In Vitro Treatment with 2-APB Inhibits the Inflammation in Nasal Polyps

Lin Lin, MD1*, Fei Dai, MM1*, Zhongchun Chen, MM1, and Lihui Cai, MM1

Abstract
Objective. Glucocorticoids are considered the main treatment option for chronic rhinosinusitis with nasal polyps (CRSwNP), but their effect rate ranges from 60.9% to 80%. Novel therapeutic means should be studied. The purpose of this study was to investigate the expression of Orai1 in nasal polyps (NPs) and the influence of intervention of Orai1 on NPs after in vitro treatment of 2-aminoethoxydiphenyl borate (2-APB).

Study Design. Prospective cross-sectional study.

Setting. University hospital.

Subjects and Methods. Nasal biopsy samples were obtained from normal subjects or subjects with CRSwNP. We studied the localization of Orai1 protein in NPs by using immunohistochemistry. Then these tissues in cultures were maintained in the absence or presence of dexamethasone (DEX) or 2-APB. Orai1 was examined by Western blot, enzyme-linked immunosorbent assay (ELISA), and real-time reverse transcription-polymerase chain reaction (RT-PCR). Inflammatory mediators including interleukin (IL)-1β, IL-5, eosinophil cation protein (ECP), leukotriene (LT)C4, interferon (IFN)-γ, and dermato-phagoides pteronyssinus (DP)-specific immunoglobulin E (sIgE) as well as mucins (MUCs) including MUC5B and MUC7 in cultures were analyzed with ELISA and real-time RT-PCR.

Results. The expression of Orai1 was localized to cytoplasmic membrane of inflammatory cells and submucosal glandular cells and was upregulated in NPs compared with normal nasal mucosa. Orai1 was decreased in NPs after in vitro treatment of 2-APB but not after DEX intervention. The levels of inflammatory mediators and mucins were reduced more after 2-APB treatment when compared with those after DEX treatment.

Conclusion. Orai1 may play crucial roles in NP formation, and the intervention of Orai1 may inhibit NP development.

Keywords
nasal polyps, inflammation, Orai1, DEX, 2-APB

The annual incidence of chronic rhinosinusitis with nasal polyps (CRSwNP) is 0.63 cases per 1000.1 This disease affects 0.5% to 4% of the world population.2 The pathogenesis of nasal polyps (NPs) remains unclear. NPs are characterized by morphological changes, such as hyperplasia and metaplasia of epithelium; infiltration of inflammatory cells consisting of mast cells, eosinophils, lymphocytes, and neutrophils3; and stromal edema and fibrosis. It is theorized that allergy, bacterial or fungal infections, and structural abnormalities all play a role in polyph formation.4

Glucocorticoids, like dexamethasone (DEX), are the most widely prescribed anti-inflammatory drugs and are shown to downregulate the proinflammatory cytokines and adhesion molecules that attract and activate eosinophils in NPs.5 However, these drugs have several side effects. The most common results of local corticosteroid use are epistaxis and nasal irritation, including itching, sneeze, dry nose, and rhinitis.6 Therefore, a novel therapeutic strategy for NPs that directly addresses their pathogenesis is urgently needed.

Intracellular Ca2+ signals regulate a diverse array of physiologic processes including cellular secretion, cell growth, and gene expression.7 Changes of cytoplasmic Ca2+ levels are caused by altered flux across plasma membrane and by release from endoplasmic reticulum. Store-operated Ca2+ entry (SOCE) is activated by depletion of Ca2+ stores in endoplasmic reticulum.8 Ca2+ release–activated Ca2+ (CRAC) channel is the characterized SOCE pathway, and Orai1 has been identified as an important component of the CRAC channel.9 Coexpression of Orai1 with stromal interaction molecule 1, an identified molecule in endoplasmic reticulum, dramatically increases SOCE and CRAC channel activity.10 When Orai1 forms the CRAC channel in CD4+ T cells, Ca2+ influx via this channel in turn results in the sustained Ca2+ signal.

