC channels.^{14,15} The aim of this study was to analyze whether the intervention of Orai1 with 2-APB was able to control upper and lower airway epithelium reactions under allergic conditions in vivo and in vitro by using mice models of AR and cultured airway epithelial cells. To the best of our knowledge, we are the first to evaluate the role of Orai1 protein in the etiology of allergic diseases.

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Disclosures: Authors have nothing to disclose.

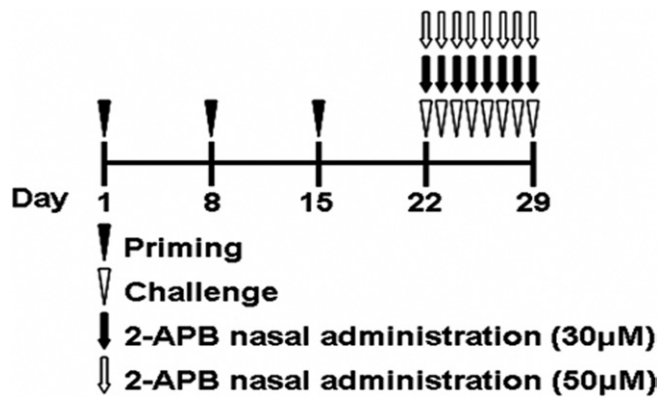


Fig. 1. Study protocols. Allergic rhinitis mice models were prepared by intraperitoneal immunization on days 1, 8, and 15 (priming) followed by daily intranasal challenge on days 22 to 29. Some groups of mice were treated with daily intranasal 2-aminoethoxydiphenyl borate (2-APB) on days 22 to 29. Nonsensitized mice were given only the last intranasal challenge on day 29.

Methods

Animals

Female BALB/c mice (6–8 weeks old) were purchased from the Chinese Academy of Sciences Shanghai Laboratory Animal Center. These mice were maintained in horizontal laminar flow cabinets and provided sterile food and water in a specific pathogen-free facility. Animal studies were approved by the Institutional Animal Care and Use Committee of Fudan University. The mice were randomly divided into 4 experimental groups ($n = 12$ for each group).

Allergic model system

According to published procedures,¹⁶ mice were administered 0.5 mg/mL of ovalbumin (grade V; Sigma-Aldrich, St. Louis, Missouri) and 20 mg/mL of aluminium hydroxide (Sinopharm Chemical Reagent Co Ltd, Shanghai, China) in saline at a dosage of 0.2 mL per mouse by intraperitoneal injection. The sensitization was repeated 3 times at weekly intervals (days 1, 8, and 15) followed by daily instillation of ovalbumin solution droplet (40 mg/mL in saline) into the nostrils (0.02 mL per mouse) using a micropipette on days 22 to 29 (challenge) (Fig 1). Some groups of mice were administered 30 or 50 μ M¹⁷ (0.02 mL per mouse) of 2-APB (Sigma-Aldrich) in saline into the nostril daily on challenging days (2-APB-treated groups), whereas another group of mice was not treated with it (control group). As a negative control, a group of mice received neither sensitization nor challenge treatment, except for the last challenge on day 29 (nonsensitized group). To avoid lung deposi-

tion of allergen, we repeatedly applied a 10- μ L droplet of ovalbumin into the noses of awake ovalbumin-sensitized mice.¹⁸ Nasal symptoms were evaluated by counting the numbers of sneezes and nasal rubs during the 10 minutes immediately after the last ovalbumin intranasal provocation on day 29.

Tissue, nasal lavage fluid, and bronchoalveolar lavage fluid

After exsanguinations of one portion of mice, the head and trachea of each mouse were obtained for immunohistochemical staining. From the other portion of the mice the nasal mucosa and trachea were obtained and cut into 2 portions: one was dissected for protein isolation and the other for messenger RNA (mRNA) extraction. All sections were frozen at -70°C .

After the mice were killed, 1 blunted, 18-gauge needle was pointed toward the heads of a portion of the mice for nasal lavage fluid (NLF). One injection of 1,000 μ L of phosphate-buffered saline was performed, and the fluid was collected with a tube under both nares of the nose for NLF. Bronchoalveolar lavage fluid (BALF) was obtained through a tracheal cannula. One injection of 1,000 μ L of phosphate-buffered saline was administered, and the fluid was collected. NLF and BALF were centrifuged for 10 minutes at 200g at 4°C . The supernatants were stored at -70°C , and the concentrations of IL-1 β , IL-6, macrophage inflammatory protein 2 (MIP-2), and TNF- α were determined using enzyme-linked immunosorbent assay (ELISA).

Immunohistochemical staining

After mice were killed, specimens, including heads and tracheas, were embedded in paraffin and sectioned at a thickness of 4 μ m for immunohistochemical staining. The staining protocol was referred to as e-Method 1 (see supplementary material). Antibody localization was detected microscopically by brown staining. The microscopic examination was performed by 2 independent observers who were masked to the sample groups using different magnifications ($\times 200$ or $\times 1,000$).

Airway epithelium culture

Primary epithelial cultures were established as previously described.¹⁹ Mice were killed, and the nasal epithelium on either side of the nasal septum was removed by dissection.²⁰ The detailed protocol was referred to as e-Method 2 (see supplementary material). Mice tracheas were also obtained. The tracheal epithelial cells were cultured according to the published protocols.¹⁹ Briefly, the nasal and tracheal epithelial cells were cultured in 24-well plates at a concentration of 2×10^5 per well in 1

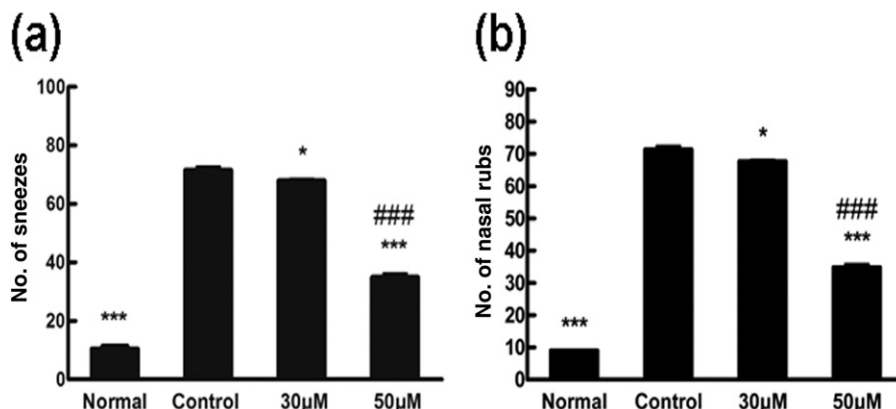


Fig. 2. Nasal allergic symptoms. The numbers of sneezes (a) and nasal rubs (b) were determined 10 minutes immediately after the final challenge. Values represent the mean (SEM) of 6 mice in each group. * $P < .05$ and *** $P < .001$ vs control group; ### $P < .001$ vs 30 μ M group. Normal indicates the nonsensitized group; control, control group; 30 μ M, 30 μ M 2-aminoethoxydiphenyl borate (2-APB)-treated group; and 50 μ M, 50 μ M 2-APB-treated group.

