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Therapeutic effects of SKF-96365 on murine allergic rhinitis induced by OVA

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Abstract

Introduction: SKF-96365 is regarded as an inhibitor of receptor-mediated calcium ion (Ca^{2+}) entry. The current study aimed to explore the effects of SKF-96365 on murine allergic rhinitis (AR).

Methods: Intranasal SKF-96365 administration was performed on OVA induced murine AR. Serum and nasal lavage fluid (NLF) from mice were harvested to assay IgE and inflammatory cytokines using ELISA method. Inflammatory cells were counted and analyzed in NLF. Nasal mucosa tissues were collected from mice and used for HE staining, immunohistochemistry (IHC) staining, and real-time PCR detection.

Results: SKF-96365 had therapeutic effects on murine AR manifesting attenuation of sneezing, nasal rubbing, IgE, inflammatory cytokines, inflammatory cells, TRPC6 immunolabeling, and TRPC6, STIM1 and Orai1 mRNA levels in AR mice.

Conclusion: SKF-96365 could effectively alleviate the symptoms of murine AR. SKF-96365 could suppress TRPC6, STIM1, and Orai1 activities, leading to the downregulation of inflammatory cytokines and inflammatory cells in murine AR.

Keywords

allergic rhinitis, SKF-96365, calcium, TRPC6, STIM1, Orai1, ovalbumin

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Introduction

Allergic rhinitis (AR) is a common allergen-induced and IgE-mediated disorder of the nasal mucosa characterized by rhinorrhea, sneezing, itching, and nasal congestion.[1,2] AR has adverse effects on the patients' quality of life and work,[3] and the pathogenesis of AR is still uncertain.[4]

Glucocorticosteroids and antihistamines are effective in the therapy of AR, however, they only have temporary reliefs and some AR patients are insensitive or tolerant to them.[5] Therefore, a novel drug with better effects is in great demand.

Considerable data indicate that calcium ion (Ca^{2+}) is an essential second messenger inducing a signaling cascade in many immune responses, and Ca^{2+} influx is mediated by Ca^{2+} channels distributed in various cells.[6] Transient receptor potential canonical channel 6 (TRPC6) and store-operated calcium entry (SOCE) mediated by stromal interaction molecule1 (STIM1) and Orai1 play pivotal roles in mediating Ca^{2+} influx in a variety of cells.[7–9] Our previous work showed that TRPC6 can synergize

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Table 1. Detailed information of murine AR model establishment and treatment.

Group	*N*	IP sensitization stage (days 1, 8, and 15)	IN challenge stage (days 22–29)	Drugs dose
Control	10	Normal saline	Normal saline	
OVA + Dex	10	OVA/Alum sensitization	OVA + Dex	100 μg
OVA + 2-APB	10	OVA/Alum sensitization	OVA + 2-APB	200 μg
OVA + SKF-400 μg	10	OVA/Alum sensitization	OVA + SKF-96365	400 μg
OVA + SKF-200 μg	10	OVA/Alum sensitization	OVA + SKF-96365	200 μg
OVA	10	OVA/Alum sensitization	OVA	
OVA + OAG	10	OVA/Alum sensitization	OVA + OAG	100 μg

AR: allergic rhinitis; N: number; IP: intraperitoneal; IN: intranasal; OVA: ovalbumin; Dex: dexamethasone; Alum: aluminum hydroxide; 2-APB: 2-aminoethoxydiphenyl borate; SKF or SKF-96365: 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl) propoxy]ethyl-1H-imidazole; OAG: 1-oleoyl-2-acetyl-glycerol.

with STIM1 and Orai1 to modulate Ca^{2+} influx of nasal epithelial cells (NECs) and then trigger inflammatory response in chronic rhinosinusitis with nasal polyp.[10]