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Ca$^{2+}$ signaling activates nuclear factor of activated T cells (NFAT), which is required for the development of a Th2 immune response in allergic airway inflammation.$^{11-13}$ In addition, CRSwNP is primarily characterized by the prevalent Th2 responses including the invasion of inflammatory cells such as Th2 cells, mast cells, and eosinophils and the release of proinflammatory mediators like interleukin (IL)-5, eosinophil cation protein (ECP), and leukotriene (LT)C4 from them.$^{14}$ So we assume that the Orai1/CRAC/NFAT pathway may play a role in the pathogenesis of NPs. If this pathway could be blocked, the formation and development of NPs would be inhibited in accordance with the above hypothesis.

It has been reported that 2-aminoethoxydiphenyl borate (2-APB) influences SOCE by regulating CRAC channel activity, and 2-APB emerges as a useful pharmacologic tool in the study of SOCE.$^{15}$ 2-APB may have an extracellular site of action$^{16}$ and it has no direct adverse effects on calcineurin, unlike cyclosporin A and FK506, which inhibit immune system cell activation through blocking calcineurin function.$^{17}$ Our previous studies have demonstrated that 2-APB administration into the nostril alleviates murine allergic rhinitis$^{18}$ and ameliorates mucin hypersecretion from the submucosal gland, which is enhanced by IL-33 in allergic mice.$^{19}$ We aimed to analyze whether intervention in the Orai1 pathway with in vitro treatment of 2-APB could control the inflammation in NPs in this context. In the experiment, we studied the expression of Orai1 protein in NP tissues, and then we administered DEX or 2-APB into the cultured NPs in vitro to investigate protein production after these 2 treatments. Finally, we examined the cultures for concentrations of inflammatory mediators including IL-1β, IL-5, ECP, LTC4, interferon (IFN)-γ, and dermatophagoides pteronyssinus (DP)-specific immunoglobulin E (sIgE) as well as mucus (MUCs) including MUC5B and MUC7. Among these mediators, IL-1β was chosen to evaluate the activation of epithelia of NPs; IL-5, ECP, LTC4, and DP-sIgE were chosen to assess the Th2 responses; and IFN-γ was chosen to examine Th1 cell activity. In addition, MUC5B and MUC7 were chosen to analyze the function of submucosal glands in NPs.

**Materials and Methods**

Functional endoscopic sinus surgery was performed to resect NPs from patients with CRSwNP, who were referred to the Department of Otorhinolaryngology–Head and Neck Surgery, Huashan Hospital of Fudan University, Shanghai, China. The diagnosis of CRSwNP was made according to the European Position Paper on Rhinosinusitis and Nasal Polyps 2012.$^5$ Patients with an established immunodeficiency, pregnancy, coagulation disorder, Churg-Strauss syndrome, classic allergic fungal sinusitis, aspirin intolerance, or cystic fibrosis were excluded from the investigation. None of the patients had received any medications (antihistamines, antibiotics, topical or oral steroids) at least 1 month before surgery. The atopic status of CRSwNP patients was evaluated according to skin reactivity to DP on skin prick test (SPT). The reaction to the SPT was considered positive if the wheal area caused by the allergen was greater than 7 mm$^2$ (diameter $\geq$ 3 mm). The normal group (normal mucosa) consisted of samples collected from the inferior turbinates of patients undergoing nasal septoplasty–inferior turbinateplasty because of nasal obstruction. Details of patients’ characteristics are included in **Table 1**. This study was approved by the ethics committee of Huashan Hospital of Fudan University, and signed informed consent was obtained from all patients.