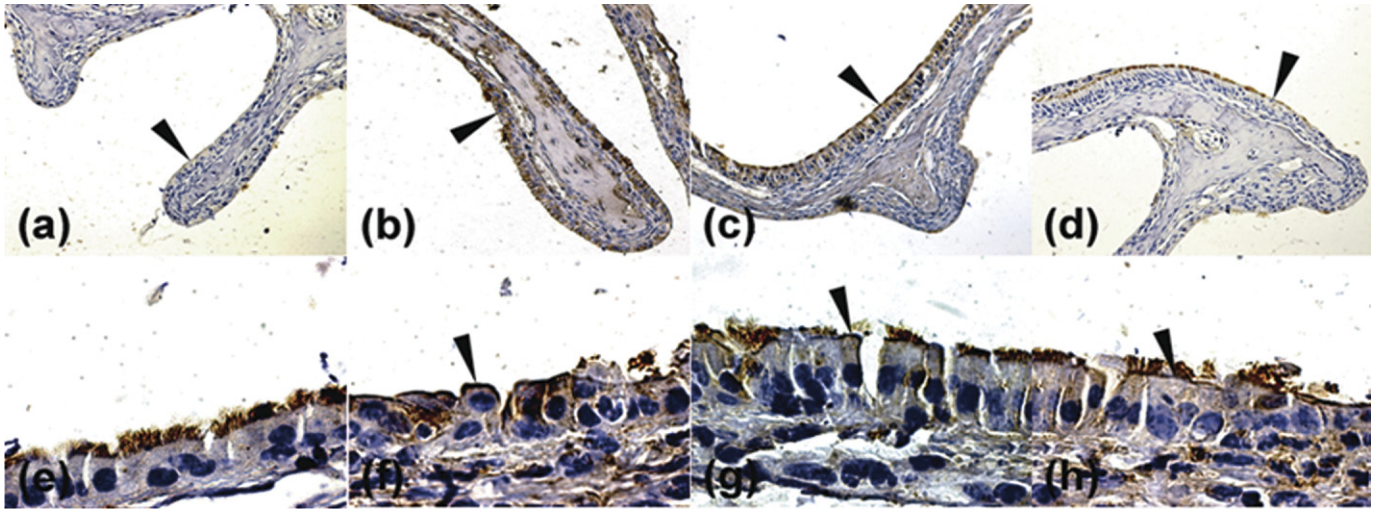


Fig. 3. Immunohistochemistry examination of Orai1. Nose (a, b, c, and d) and trachea (e, f, g, and h) sections were stained with immunohistochemical staining. a and e, Nonsensitized mice; b and f, control mice; c and g, 30 μ M-treated mice; d and h, 50 μ M-treated mice. The arrowheads indicate the immunohistochemical staining of Orai1 (a, b, c, and d, original magnification $\times 200$; e, f, g, and h, original magnification $\times 1,000$).

mL of basal medium. Six wells were cultured with either media alone (nonsensitized group) or media containing 10 μ g/mL of ovalbumin (control group) or 10 μ g/mL of ovalbumin plus 30 μ M 2-APB (30 μ M group) or 10 μ g/mL of ovalbumin plus 50 μ M 2-APB (50 μ M group), and the protocol was continued for periods up to

7 days. Supernatants were removed and stored at -70°C for cytokine analysis. Sheets of nasal and tracheal epithelium were obtained after dispase treatment, stored at -70°C , and prepared for analysis with Orai1 protein, its mRNA, and the mRNAs of the cytokines.

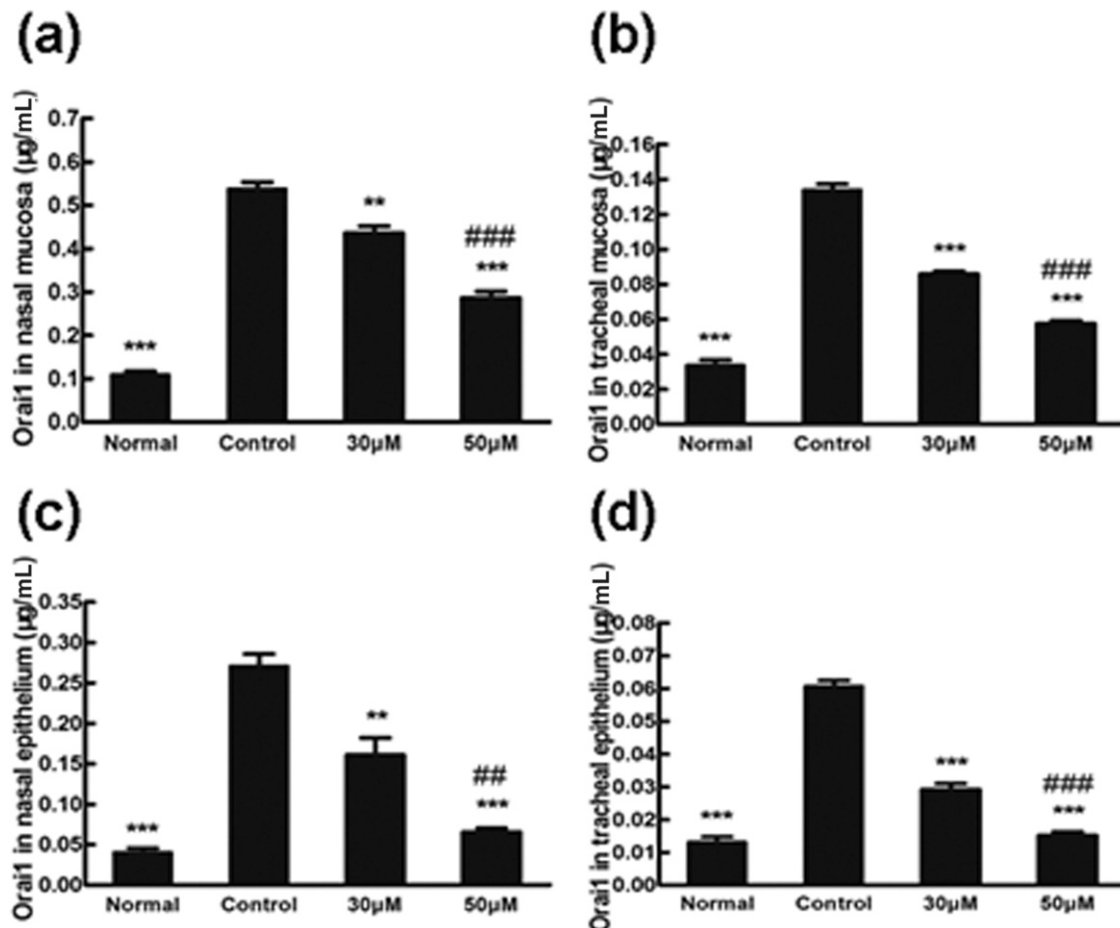


Fig. 4. Orai1 protein expression in mice nasal (a) and tracheal (b) mucosa and in cultured nasal (c) and tracheal (d) epithelium. Each value in the enzyme-linked immunosorbent assay represents the mean (SEM) of 6 mice in each group. ** $P < .01$, *** $P < .001$ vs control group; ### $P < .001$ vs 30 μ M group. Normal indicates nonsensitized group; control, control group; 30 μ M, 30 μ M 2-aminoethoxydiphenyl borate (2-APB)-treated group; and 50 μ M, 50 μ M 2-APB-treated group.