1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl) propoxy]ethyl-1H-imidazole (SKF-96365) is a small molecule compound acting as a non-selective inhibitor of TRP channels, SOCE and voltage-gated Ca^{2+} channels, and SKF-96365 has been proved to suppress cellular Ca^{2+} influx via inhibiting these Ca^{2+} channels.[11–13] There is ample evidence that SKF-96365 is widely used to study the function of TRPC channels.[12–17] Previous reports indicate that SKF 96365 could suppress cardiomyocyte hypertrophy, and inhibit spontaneous nociception and pain hypersensitivity induced by melittin via inactivating TRPC channels and Ca^{2+} influx.[12,18] In cultured NECs, TRPC6, STIM1, Orai1, Ca^{2+} MFI levels, and inflammatory mediators were upregulated by lipopolysaccharide (LPS) and 1-oleoyl-2-acetyl-glycerol (OAG, diacylglycerol (DAG) analog, TRPC6 activator), but were inhibited by SKF-96365.[10] In addition, 2-aminoethoxydiphenyl borate (2-APB) is a chemical which could inhibit SOCE and TRP channels,[19,20] and one previous report has indicated that 2-APB administration intranasally could reduce numbers of sneezing, nasal rubbing, and eosinophils in murine AR.[21] However, little is known about the effects of SKF-96365 on murine AR. Therefore, we conducted this study to explore the therapeutic effects of SKF-96365 on murine AR.

Materials and methods

Animals

Female BALB/c mice were purchased and maintained under a pathogen-free condition. To exclude off-target effects and prove the specificity of the findings, we included a second inhibitor 2-APB, TRPC6 activator OAG, and dexamethasone (Dex) as controls. We classified the mice into seven groups ($n = 10$ each group): normal control group, 100 μg Dex treated AR group, 200 μg 2-APB treated AR group, 400 μg SKF-96365 treated AR group, 200 μg SKF-96365 treated AR group, untreated AR group, and 100 μg OAG treated AR group. Detailed information of murine AR model establishment and treatment was listed in Table 1. This study was approved by the Animal Ethics Committee of Shanghai Sixth People's Hospital.

Murine AR model was established according to a previously used protocol.[22] Briefly, mice were sensitized intraperitoneally with 100 μg ovalbumin (OVA) and aluminum hydroxide (4 mg) in 0.2 ml sterile saline on days 1, 8, and 15 (Figure 1). Then, on days 22–29, the mice were challenged intranasally with 40 mg/ml OVA diluted in 20 μl sterile saline. In addition, on days 22–29, SKF-96365 (200 or 400 μg, Abcam, Figure 1), 2-APB (200 μg, Abcam), OAG (100 μg, Abcam), or Dex (100 μg, Sigma-Aldrich) was administrated intranasally on AR mice 1 h before OVA challenge. The subjective measurements including sneezing, inflammatory cells, eosinophils, and TRPC6 immunolabeling were evaluated by the investigators blinded to the study. More detailed protocols were provided in the Supplemental Material.

Evaluation of nasal symptoms and sample collection

Nasal symptoms were evaluated by counting the frequencies of sneezing and nasal rubbing during the 15-min period 10 min after final OVA challenge on day 29. Then, blood, NLF, and nasal mucosas were collected from mice to perform experiment

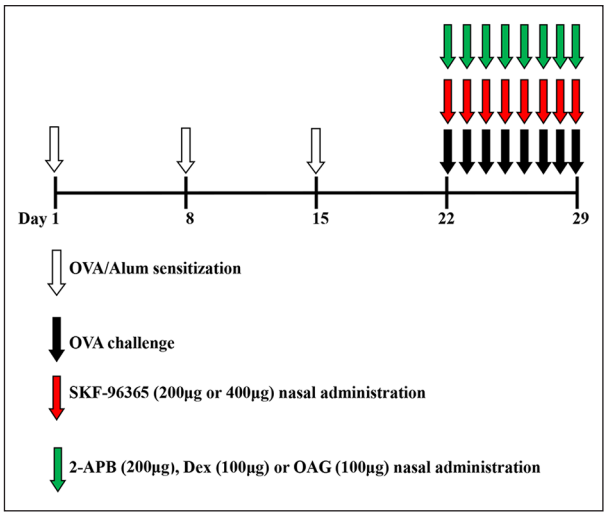

Figure 1. Schematic diagram demonstrating the process of AR mouse model construction and SKF-96365 treatment.

research. More detailed protocols were provided in the Supplemental Material.

ELISA and inflammatory cells counting

OVA-specific IgE, histamines, and LTC4 concentrations (BlueGene Biotech, Shanghai, China) in serum samples and OVA-specific IgE, IL-4, IL-5, IL-6, IL-13, and IL-33 concentrations (BlueGene Biotech) in NLF samples were detected by ELISA. Additionally, total cells, eosinophils, macrophages, neutrophils, and lymphocytes in the NLF were counted after Wright-Giemsa staining. More detailed protocols were provided in the Supplemental Material.