**Table 1. Patient Characteristics.**

<table>
<thead>
<tr>
<th></th>
<th>Normal Mucosa</th>
<th>Nasal Polyps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of subjects</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>Median age, y (range)</td>
<td>46 (24-55)</td>
<td>50.5 (27-68)</td>
</tr>
<tr>
<td>Female/male</td>
<td>5/7</td>
<td>7/11</td>
</tr>
<tr>
<td>History of asthma</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Positive skin prick test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use of antihistamines</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Use of antibiotics</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aspirin intolerance</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Use of topical steroid</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Use of oral steroid</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*aAll values, except for age, are numbers of subjects.*

Samples from inferior turbinates and NPs were obtained and cut into 3 portions: 1 portion was frozen at −70°C for extraction, 1 portion was immersed in 10% neutral buffered formalin for immunohistochemical staining, and 1 portion was cultured for in vitro treatments.

**Organ Culture of Normal Mucosa and NPs**

Normal mucosa and NPs were cultured using an air-liquid interface method.$^{20}$ Blades were used to cut the previously described tissues into 2- to 3-mm$^3$ pieces under sterile conditions. Tissue fragments were washed 3 times with phosphate-buffered saline (PBS) containing an antimycotic (5 μg/mL fungizone) and an antibiotic (300 μg/mL penicillin G) and then were rinsed with 98% Dulbecco’s minimum essential medium (DMEM) supplemented with 10% calf serum and gentamicin (20 μg/mL). To determine the effects of DEX or 2-APB treatment on NPs, the above tissues were next saturated for 1 hour in culture medium (DMEM + 10% calf serum + 10 μg/mL gentamicin) as follows: (1) with 100 μM DEX (the DEX treatment group),$^{21}$ (2) with 50 μM 2-APB (the 2-APB treatment group),$^{18}$ and (3) with neither DEX nor 2-APB (the NP control group). Then the tissues were placed on hydrated 1 × 1-cm gelatin sponge with the mucosa facing upward and the submucosa downward.

**Immunohistochemistry**

Sections were deparaffinized and rehydrated with water. Normal goat serum (Vector Laboratories, Burlingame, California) was used to block nonspecific binding sites, and the primary Orai1 antibody (New East Biosciences,
Malvern, Pennsylvania) in antibody diluent (Dako, Carpinteria, California) at 1:100 dilution was immediately applied to the sections and incubated for 45 minutes. Biotinylated antirabbit polyclonal immunoglobulin G (IgG) (Vector Laboratories) was applied to each section for 30 minutes, followed by 20 minutes of incubation with horseradish peroxidase streptavidin (Vector Laboratories). Slides were washed in PBS, stained with 3,3'-diaminobenzidine chromogen (DAB) (Sigma-Aldrich, St Louis, Missouri) for 5 minutes, and counterstained with hematoxylin. For Orai1 staining, the intensities were scored as no staining (0), weak staining (1+), moderate staining (2+), or strong staining (3+). The percentage of staining area was classified as 0 (0%), 1 (1%-10%), 2 (11%-50%), or 3 (51%-100%). The intensity and percentage scores were multiplied to give a composite score of 1 to 9 for each specimen. Composite scores of 1 to 3 were considered as low Orai1 expression, and scores of 4 to 9 were considered high expression of Orai1. Two independent observers who were blinded to subject groups performed microscopic examination with different magnifications (×200 or ×400) and scored the degrees of staining.

Western Blot Analysis
Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Sigma-Aldrich). The membrane was then blocked for 30 minutes in Tris-buffered saline (TBS) with 0.2% (vol/vol) Tween-20 (TBS-T) and 5% (wt/vol) nonfat dry milk and then incubated at 4°C overnight with primary Orai1 antibody (New East Biosciences) at a dilution of 1:1000. After incubation, the membrane was washed 3 times for 5 minutes in TBS-T buffer and then treated with peroxidase-conjugated secondary antihuman IgG antibody for 1 hour at room temperature. Chemiluminescence was detected using an ECL kit (Amersham Life Science, Buckinghamshire, UK) and the multiple Gel DOC system. Data were analyzed using Image J 1.38 software (NIH, Bethesda, Maryland). β-Actin expression was analyzed using the β-actin antibody (Sigma-Aldrich).