Enzyme-linked immunosorbent assay

We quantified Orai1 protein in frozen tissue samples using ELISA. The ELISA protocol was referred to as e-Method 3 (see supplementary material). Concentrations of IL-1 β , IL-6, MIP-2, and TNF- α were evaluated using corresponding ELISA kits purchased from Cell Applications Inc (San Diego, California). The ELISAs were performed in accordance with the manufacturers' protocols.

Real-time reverse transcription–polymerase chain reaction

Primers were designed using Primer Express software (Applied Biosystems, Foster City, California) from sequence available in Genbank and were synthesized (Geneland Biotech, Shanghai, China). Real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed to detect mRNAs of Orai1, IL-1 β , IL-6, MIP-2, and TNF- α . Orai1 primers were as follows: forward primer 5'-TTTGCCTCATGATCAGCAC-3' and reverse primer 5'-TTGGCGACGATGACTGATTC-3'. IL-1 β primers were as follows: forward primer 5'-TCCAGGATGAGGACATGAGCAC-3' and reverse primer 5'-GAACGTCACACCAGCAGGTTA-3'. IL-6 primers were as follows: forward primer 5'-CCACTTCACAAGTCGGAGGCTTA-3' and reverse primer 5'-GCAAGTGCATCATCGTTGTTTCATAC-3'. MIP-2 primers were as follows: forward primer 5'-CCACTCTCAAGGGCGGTCAA-3' and reverse primer 5'-CACTTTCTCTGACCCCTATTCCCC-3'. TNF- α primers were as follows: forward primer 5'-ATGAGCACAGAAAGCATGATC-3' and reverse primer 5'-TCCACTTGGTGGTTTGCTACG-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also examined to control the sample-to-sample variation in RNA isolation and integrity by using a pair of primers: forward primer 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer 5'-TCCAC-CACCCTGTTGCTGTA-3'. The real-time RT-PCR protocol was referred to as e-Method 4 (see supplementary material). Specificity of PCR products was evaluated by melting curve analysis and by size in agarose gels. Using 3 dilutions of complementary DNA, we controlled for linearity of PCR amplification. Evaluation of data was performed using threshold cycle values with GAPDH as the internal standard.

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Statistical analysis

Statistical analysis was performed using commercially available statistical software (Prism 4.0; GraphPad Software Inc, San Diego, California). Data were analyzed with the unpaired *t* test with or without Welch correction and presented as mean (SEM). Significance of a difference was accepted at the 5% level of confidence. *P* < .05 was considered statistically significant.

Results

Improved symptoms in AR mice after 2-APB treatment

Numbers of sneezes and nasal rubs were counted after the last antigen challenge. Control mice showed an increase in numbers of sneezes and nasal rubs compared with those in nonsensitized mice (Fig 2). However, the numbers for 2-APB-treated allergic groups was less than those for the control group (Fig 2). The results clearly indicate

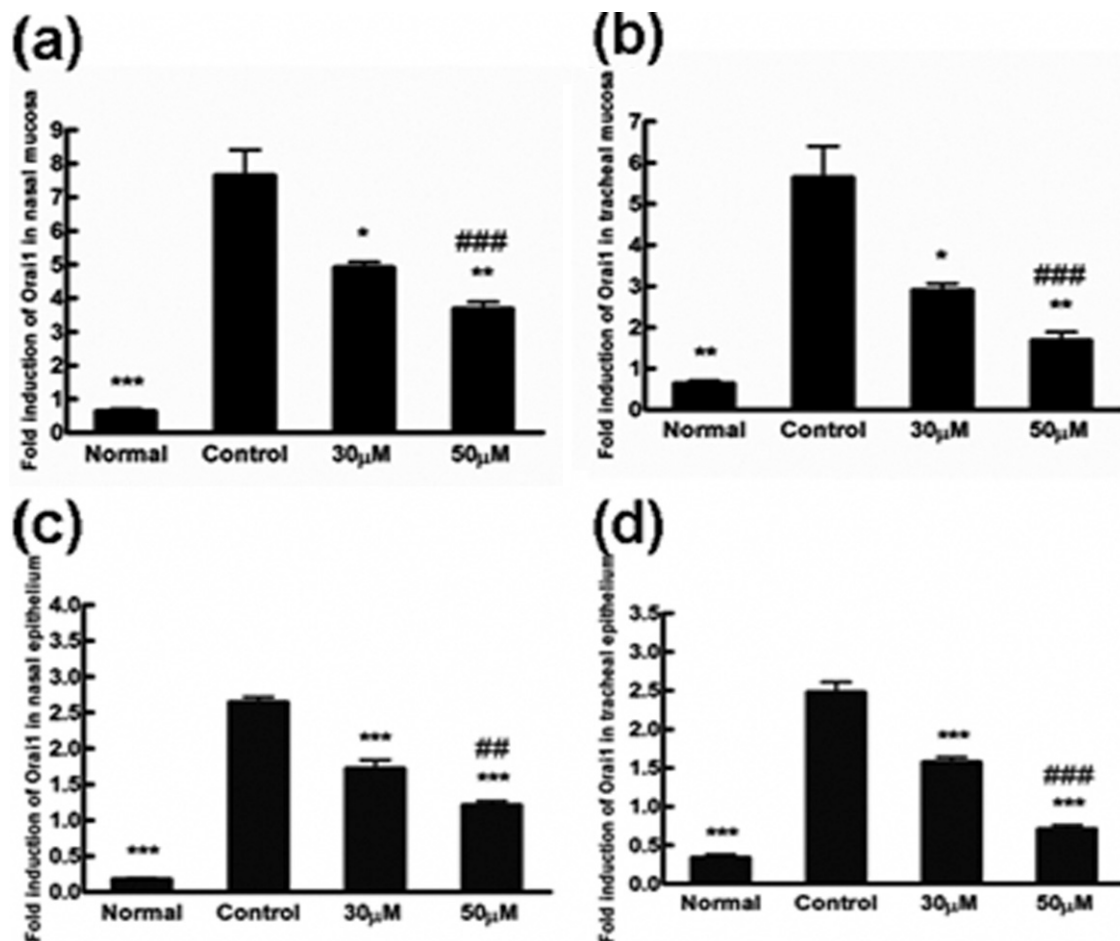


Fig. 5. Orai1 messenger RNA expression in mice nasal (a) and tracheal (b) mucosa and in cultured nasal (c) and tracheal (d) epithelium. Each value in the real-time reverse transcription–polymerase chain reaction graphs represents the mean (SEM) of 6 mice in each group. **P* < .05, ***P* < .01, ****P* < .001 vs control group; ##*P* < .01, ###*P* < .001 vs 30 μ M group. Normal indicates nonsensitized group; control, control group; 30 μ M, 30 μ M 2-aminoethoxydiphenyl borate (2-APB)-treated group; and 50 μ M, 50 μ M 2-APB-treated group.