HE and IHC staining

Eosinophils and TRPC6 immunoreactivity in nasal mucosas of mice were evaluated by HE and IHC staining. More detailed protocols were provided in the Supplemental Material.

Real-time reverse transcriptions PCR

Real-time PCR was used to detect TRPC6, STIM1, and Orai1 mRNA levels in nasal mucosas of mice. More detailed protocols were provided in the Supplemental Material.

Statistical analysis

Values were noted as mean ± standard error of mean (SEM). One-way ANOVA with Bonferroni post hoc test was employed for intergroup comparison. Two-sided P value <0.05 was deemed statistically significant.

Results

Effects of SKF-96365 on frequencies of sneezing and nasal rubbing in AR mice

Of note, untreated AR mice exhibited significantly elevated frequencies of sneezing and nasal rubbing compared with normal control mice, and SKF-96365 treated AR mice (200 or 400 μg), 2-APB and Dex treated AR mice manifested significantly decreased frequencies of sneezing, and nasal rubbing compared to untreated AR mice (Figure 2a and b). Nonetheless, OAG treated AR mice manifested obviously elevated frequencies of sneezing and nasal rubbing compared to untreated AR mice (Figure 2a and b). In addition, SKF-96365 (400 μg) was more effective in comparison to SKF-96365 (200 μg) (Figure 2a and b).

ELISA assay in the serum and NLF and inflammatory cells in the NLF

Notably, untreated AR mice manifested significant elevation of OVA-specific IgE, histamines, and LTC4 levels in serum, and OVA-specific IgE, IL-4, IL-5, IL-6, IL-13, and IL-33 levels in NLF compared to normal control mice, and these measurements were obviously decreased in SKF-96365 treated AR mice (200 or 400 μg), 2-APB and Dex treated AR mice in comparison to untreated AR mice (Figure 2c–k). Nonetheless, these measurements were obviously increased in OAG treated AR mice in comparison to untreated AR mice (Figure 2c–k). Furthermore, these effects were more obvious in SKF-96365 (400 μg) treated AR mice in comparison to SKF-96365 treated (200 μg) AR mice (Figure 2c–k).

Effects of SKF-96365 on inflammatory cells in the NLF

Furthermore, untreated AR mice manifested significant elevation of total cells, eosinophils, macrophages, neutrophils, and lymphocytes numbers in NLF compared to normal control mice (Figure 3a–e). Additionally, these cells were diminished in SKF-96365 treated AR mice (200 or 400 μg), 2-APB and Dex treated AR mice compared to