ELISA and Real-Time RT-PCR
The protocols for enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcription-polymerase chain reaction (RT-PCR) are described in Appendices 1 and 2, respectively (available at www.otojournal.org/supplemental).

Statistical Analysis
Statistical analysis was performed using a commercially available statistical software, Prism 6.0 (GraphPad Software Inc, San Diego, California). We used Cohen’s κ as a measure of agreement for scoring the degrees of staining between the 2 observers. Kruskal-Wallis test was performed for comparisons between patient groups. A nonparametric Mann-Whitney test was applied as the initial Kruskal-Wallis test was significant. \( P < .05 \) was considered statistically significant.

Results
Immunohistochemical Analysis of Orai1 in NPs
As shown in Figure 1, we detected Orai1 staining mainly in submucosal inflammatory cells (Figure 1A and 1B) and glandular cells (Figure 1C and 1D), and the protein was localized primarily in the plasma membrane of these tissue cells. Higher expression of Orai1 was observed in the infiltrated inflammatory cells (score, 4-9) and in the ductal epithelium of the submucosal glands (score, 4-9) of NPs compared with normal mucosa (score, 1-3) (Table 2). All \( κ \) values for scoring of staining degrees were equal to 1, which indicated a very good level of agreement between the 2 observers. Furthermore, we found that Orai1+ inflammatory cell numbers were highly elevated in NPs (Figure 1B). We counted the number of these cells using a semiquantitative method and confirmed that their numbers were significantly increased in NPs compared with normal mucosa from inferior turbinate (\( P < .0001, \) Figure 1E).

Upregulation of Orai1 Protein in NPs
The study showed an apparent immunoreactive band with a molecular mass of approximately 35 kDa reacting with Orai1 antibody in NPs and a less apparent band with the same molecular mass in normal mucosa (Figure 2A). The result also demonstrated that Orai1 and its mRNA were significantly increased in NP tissues from patients with CRSwNP in
comparison with normal samples (Figure 2B, \( P < .0001 \), normal vs NPs; Figure 2C, \( P < .0001 \), normal vs NPs).

### Orai1 Expression in the Cultured NPs after In Vitro DEX or 2-APB Administration

The present study showed that in NPs as well as in normal nasal mucosa there was an apparent immunoreactive band of approximately 35 kDa molecular mass, and the study also demonstrated that there were no obvious differences in intensity between the NP control group and the DEX treatment group. A less apparent band was detected in the cultured NPs after in vitro 2-APB administration compared with the NP or DEX treatment polyp tissues (Figure 3A). As shown in Figure 3B and 3C, Orai1 protein and mRNA were upregulated in NPs compared with normal mucosa (\( P < .0001 \) and \( P < .0001 \), respectively, normal vs NPs). DEX did not affect Orai1 production whether in protein or mRNA. However, 2-APB administration reduced their expressions (\( P < .0001 \) and \( P < .0001 \), respectively, 2-APB vs NPs). Additionally, there were statistical differences between the DEX and 2-APB treatment groups (\( P < .0001 \) and \( P < .0001 \), respectively, 2-APB vs DEX).

### ELISA Analysis of IL-1β, IL-5, ECP, LTC4, IFN-γ, DP-sIgE, MUC5B, and MUC7 in the Culture after DEX or 2-APB Treatment

The details of ELISA analysis are found in Appendices 3 and 5 and Supplemental Figure S1 (available at www.otojournal.org/supplemental). The results showed that 2-APB treatment might prohibit the development of NPs, and the in vitro effects of this drug were better than DEX in the treatment of NPs.

