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Original Article

Cinnamaldehyde reduces inflammatory responses in chronic rhinosinusitis by inhibiting TRPM8 expression

Yi Cai, Mingjian Xie, Zhoumu Fang, Yanfei Bai, Jiang Bian

Department of Otolaryngology-Head and Neck Surgery, Jinhua People's Hospital, Jinhua 321000, Zhejiang, China

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Abstract: Objective: This study aimed to investigate the effects of cinnamaldehyde (CA) intervention on transient receptor potential melastatin 8 (TRPM8) expression in human nasal epithelial cells (HNECs) and mouse models of chronic rhinosinusitis (CRS) and determine the alleviating effects of CA on CRS. Methods: HNECs were treated with CA, and the protein levels and mRNA expression of pro-inflammatory cytokines, namely, interleukin-25 (IL-25), IL-33, and thymic stromal lymphopoietin (TSLP), were measured by enzyme-linked immunosorbent assay and real-time reverse-transcription polymerase chain reaction (RT-PCR). TRPM8 expression levels were examined by RT-PCR and western blot. The C57BL/6 mice were randomly divided into three groups (control group, model group, CA group). The model and CA groups were induced by intranasal drip intervention of ovalbumin (OVA) three times a week for 9 weeks. Each mouse was individually observed in a single cage to record the frequency of nose scratching and sneezing within 10 minutes. Histologic examination of nasal mucosa in mice was done using hematoxylin-eosin staining to compare the degree of inflammation. Pro-inflammatory cytokine levels and TRPM8 expression levels were measured in mouse nasal lavage fluid. Results: In vitro experiments demonstrated that CA intervention in HNECs significantly reduced the protein and mRNA of IL-25, IL-33, TSLP, and TRPM8. In vivo analysis showed that the CA group exhibited fewer nose scratching and sneezing symptoms and reduced nasal mucosal inflammation as well as lower levels of IL-25, IL-33, and TSLP in nasal lavage fluid and tissues than the model group.

Keywords: Cinnamaldehyde, chronic rhinosinusitis, transient receptor potential melastatin 8

Introduction

Chronic rhinosinusitis (CRS) refers to an inflammatory condition of the nasal cavity and paranasal sinuses mucosa, and can be classified into CRS with or without nasal polyps. The clinical manifestations of CRS include purulent nasal discharge, nasal congestion or discomfort, and diminished sense of smell [1]. This condition occurs in approximately 5%-12% of the population, substantially affects the physical health and quality of life of patients, and imposes societal burdens [2]. Current treatment modalities for CRS often fail to achieve an optimal outcome because of its heterogeneity and complex pathogenesis.

With the development of modern traditional Chinese medicine (TCM), the therapeutic involvement of effective components in Chinese herbal medicine has been integrated into the treatment of CRS. Chinese patent medicine (CPM) treatments for CRS are not only significantly effective but also convenient to administer. Currently, various types of CPMs are utilized clinically for the treatment of CRS [3]. Cinnamaldehyde (CA) is an allyl aldehyde organic compound extracted from cinnamon trees and a principal component of their essential oils [4]. Pharmacological studies have demonstrated that CA exhibits multiple pharmacologic activities including anti-inflammatory, antipyretic, analgesic, anti-tumor, antibacterial, hypoglycemic, and anti-obesity effects [5-9]. The anti-inflammatory activity of CA is of particular importance.

Transient receptor potential (TRP) channels are non-voltage-gated cation channels widely expressed in various tissues of mammals and participate in numerous essential physiologic functions. TRPM8, a member of the TRP superfamily, regulates a spectrum of physiologic and pathologic processes in organisms, including

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cold sensation, pain perception, inflammatory responses, immune modulation, and cellular growth regulation [10-12]. Limited studies have been reported on TRPM8 and airway diseases. TRPM8 expression has been identified in the airway macrophages of patients with bronchial asthma and the epithelial cells of nasal mucosa, where it participates in excessive mucin protein secretion in chronic airway diseases [13, 14]. Furthermore, no studies have yet investigated the mechanisms of CA and TRPM8 for the treatment of CRS. The present study aims to determine the influence of CA intervention on TRPM8 in HNECs and CRS from a mouse model, so as to provide new insight into the prevention and treatment of CRS.

Materials and methods

Cells

HNECs were purchased from Wuhan Pricella Biotechnology Co., Ltd. (Cat# CP-H252). The cells were cultured in DMEM/F12 medium (Cat# 11320033, Gibco, Carlsbad, CA, USA) containing 10% fetal calf serum (FBS, Cat# 10099158, Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Cat# 15140-122, Gibco, Carlsbad, CA, USA). They were maintained in a cell culture incubator at 37°C with 5% CO_2. Cells in the logarithmic growth phase and under good condition were used for subsequent experiments.

Intervention on HNECs

During cell passage, HNECs were digested with trypsin until they appeared rounded. Digestion was terminated by adding FBS, after which the cells were scraped, resuspended, and washed twice before being re-seeded into six-well plates.

After seeding the cells for 12 h and ensuring complete adhesion, the nasal epithelial cells were treated with CA (99.10%; Cat# HY-W019711, MedChemExpress, New Jersey, USA) dissolved in DMSO (HY-Y0320, MedChemExpress, USA) at final concentrations of 0.2 and 2 mM. The cells were exposed to the treatment for 3, 6, 12, and 24 h.