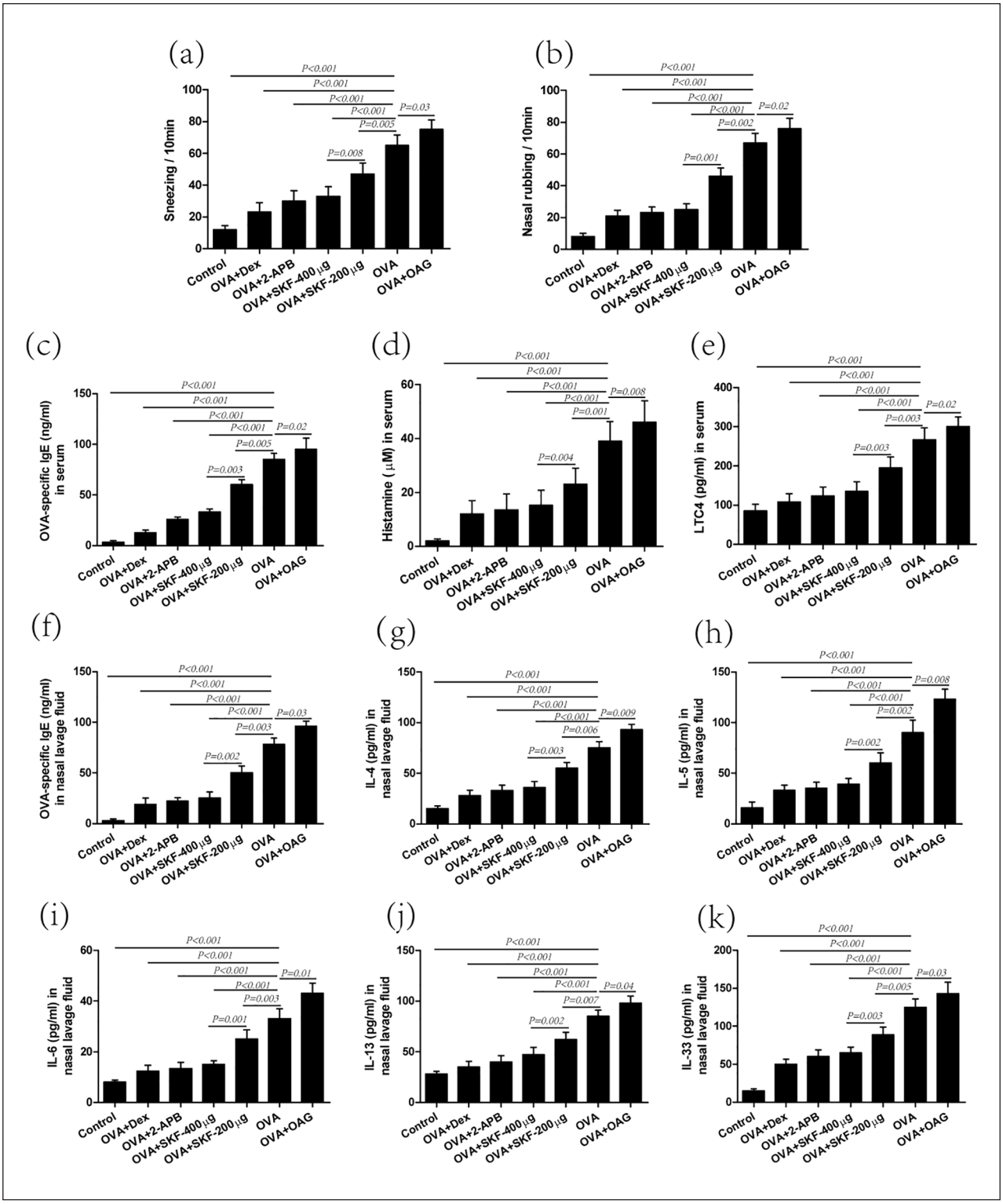

Figure 2. (a)–(k) Sneezing, nasal rubbing and OVA-specific IgE, histamines and LTC4 levels in the serum, and OVA-specific IgE, IL-4, IL-5, IL-6, IL-13, and IL-33 levels in the NLF from normal control group, 100 μg Dex treated AR group (OVA + Dex), 200 μg 2-APB treated AR group (OVA + 2-APB), 400 μg SKF-96365 treated AR group (OVA + 400 μg SKF), 200 μg SKF-96365 treated AR group (OVA + 200 μg SKF), untreated AR group (OVA), and 100 μg OAG treated AR group (OVA + OAG).