### Real-Time RT-PCR Analysis of IL-1β, IL-5, EAR3, LTC4S, IFN-γ, Germline Cε Transcripts, MUC5B, and MUC7 in the Cultures after DEX or 2-APB Treatment

The details of real-time RT-PCR analysis are found in Appendices 4 and 6 and Supplemental Figure S2 (available at www.otojournal.org/supplemental). These findings clearly demonstrated that 2-APB treatment effectively ameliorated the mediators and mucus in polypoid lesions and might produce better efficacy in the control of NP formation than does DEX.

### Discussion

According to the study, Orai1+ inflammatory cells were elevated in NPs (Figure 1B and 1E) in comparison with normal mucosa (Figure 1A and 1E). The result demonstrated that NPs exhibited infiltration by a high number of inflammatory cells and an enhancement of their activation because of the expression of Orai1. As a result, these cells released proinflammatory cytokines, which played important roles in the persistence of mucosal inflammation associated with NPs.22,23 In the present study, Orai1 production was elevated in the cultured NPs compared with the cultured normal mucosa and was decreased after in vitro 2-APB administration. The present study showed that DEX treatment did not affect Orai1 production whether in protein or mRNA. However, 2-APB administration reduced their expressions (\( P < .0001 \) and \( P < .0001 \), respectively, 2-APB vs NPs). Additionally, there were statistical differences between the DEX and 2-APB treatment groups (\( P < .0001 \) and \( P < .0001 \), respectively, 2-APB vs DEX).
application. Nevertheless, DEX had no specific effect (Figure 3A-C).

In NPs, IL-1β is mainly released from nasal structural cells such as epithelial cells. As shown in Supplemental Figures S1A and S2A, IL-1β was increased in the NP group compared with the normal group and was decreased after in vitro DEX treatment. DEX inhibited this cytokine production by binding to its corresponding receptor in epithelial cells. In addition, IL-1β and its mRNA were reduced in the 2-APB-treated group, and their expressions were decreased more than those in the DEX-treated group. However, the nasal epithelium was negatively immunostained for Orai1 protein; thus, 2-APB could not control IL-1β release from epithelial cells directly. It has been demonstrated that Th2 cytokines like IL-4, IL-5, and IL-13 can inhibit the nuclear factor-κB system, which regulates IL-1β expression in airway epithelial cells. In addition, IL-5 was decreased after 2-APB treatment in this study, which might result in downregulation of IL-1β.

The tissue of NPs is characterized by intense eosinophilic infiltration and a Th2-based cytokine profile including IL-5 and eotaxin. IL-5 significantly increased tissue eosinophils. IL-5 was found to be significantly raised in NPs, and it diminished after DEX administration according to the study (Supplemental Figures S1B and S2B). Glucocorticoids increased apoptosis of T cells in NP and inhibited corresponding gene expression in T cells, which might be the mechanisms of DEX treatment. However, 2-APB treatment also produced an inhibitory effect on IL-5 in NPs, and the decrease of IL-5 was more than that after DEX treatment in protein and mRNA (Figure 4B and 5B). This might be caused by the intervention of Orai1 protein by 2-APB, which in turn controlled the activation of T cells.

One of the primary cationic proteins from eosinophils, ECP, is toxic to respiratory epithelium. Eosinophil infiltration and activation may cause tissue damage through the release of ECP, and the elevated ECP level characterizes eosinophilic inflammation in polyp tissues. This investigation demonstrated a higher level of ECP in NPs than in normal mucosa (Supplemental Figures S1C and S2C). However, the ECP content was reduced by in vitro DEX administration, which indicated that DEX could prevent eosinophils from activating and could increase their apoptosis in NPs. The data also showed the amelioration of ECP in the culture after 2-APB treatment. However, no confirmed reports have shown Orai1 expression in eosinophils to date. So it was assumed that 2-APB could not influence the activation and degranulation of eosinophils directly. This drug might diminish IL-5 release from T cells, which then decreases the ECP concentration in NPs.