that the nasal allergic model was constructed successfully and 2-APB treatment effectively improved allergic symptoms in this model system.

Orai1 expression in airway mucosa and cultured epithelium after 2-APB intervention

Immunohistochemical staining of Orai1 showed that this protein localized in the plasma membrane of nasal and tracheal mucosa epi-

thelium in AR mice (Fig 3b and f) but was less detectable in nonsensitized mice and 2-APB-treated groups (Fig 3a, c, and d, and Fig 4e, g, and h). According to ELISA and real-time RT-PCR, Orai1 protein and its mRNA were increased in airway mucosa and cultured airway epithelium of AR groups compared with those of nonsensitized groups and were decreased in treated groups (Figs 4 and 5). The results indicated

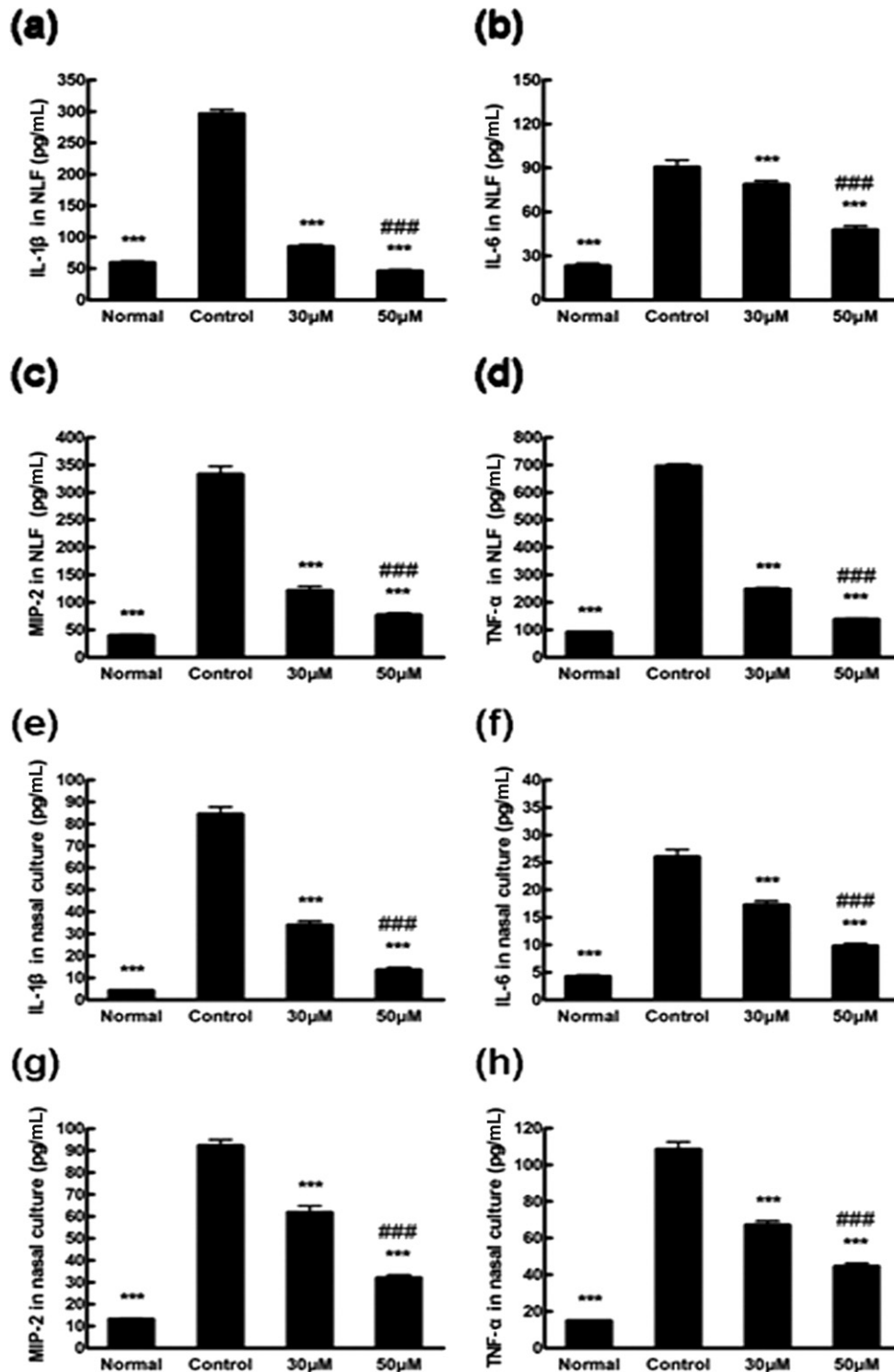


Fig. 6. Enzyme-linked immunosorbent assay (ELISA) analysis of mice nasal lavage fluid (NLF) and nasal culture supernatant. The NLF and nasal culture supernatant were collected for ELISA analysis of interleukin (IL) 1 β (a), IL-6 (b), macrophage inflammatory protein 2 (MIP-2) (c), and tumor necrosis factor α (TNF- α) (d) in NLF and IL-1 β (e), IL-6 (f), MIP-2 (g), and TNF- α (h) in nasal culture supernatant. Each value represents the mean (SEM) of 6 mice in each group. *** P < .001 vs control group; ### P < .001 vs 30 μ M group. Normal indicates nonsensitized group; control, control group; 30 μ M, 30 μ M 2-aminoethoxydiphenyl borate (2-APB)-treated group; and 50 μ M, 50 μ M 2-APB-treated group.

that Orai1 in airway epithelium was improved under allergic conditions and was reduced after prevention of 2-APB.