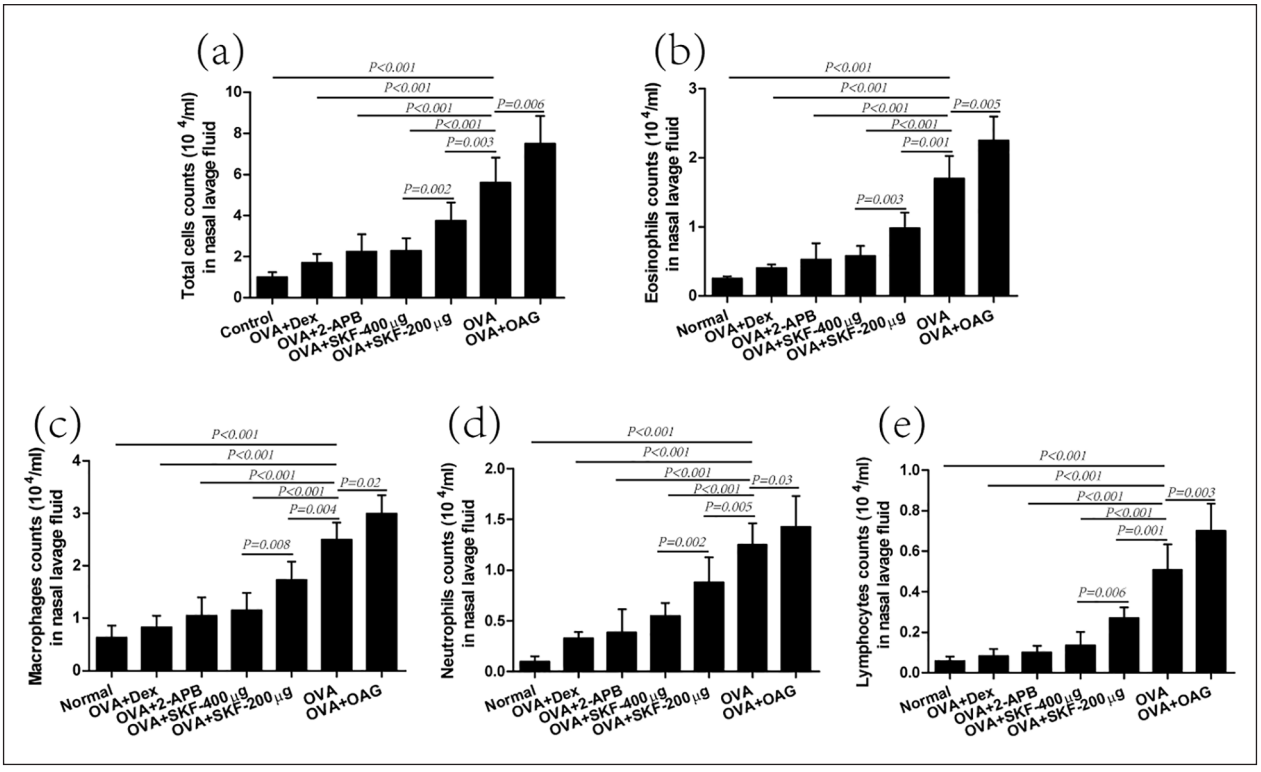

Figure 3. (a)–(e) Total cells, eosinophils, macrophages, neutrophils, and lymphocytes in the NLF from normal control group, 100 μg Dex treated AR group (OVA + Dex), 200 μg 2-APB treated AR group (OVA + 2-APB), 400 μg SKF-96365 treated AR group (OVA + 400 μg SKF), 200 μg SKF-96365 treated AR group (OVA + 200 μg SKF), untreated AR group (OVA), and 100 μg OAG treated AR group (OVA + OAG).

untreated AR mice (Figure 3a–e). Nonetheless, these cells numbers were obviously increased in OAG treated AR mice in comparison to untreated AR mice (Figure 3a–e). Furthermore, these effects were more obvious in SKF-96365 (400 μg) treated AR mice in comparison to SKF-96365 treated (200 μg) AR mice (Figure 3a–e).

Effects of SKF-96365 on eosinophils in nasal mucosas

Of interest, untreated AR mice manifested significant elevation of eosinophils numbers in nasal mucosas compared to normal control mice, and SKF-96365 treated AR mice (200 or 400 μg), 2-APB and Dex treated AR mice exhibited marked decline of eosinophils numbers compared to untreated AR mice (Figure 4a–h). Nonetheless, eosinophils numbers were obviously increased in OAG treated AR mice in comparison to untreated AR mice (Figure 4a–h). Furthermore, these effects were more obvious in SKF-96365 (400 μg) treated AR mice in comparison to SKF-96365 treated (200 μg) AR mice (Figure 4a–h).

Effects of SKF-96365 on TRPC6 immunolabeling in nasal mucosas

TRPC6 positive cells were mainly epithelial cells and sub-mucosal inflammatory cells in the nasal mucosa (Figure 5a–g). Of note, untreated AR mice manifested significant elevation of TRPC6 immunolabeling compared to normal control mice, and TRPC6 immunolabeling was significantly downregulated in SKF-96365 treated AR mice (200 or 400 μg), 2-APB and Dex treated AR mice compared to untreated AR mice (Figure 5a–i). Nonetheless, TRPC6 immunolabeling was obviously increased in OAG treated AR mice in comparison to untreated AR mice (Figure 5a–i). In addition, these effects were more obvious in SKF-96365 (400 μg) treated AR mice in comparison to SKF-96365 treated (200 μg) AR mice (Figure 5a–i).