Many studies have revealed the abundance and distribution of mast cells in NPs and have shown that LTC4 release from these cells can produce profound tissue edema. LTC4 and LTC4S mRNA were enhanced in polyp cultures in comparison with normal mucosa cultures, and all were downregulated after DEX treatment (Supplemental Figures S1D and S2D). DEX might restrain the degranulation of mast cells through binding to its receptor in them, which then would lower the release of LTC4 by these cells. LTC4 and LTC4S mRNA were also decreased after 2-APB administration. However, there were statistical differences between DEX and 2-APB treatment. The above substances were abated more in the 2-APB-treated group. This might have occurred because the intervention of Orai1 protein in mast cells reduced the degranulation of LTC4.

It has been reported that NPs from south Chinese patients show an increase of T-bet expression and IFN-γ protein formation. In this investigation, IFN-γ was significantly upregulated in NPs versus the level seen in normal tissues and was downregulated after DEX treatment (Supplemental Figures S1E and S2E). DEX induced apoptosis of T lymphocytes in NPs and suppressed corresponding gene expression, which might result in a decrease in IFN-γ. Meanwhile, 2-APB treatment also inhibited IFN-γ expression in NPs, and the decrease of IFN-γ after 2-APB treatment was more than that after DEX treatment. This might be attributable to the intervention of Orai1 protein by 2-APB in Th1 cells, which in turn controlled the cytokine release from these cells.

A few studies have demonstrated increased production of DP-sIgE within NP tissues. In the present study, DP-sIgE and germline Cε transcript were upregulated in NPs versus normal mucosa and were downregulated after DEX treatment (Supplemental Figures S1F and S2F). The inhibitory effect by DEX might be caused by its influence on IgE-secreting cells in combination with its receptor in these cells. Similarly, 2-APB also limited their production of DP-sIgE and germline Cε transcript, and their contents were obviously less than those after DEX treatment. DP-sIgE is secreted from plasma cells after B-cell differentiation in NPs. It is possible that 2-APB fulfills this function by controlling Orai1 protein expression in the plasma membrane of these cells. The downregulation of the specific immunoglobulin also led to the decrease of ECP production in this research due to the effect of DP-sIgE on eosinophils.

MUC5B and MUC7 originate mainly from submucosal glands, and the expression of both of them is higher in NPs. These MUCs were all increased in polypoid tissues compared with normal tissues, and all decreased after DEX administration in this study (Supplemental Figures S1G, S1H, S2G, and S2H). We speculate that DEX reduced the enhanced expression of these glycoproteins and their mRNAs by combining its corresponding receptor in glandular cells. As shown in the figures, 2-APB in vitro treatment downregulated these proteins, and the decreases were more than those after DEX treatment. Direct interference with Orai1 protein in the glandular epithelium by 2-APB contributed to the decreased secretion of mucins.

In conclusion, Orai1 may play a crucial role in NP formation, and intervention with this protein may inhibit the development of this disease. It should be emphasized that administration with 2-APB may offer novel and effective therapeutic means to treat NPs by interfering with the local expression of Orai1 and release of IL-1β, IL-5, ECP, LTC4, IFN-γ, and DP-sIgE and MUCs like MUC5B and MUC7.
which are significant events in the pathogenesis of CRSwNP. However, 2-APB has been widely used only in basic science and has not been applied to treat human diseases. This study may provide the laboratory basis and theoretical evidence for exploring medical interventions to directly control the expression of Orai1 protein in NPs for the treatment of CRSwNP.

Author Contributions

Lin Lin, conception, study design, data collection, data analysis, drafting and revising manuscript, final approval; Fei Dai, conception, study design, data collection, data analysis, drafting and revising manuscript, final approval; Zhongchun Chen, data collection, data analysis, drafting and revising manuscript, final approval; Lihui Cai, data collection, data analysis, drafting and revising manuscript, final approval.

Disclosures

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Supplemental Material

Additional supporting information may be found at http://otojournal.org/supplemental.

References