NLF, BALF, and culture supernatant concentrations of IL-1 β , IL-6, MIP-2, and TNF- α after orai1 intervention

We examined indicated mediators in NLF, BALF, and culture supernatants among groups. The immunization in vivo and co-

culture in vitro with ovalbumin heightened NLF and nasal culture supernatant levels of these mediators in control groups compared with those in nonsensitized ones (Fig 6). In contrast, the administration of 2-APB reduced their concentrations in treated groups compared with those in control groups (Fig 6). As for BALF and tracheal culture supernatant, the challenge and

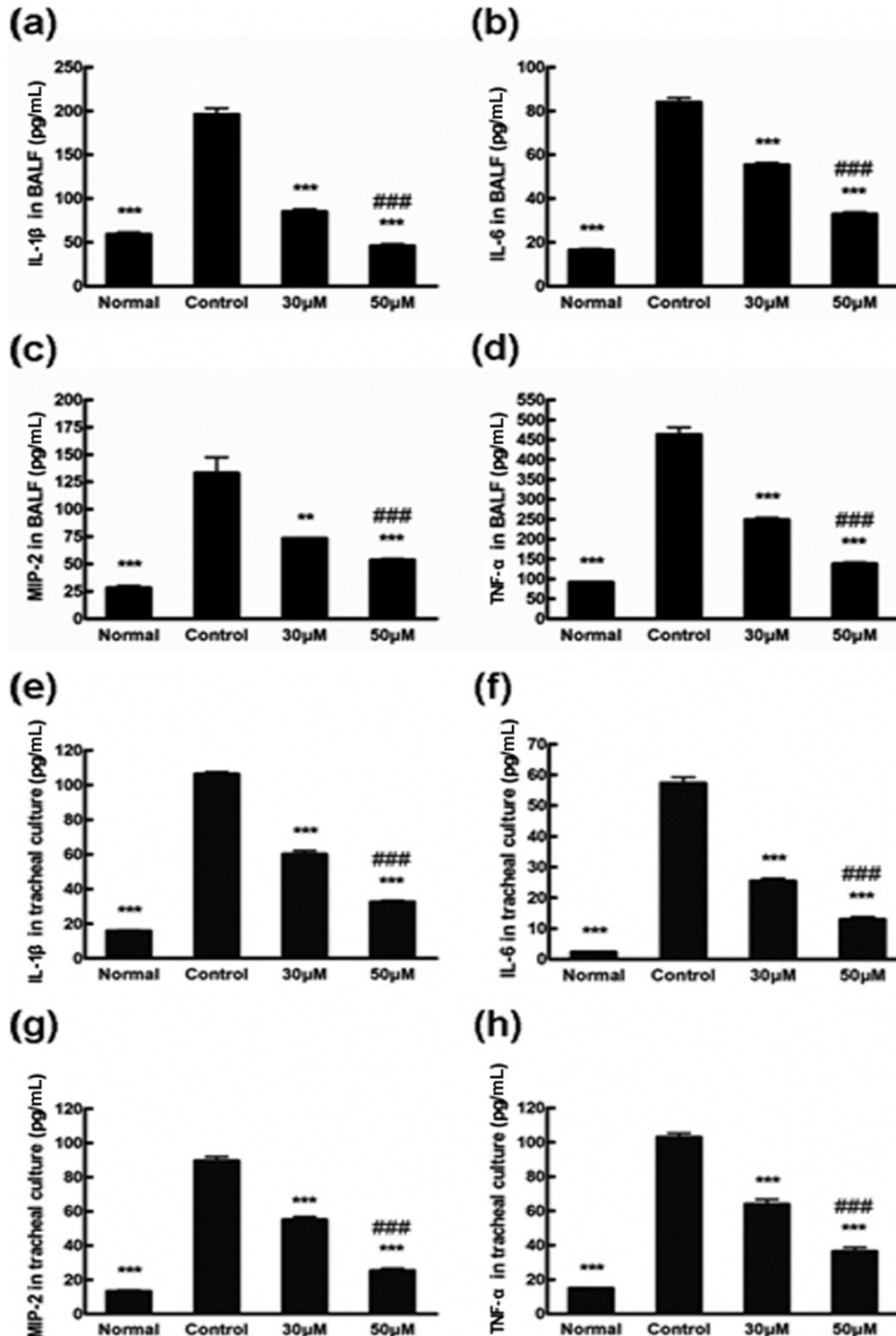


Fig. 7. Enzyme-linked immunosorbent assay (ELISA) analysis of mice bronchoalveolar lavage fluid (BALF) and tracheal culture supernatant. The BALF and tracheal culture supernatants were collected for ELISA analysis of interleukin (IL) 1 β (a), IL-6 (b), macrophage inflammatory protein 2 (MIP-2) (c), and tumor necrosis factor α (TNF- α) (d) in BALF and IL-1 β (e), IL-6 (f), MIP-2 (g), and TNF- α (h) in tracheal culture supernatant. Each value represents the mean (SEM) of 6 mice in each group. ** P < .01, **** P < .001 vs control group; **** P < .001 vs 30μM group. Normal indicates nonsensitized group; control, control group; 30μM, 30μM 2-aminoethoxydiphenyl borate (2-APB)-treated group; and 50μM, 50μM 2-APB-treated group.