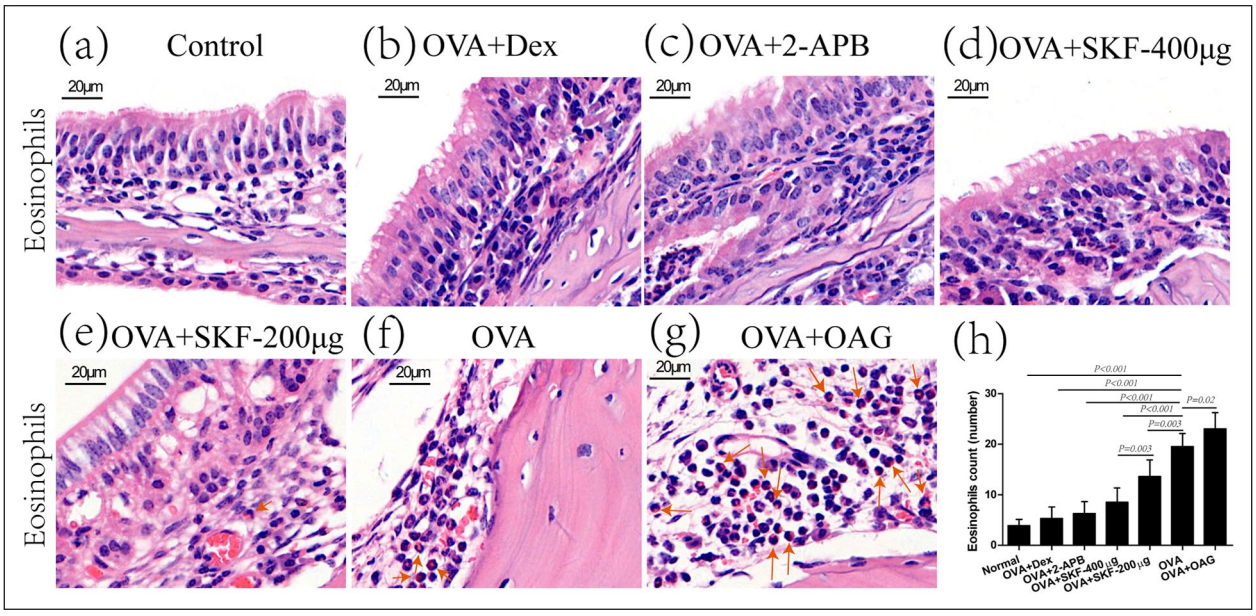

Figure 4. (a)–(h) HE staining for eosinophils in nasal tissues from normal control group, 100 μg Dex treated AR group (OVA + Dex), 200 μg 2-APB treated AR group (OVA + 2-APB), 400 μg SKF-96365 treated AR group (OVA + 400 μg SKF), 200 μg SKF-96365 treated AR group (OVA + 200 μg SKF), untreated AR group (OVA), and 100 μg OAG treated AR group (OVA + OAG). Red arrows indicate eosinophils having two-lobed nuclei connected by a band of nuclear material. Scale bar = 20 μm.

Effects of SKF-96365 on TRPC6, STIM1 and Orai1 mRNA levels in nasal mucosas

Of note, untreated AR mice manifested significant elevation of TRPC6, STIM1, and Orai1 mRNA levels compared to normal control mice, and these measurements were obviously decreased in SKF-96365 treated AR mice (200 or 400 μg), 2-APB and Dex treated AR mice compared to untreated AR mice (Figure 5j–l). Nonetheless, these measurements were obviously elevated in OAG treated AR mice in comparison to untreated AR mice (Figure 5j–l). Furthermore, these effects were more obvious in SKF-96365 (400 μg) treated AR mice in comparison to SKF-96365 treated (200 μg) AR mice (Figure 5j–l).

Discussion

It is generally acknowledged that Ca^{2+} signaling and related Ca^{2+} channels are essential to elicit allergic and inflammatory responses.[23–25] Our previous studies demonstrated that KCa3.1, STIM1, and Orai1 play pivotal roles in mediating Ca^{2+} signaling in the pathogenesis of AR.[26] TRPC6, one type of Ca^{2+} channels, has been proved to play key roles in mediating intracellular Ca^{2+} homeostasis in the processes of cell growth, cell migration, and cell activation.[8] It has been revealed that TRPC6 and Ca^{2+} influx in bronchial epithelial cells could be increased by LPS and then inflammatory response was triggered.[27] In addition, TRPC6 is involved in the etiology and pathophysiology of asthma, COPD, lung fibrosis, and lung edema.[28,29] Our previous work showed that TRPC6 modulates cellular Ca^{2+} influx via collaborating with STIM1 and Orai1 in nasal epithelial cells, and these effects could be enhanced by OAG and suppressed by SKF-96365.[10]

Considerable data indicate that SKF-96365 is a calcium antagonist which plays key roles in the inhibition of cellular Ca^{2+} influx via inactivating TRPC channels and SOCE in a variety of cells, and has been widely used to explore the pathophysiological function of TRPC channels and SOCE in non-excitable cells and excitable cells.[30,31] Accumulating evidence indicates that SKF-96365 could suppress hypoxic pulmonary vasoconstriction,[17] inhibit in vitro cultured mouse airway smooth muscle cells proliferation induced by ESI-09,[32] and regulate apoptosis and autophagy in colorectal cancer cells.[31] However, there is a lack of data regarding the effects of SKF-96365 on murine AR. Thus, we performed this study to explore the therapeutic effects of SKF-96365 on murine AR. To exclude off-target effects and prove the specificity of the findings, we included a second inhibitor 2-APB, TRPCs activator OAG, and Dex as controls. As described previously[33,34], 2-APB could exert its

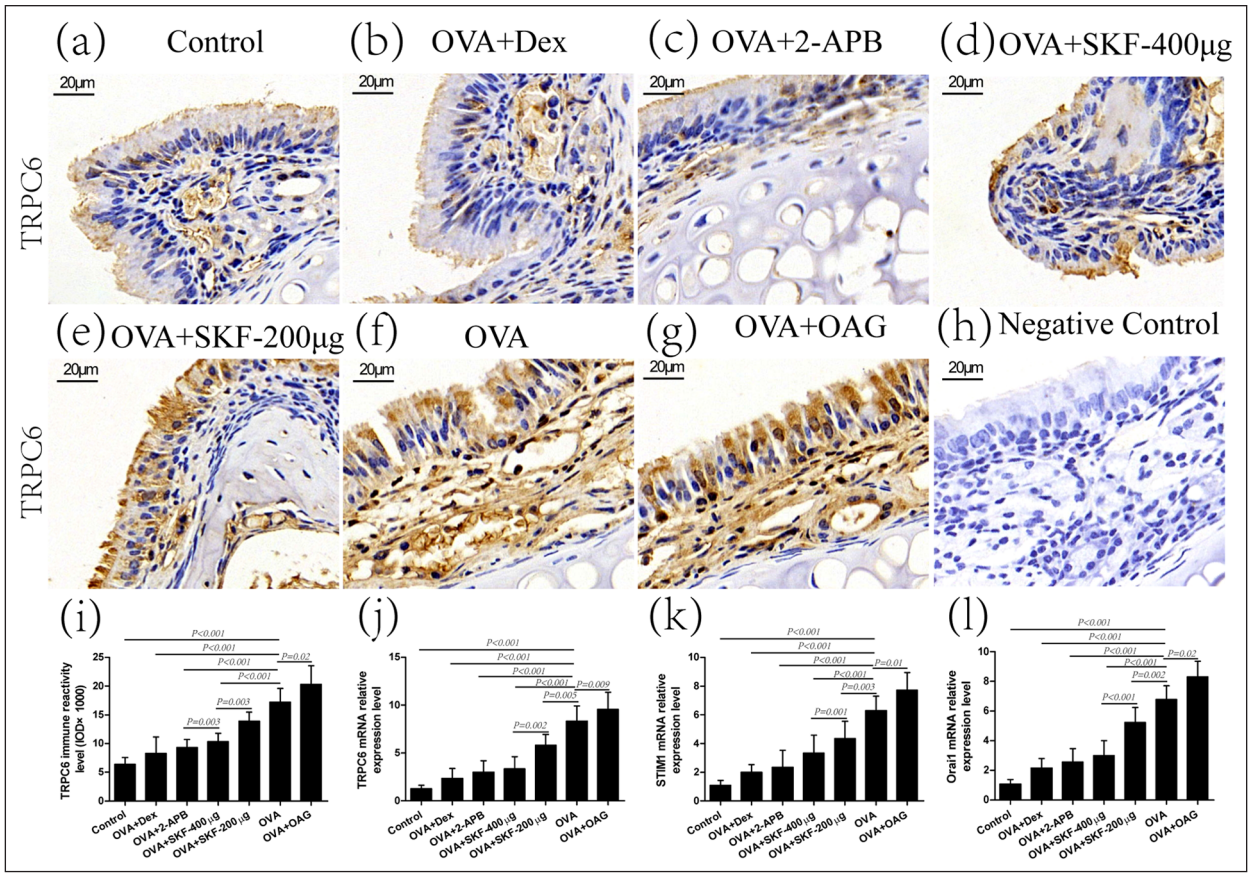

Figure 5. (a)–(l) TRPC6 immunolabeling, and mRNA levels of TRPC6, STIM1 Orai1 in nasal tissues from normal control group, 100 μg Dex treated AR group (OVA + Dex), 200 μg 2-APB treated AR group (OVA + 2-APB), 400 μg SKF-96365 treated AR group (OVA + 400 μg SKF), 200 μg SKF-96365 treated AR group (OVA + 200 μg SKF), untreated AR group (OVA), and 100 μg OAG treated AR group (OVA + OAG).
Scale bar = 20 μm.

role at the concentration of 5–50 μM. As the molecular weight of 2-APB is 225.09, doses range = (5–50 μM) × 0.0301 = (5 × 225.09–50 × 225.09 μg/l) × 0.0301 = 33.7635 μg–337.635 μg, the 200 μg dose of 2-APB used in our study was among the range (approximately 29.6178 μM which is close to the dose of 2-APB (30 μM) used to treat murine AR in the previous report[21]), and we also found 2-APB was effective in the treatment of murine AR and the findings are partially in line with this previous report.[21]

The in vivo findings indicated that untreated AR mice manifested significant elevation of sneezing, nasal rubbing, OVA-specific IgE, histamines and LTC4 levels in serum, OVA-specific IgE, IL-4, IL-5, IL-6, IL-13, IL-33 and inflammatory cells levels in NLF, eosinophils, TRPC6 immunolabeling, and TRPC6, STIM1 and Orai1 mRNA levels compared to normal control mice, and these effects were markedly decreased in SKF-96365 treated AR mice (200 or 400 μg), 2-APB and