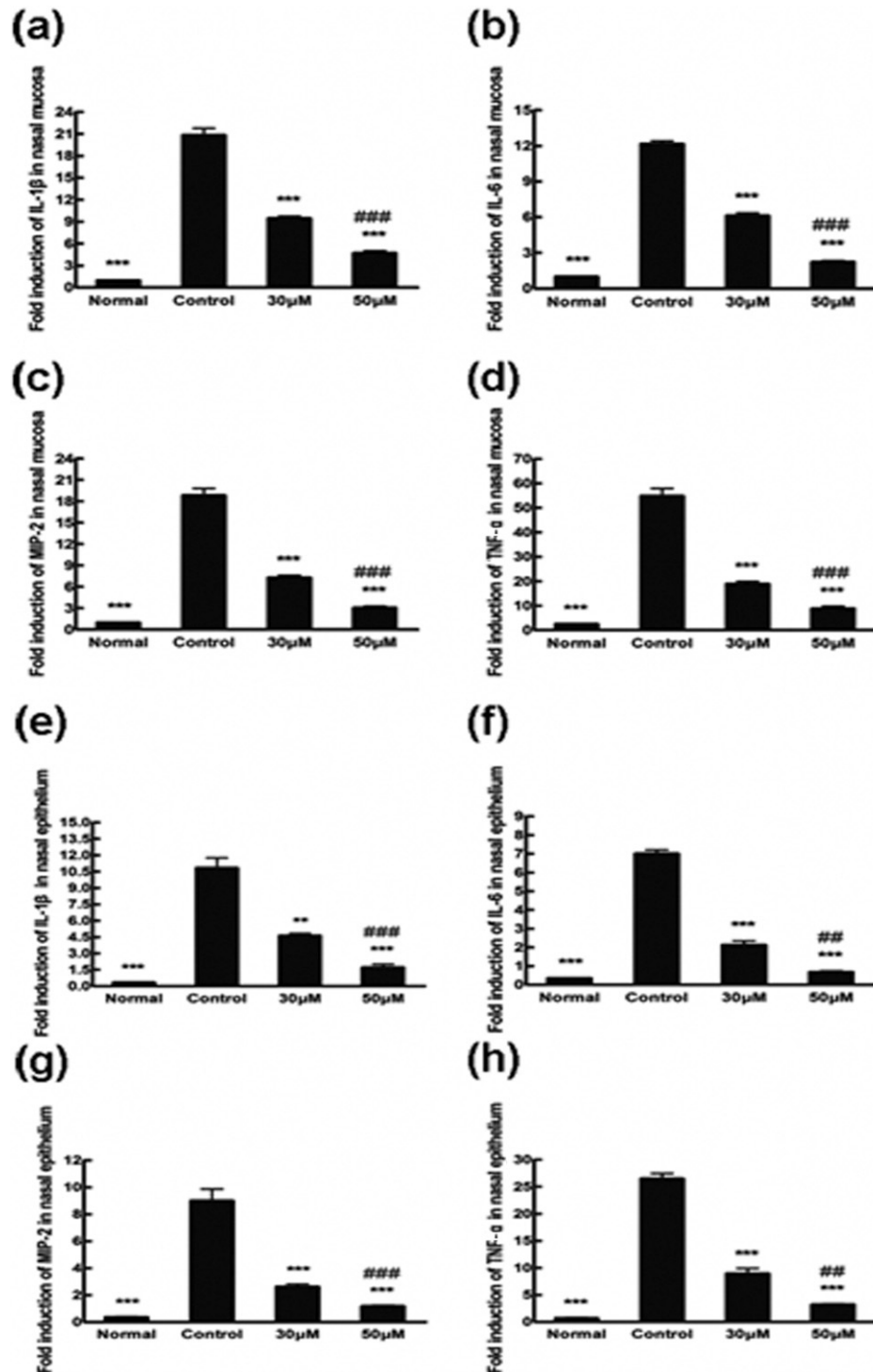


Fig. 8. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of interleukin (IL) 1 β , IL-6, macrophage inflammatory protein 2 (MIP-2), and tumor necrosis factor α (TNF- α) in nasal mucosa and cultured nasal epithelium. Real-time RT-PCR was performed to detect the messenger RNAs (mRNAs) of IL-1 β (a), IL-6 (b), MIP-2 (c), and TNF- α (d) in nasal mucosa and mRNAs of IL-1 β (e), IL-6 (f), MIP-2 (g), and TNF- α (h) in cultured nasal epithelium. Each value represents the mean (SEM) of 6 mice in each group. ** P < .01 and *** P < .001 vs control group; ## P < .01 and ### P < .001 vs 30μM group. Normal indicates nonsensitized group; control, control group; 30μM, 30μM 2-aminoethoxydiphenyl borate (2-APB)-treated group; and 50μM, 50μM 2-APB-treated group.

coculture with specific antigen enhanced the contents in allergic groups compared with those in the nonsensitized groups (Fig 7). On the contrary, 2-APB treatment alleviated their contents in treated groups in contrast with those in control groups (Fig 7). These results showed that the intervention of Orai1 prohibited airway allergic reactions at the protein level in vivo and in vitro.

Induction of mRNAs of IL-1 β , IL-6, MIP-2, and TNF- α in airway mucosa and cultured epithelium after 2-APB administration

As shown in Figure 8, control groups showed an increased expression of mRNAs of these mediators in nasal mucosa and cultured nasal epithelium compared with those of nonsensitized ones. The 2-APB intervention reduced the mRNAs of these mediators (Fig 8). In Figure 9, the graphs reveal that the control groups showed an