Dex treated AR mice compared to untreated AR mice. Nonetheless, these effects were markedly enhanced in OAG treated AR mice compared to untreated AR mice. Moreover, these effects were more obvious in SKF-96365 (400 μg) treated AR mice in comparison to SKF-96365 treated (200 μg) AR mice. The results demonstrate that SKF-96365 might suppress TRPC6, STIM1, and Orai1expression in nasal mucosas in AR mice, leading to the inhibition of allergic and inflammatory reactions manifesting decreased inflammatory cells numbers and inflammatory cytokines levels. These findings are partially in line with previous studies suggesting that administration of SKF-96365 could diminish TRPC6 protein expression in the hyperalgesia in diabetic rats,[35] and modulate inflammatory reactions elicited by LPS and IFN-γ treatment in murine peritoneal macrophages.[36] Of interest, our results are also in agreement with previous reports demonstrating that SKF-96365 could suppress human non-small cell lung cancer cell

proliferation by blocking TRPC6,[37] and reduce airway smooth muscle cells (ASMCs) proliferation via inhibiting STIM1/Orai1-mediated SOCE to decrease Ca^{2+} influx in ASMCs.[38]

Some limitations of our study should be noted. First, due to the cell diversity of nasal tissues, specific cells including eosinophils, mast cells, lymphocytes, dendritic cells or macrophages need to be isolated to further precisely assess the effects, and mechanism of SKF-96365 on the allergic response of AR. Second, due to the lack of specific inhibitors of TRPC6 and SOCE, we used the non-specific inhibitor SKF-96365 to treat murine AR, other possible off-target effects need to be further explored deeply in the future.

Conclusion

In summary, SKF-96365 could effectively alleviate the symptoms of murine AR. SKF-96365 could suppress TRPC6, STIM1, and Orai1 activities, leading to the downregulation of inflammatory cytokines and inflammatory cells in murine AR.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

Ethical approval for this study was obtained the Animal Ethics Committee of Shanghai Sixth People's Hospital (No. 2020-0201).

Animal welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation of the country.

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Supplementary material

Supplemental material for this article is available online.

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