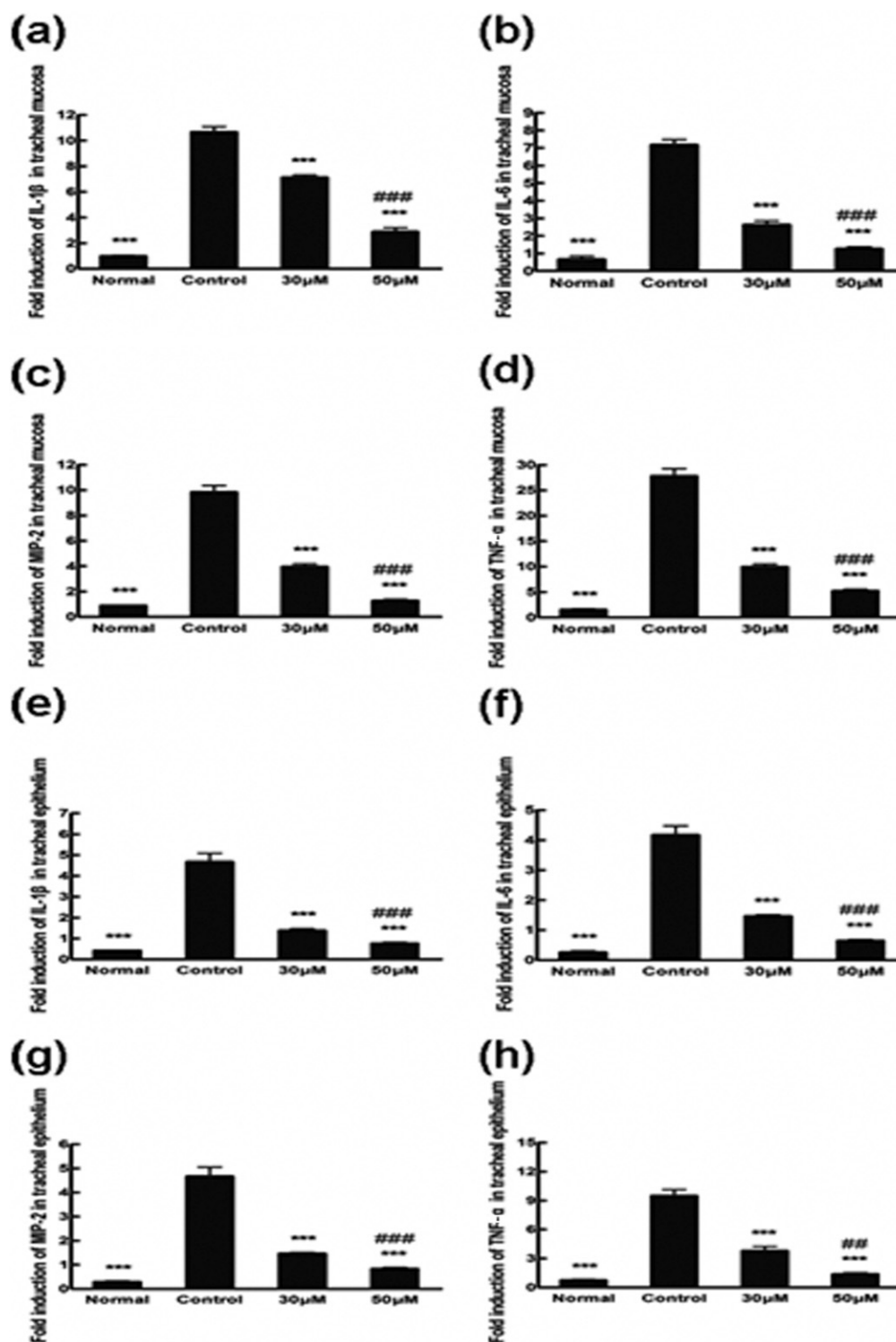


Fig. 9. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of interleukin (IL) 1 β , IL-6, macrophage inflammatory protein 2 (MIP-2), and tumor necrosis factor α (TNF- α) in tracheal mucosa and cultured tracheal epithelium. Real-time RT-PCR was performed to detect the messenger RNAs (mRNAs) of IL-1 β (a), IL-6 (b), MIP-2 (c), and TNF- α (d) in tracheal mucosa and mRNAs of IL-1 β (e), IL-6 (f), MIP-2 (g), and TNF- α (h) in cultured tracheal epithelium. Each value represents the mean (SEM) of 6 mice in each group. *** P < .001 vs control group; ### P < .001 vs 30 μ M group. Normal indicates nonsensitized group; control, control group; 30 μ M, 30 μ M 2-aminoethoxydiphenyl borate (2-APB)-treated group; and 50 μ M, 50 μ M 2-APB-treated group.

enhanced expression of mRNAs of the mediators in tracheal mucosa and cultured tracheal epithelium compared with those of the nonsensitized groups. The 2-APB treatment buffered their mRNAs (Fig 9). These findings indicate that 2-APB restrained allergic responses in the airway at the mRNA level in vivo and in vitro.

Different effects on epithelium responses after administration of different concentrations of 2-APB

When allergic groups were administered 2-APB at a concentration of 30 μ M or 50 μ M during the ovalbumin challenge period, the numbers of sneezes and nasal rubs were lessened in the latter

groups (Fig 2). In the meantime, Orai1 protein and mRNA production in the 50 μ M 2-APB-treated groups was less than that in the 30 μ M 2-APB-treated groups in the airway mucosa and cultures (Figs 4 and 5). Similarly, statistical differences were found between 2 groups in protein and mRNA concentrations of the mediators in mice airway and culture (Figs 6–9). This finding implies that the effect of 2-APB treatment on mice airway epithelium reactions may rely on the concentration administered.

Discussion

Nasal mucosa plays a crucial role in the initiation and development of AR. Recent studies indicate that nasal epithelial cells perform a broad scope of functions, such as release of cytokines and chemokines^{1,2} and presentation of antigens to T cells.²¹ However, the performances of epithelial cell activities, such as secretion, gene transcription, and proliferation, are dependent on the Ca^{2+} signaling process. Orai1 protein, essential to CRAC channels, which regulate SOCE through depletion of Ca^{2+} from ER stores, may modify the concentration of cytoplasmic Ca^{2+} to control these cellular activities. On the basis of our animal experiment, we characterized upregulation of Orai1 protein and mRNA in the nasal mucosa of AR mice and in a control group of cultured nasal epithelium in contrast to those in nonsensitized mice and in nonsensitized cultured nasal epithelium (Fig 4a and c, and Fig 5a and c). The Orai1 molecule may participate in the forming of a CRAC channel pore at the surface of the nasal epithelial cells, which induced the activation of CRAC channels and subsequent substantial SOCE. Upregulation of cytoplasmic Ca^{2+} may accelerate cellular activities such as secretion of cytokines, chemokines, and other molecules, translocation of transcription factors into nucleus, and transcription of relevant genes. The activation of nasal epithelium in the allergic state may, as a result, arouse and enhance allergic reactions in vivo and in vitro through more release of inflammatory mediators, such as IL-1 β , IL-6, MIP-2, and TNF- α , in our research (Figs 6 and 8). Furthermore, it has been reported that the increased expression of HLA-DR and CD86 in the nasal epithelial cells of AR mice deriving from augmentation of Ca^{2+} concentration promoted presentation of antigen to T cells.²¹ Therefore, 2-APB treatment may suppress nasal epithelium activation directly to prohibit antigen presentation through blockade of the Orai1 protein.

The fundamental relationship between AR and asthma is that these 2 conditions represent 1 entity in 2 different parts of the respiratory tract.²² We used a mouse model system in which a compartmentalized allergic $\text{T}_\text{H}2$ response was exclusively induced in the nose¹⁸ to study the interactions between the upper and lower airways and to identify the possible underlying mechanisms. Our experiment indicated that Orai1 protein and mRNA were upregulated in tracheal mucosa without direct interaction with allergen (Figs 4b and 5b), and IL-1 β , IL-6, MIP-2, and TNF- α levels in BALF and tracheal mucosa were also enhanced after nasal challenge (Fig 7a, b, c, and d, and Fig 9a, b, c, and d). According to the hypothesis that systemic inflammation triggered by nasal provocation led to bronchial allergic responses,²³ the mediators and $\text{T}_\text{H}2$ profile cytokines released from the corresponding cells in allergic nasal mucosa entered into blood circulation and were likely responsible for the allergic reactions of lower airway epithelial cells. There may be other factors, such as certain chemokine receptors and/or pattern recognition receptors,²⁰ residing in airway mucosa epithelium that contribute to the upregulation of Orai1 and modulation of cytoplasmic Ca^{2+} in this cell type. In any case, increase of Orai1 protein in our study may lead to the establishment and activation of CRAC channels in the plasma membrane of tracheal epithelial cells. These cells may in turn aggravate allergic reactions in the lower airway through release of a variety of cytokines and chemokines, such as IL-1 β , IL-6, MIP-2, and TNF- α .^{1,2} These inflammatory mediators can aggravate the destroyed epithelial cilia, thickening of the

basement membrane, and infiltration of inflammatory cells in tracheal mucosa (Fig 3f). However, 2-APB administration into the nostril decreased production of Orai1 protein and its mRNA in tracheal mucosa (Figs 4b and 5b) and indicated mediators in BALF and tracheal mucosa in vivo in our work (Fig 7a, b, c, and d, and Fig 9a, b, c, and d). The detailed mechanisms of relationships between the upper and lower airway should be further elucidated.

On the other hand, we performed an in vitro study to investigate the effect of 2-APB on the cultured tracheal epithelium. Similar to nasal epithelium in cultures, we verified upregulation of Orai1 in the tracheal epithelium of a control group in contrast to that in a nonsensitized group in our experiment (Figs 4d and 5d). The participation of Orai1 protein induced the activation of CRAC channels and the subsequent cellular activities of tracheal epithelial cells in the allergic state. These cells may in turn initiate and promote allergic reactions through more production of inflammatory mediators (Fig 7e, f, g, and h, and Fig 9e, f, g, and h).

The impact of 2-APB on airway epithelium reactions was dependent on the concentrations of the administration in this current experiment. As shown in Figures 2–9, 50 μ M treatment diminished production of Orai1 and relevant mediators in vivo and in vitro and improved allergic symptoms compared with those after 30 μ M treatment. Furthermore, this agent has been used as a pharmacologic means in the study of SOCE in vivo (mouse¹⁷ and bovine²⁴) and in vitro (human cell strain¹⁴) for many years, and its toxic trait has not yet been found. As for the human body, no relevant reports have been made about this point, yet 2-APB is a potential drug that might alleviate allergic responses and improve allergic symptoms in allergic diseases.

There may be other Ca^{2+} channels, such as the voltage-sensitive Ca^{2+} -conducting channel in the airway epithelial cells membrane.²⁵ However, no report has indicated that this kind of channel could perform a relevant function in allergic diseases. According to our data, CRAC channels may play a role in causing changes of intracellular Ca^{2+} of airway epithelium in allergic inflammation.

In summary, the present study has clarified Orai1 expression in mice airway epithelium and the influence of exogenous 2-APB on upper and lower airway epithelium responses in vivo and in vitro. Intranasal administration with 2-APB may offer novel and effective therapeutic means to treat AR and allergic asthma by intervening expression of Orai1 and relevant mediators.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.anai.2011.09.013](https://doi.org/10.1016/j.anai.2011.09.013).

e-METHODS**1. Immunohistochemical staining**

After mice were killed, specimens, including heads and tracheas, were embedded in paraffin and sectioned at a thickness of 4 μm for immunohistochemical staining. The paraffin sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes. Normal goat serum was used to block nonspecific binding sites, and Orai1-L1 rabbit polyclonal antibody (100 $\mu\text{g}/150\ \mu\text{L}$; NewEast Biosciences, Malvern, Pennsylvania) in antibody diluent at 1:100 dilution was immediately applied to the sections and incubated for 45 minutes. After washing, biotinylated anti-rabbit polyclonal IgG (Vector Lab, Burlingame, California) was applied to each section for 30 minutes, followed by 20 minutes of incubation with horseradish peroxidase streptavidin (Vector Lab). Slides were then stained with 3, 3'-diaminobenzidine chromogene (Sigma-Aldrich, St. Louis, Missouri) for 5 minutes and counterstained with hematoxylin.

2. Airway epithelium culture

Mice were killed, and the nasal epithelium on either side of the nasal septum was removed by dissection, transferred to 1 mL of dissociation medium (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid and Dulbecco modified Eagle medium [HEPES-DMEM], with 0.05% [wt/vol] Protease XIV [Sigma-Aldrich], 1% penicillin-streptomycin), and kept for 24 hours at 4°C. Dissociation was stopped after 30 seconds of centrifugation at 14g, and the tissue resuspended in storage medium (HEPES-DMEM, 10% fetal calf serum, 1% penicillin-streptomycin) at 4°C. The dissociated epithelium was viewed under phase-contrast optics (original magnification $\times 200$) and epithelial sheets containing approximately 50 to 100 beating ciliated cells were separated from the main body of the tissue using sharpened stainless needles. These were transferred by micropipette into 150 μL of isolation medium (145mM sodium chloride, 45 mM potassium chloride, 2.0mM ethylene glycol tetraacetic acid, 10mM glucose, 10mM HEPES, pH 7.4) in a microwell (16-mm-diameter, glass coverslip glued to a 1-mm plastic ring of equal diameter) and incubated for 10 minutes at room temperature. Nasal epithelia cells were incubated in 200 μL of culture media per semipermeable support membrane (Costar Transwell Clear, tissue culture-treated, polyester membrane, 24-well plate inserts, 0.4- μm pore; Corning Costar, High Wycombe, England), precoated with human placental collagen, type VI (Sigma-Aldrich), with 600 μL of culture media in the basal compartment. Cultures

were incubated at 37°C in 6% carbon dioxide in a humidified incubator for 3 days, before medium on the apical surface was removed and the basal medium was replaced with 600 μL of differentiation media (collection media with 2% Ultrosor-G serum substitute), replaced twice weekly.

3. Enzyme-linked immunosorbent assay

We quantified Orai1 protein in frozen tissue samples using enzyme-linked immunosorbent assay. The frozen tissues were sliced, lysed, and stored at -70°C . Orai1 protein was measured using a 96-well microplate coated with 1 $\mu\text{g}/\text{mL}$ of Orai1 rabbit polyclonal antibody (NewEast Biosciences). The microplate was incubated in 3% bovine serum albumin at 37°C for 1 hour. Samples at 1:10 dilution were then added followed by incubation at 37°C for 1 hour. After washing, Orai1 antibody that had been biotinylated using a biotinylation kit (American Qualex International Inc, San Clemente, California) was then added at 1 $\mu\text{g}/\text{mL}$ and allowed to incubate at 25°C for 1 hour. After washing, 1.5 $\mu\text{g}/\text{mL}$ of streptavidin peroxidase was added followed by incubation at 25°C for 1 hour. After washing, tetramethylbenzidine (TMB) substrate (12.5 mL of citric-phosphate buffer, 200 μL of TMB stock solution [6 mg/mL in dimethyl sulfoxide], 100 μL of 1% hydrogen peroxide; Fluka Chemical Co, Ronkonkoma, New York) was added to produce a color reaction. The reaction was terminated by the addition of 6 N sulfuric acid. Optical density was determined at 450 nm using a microplate reader (Microplate Reader MTP-32; Corona Electric, Ibaraki, Japan).

4. Real-time reverse transcription-polymerase chain reaction

Total cellular RNA was extracted with Trizol (Invitrogen, Carlsbad, California) and treated with RNase-free DNase I (Ambion, Austin, Texas) twice at 37°C for 30 minutes to eliminate contaminating genomic DNA. For reverse transcription, 2 μg of the RNA was reversely transcribed with random hexamers (Invitrogen), and complementary DNA (cDNA) was amplified according to the manufacturer's instructions. After initial denaturation at 95°C for 10 minutes, the amplification profile was 15 seconds of denaturation at 95°C and 1 minute of annealing and extension at 60°C for 45 cycles. Negative control reverse transcription reaction mixtures contained no reverse transcriptase and no cDNA in the polymerase chain reaction (PCR) amplification mixtures. For measurement, 2 μL of diluted cDNA was amplified in a total reaction volume of 20 μL by using a 7500 real-time PCR System (Applied Biosystems, Foster City, California) with 20 $\times$ SYBR Green mixture (Invitrogen). Specificity of PCR products was evaluated by melting curve analysis and by size in agarose gels.