Following cell seeding for 12 h and complete adhesion, the cells were pre-treated with 10 μmol/L NF-κB pathway activator Prostratin (Cat# P0077, Sigma-Aldrich, St. Louis, MO, USA) for 30 min before the addition of CA for intervention.

Animals

Eighteen Specific Pathogen Free-grade male C57BL/6 mice (body weight 18-22 g) were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). The mice were given adaptive feeding for one week at an ambient temperature of (25±2)°C. During the experiment, the mice had free access to food and drinking water. The experimental design was approved by the Animal Experiment Committee of Jinhua Institute for Food and drug testing (AL-JSYJ202304). The ethical guidelines described in the committee's guidelines for the care and use of laboratory animals were followed throughout the experiments. The mice were randomly divided into three groups: control group, model group, and CA group, with six mice in each group. For the first three weeks, on days 0, 7, and 14, the model and CA groups received intraperitoneal injections of ovalbumin (OVA, Cat# A003049, Sangong Biotech, Shanghai, China) nasal drops (1 mg/mL), with an injection volume of 100 μL per mouse (50 μg OVA per mouse). The control group received intraperitoneal injections of sterile normal saline with an injection volume of 100 μL per mouse. Starting from the fourth week, nasal drops were administered once daily to induce and maintain inflammation. Each mouse in the model and CA groups received 10 μL of OVA solution (100 mg/mL) in each nostril, followed by a brief inversion of the mouse by its tail. The control group received the same procedure with 10 μL of sterile normal saline. From the fifth week to the twelfth week, nasal drops were administered three times per week (every Monday, Wednesday, and Friday). During these administrations, each mouse in the model group received 10 μL of OVA solution in each nostril, followed by inversion. The control group received 10 μL of sterile normal saline in the same manner. In the CA group, in addition to the OVA administration as in the model group, CA was administered every Tuesday, Thursday, and Saturday, with each mouse receiving 10 μL of CA solution (2 mmol/L) in each nostril. The control and model groups received 10 μL of sterile normal saline on these days. After 12 weeks of the experiment intervention, the mice

Table 1. Primer sequences

Gene	Forward primer sequences (5'-3')	Reverse primer sequences (5'-3')
IL-25	CTCAACAGCAGGGCCATCTC	GTCTGTAGGCCACGCAGTGTG
IL-33	GCACGGTGTCATGGTAAGAT	AGCTCCACAGAGTGTTCCTTG
TSLP	ATGTTCGCCACAAAACTAAGGC	GCGACGCCACAATCCTTGTA
TRPM8	CTCCACGACGTGTCTCCTATTA	CCAGCAGCATCATGTCGTTCT
β-actin	CGTGGGCCGCCCTAGGCACCA	TTGGCCTTAGGGTTCAGGGGG

IL-25: Interleukin-25, IL-33: Interleukin-33, TSLP: thymic stromal lymphopoietin, TRPM8: transient receptor potential melastatin 8.

were euthanized by cervical dislocation under deep anesthesia with 2% sodium pentobarbital. The mouse heads were collected, and nasal lavage was performed by inserting a 1 mL syringe filled with 1 mL PBS into the nasopharynx via the trachea to collect lavage fluid. The heads were then skinned, and nasal and sinus mucosa were harvested. The samples were stored at -20°C.

Evaluation of nasal symptoms in mice

After the nasal drop administration, experimental observations were recorded. After allowing the mice to rest for at least 10 minutes, each mouse was placed in an individual cage for observation. The number of nose scratches and sneezes was recorded over a 10-minute period. The results are presented as the average number of nose scratches/sneezes per minute.

Histopathologic morphology of mouse nasal mucosa

The fixed mice nasal mucosal tissue was routinely dehydrated, embedded in paraffin, and sectioned into 4 μm consecutive slices. These sections were dried in an oven at 60°C for 1 h. Hematoxylin and eosin (H&E) staining was performed according to the instructions of the staining kit (Cat# M027, Shanghai Gefan Biotechnology Co., Ltd., Shanghai, China). Observations were made under a light microscope (ECLIPSE Ts2, Nikon, Tokyo, Japan) at 200× magnification.

RT-PCR

Total RNA from cells/mouse nasal mucosal tissues in each group was extracted using Trizol (Cat# R0016, Beyotime, Shanghai, China). Complementary DNA (cDNA) was synthesized according to the instructions of the reverse transcription kit (Cat# RR037Q, Takara, Otsu, Shiga, Japan). Reverse-transcription polymerase chain reaction (RT-PCR) was then performed (Cat# RR014A, Takara, Otsu, Shiga, Japan) by using a real-time PCR instrument (CFX96 Touch, Bio-Rad, Hercules, CA, USA). mRNA expression levels were analyzed by the $2^{-\Delta\Delta CT}$ method. The primers used in the experiment are listed in **Table 1**.

ELISA

The supernatant from the HNEC cultures and nasal lavage fluid from mice was collected. The levels of IL-25 (Cat# ab242241), IL-33 (Cat# ab213475), and TSLP (Cat# ab316259) were measured according to the instructions of the ELISA kit (Abcam, Cambridge, MA, USA). Absorbance was determined using a microplate reader (EnSight, PerkinElmer, Waltham, MA, USA).

Western blot analysis

Total protein was extracted from HNECs/mouse nasal mucosal tissues by using RIPA lysis buffer (89901, Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration was determined using the BCA Total Protein Quantitative Kit (P0012, Beyotime, Shanghai, China). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred onto a PVDF membrane. The membrane was blocked with 5% non-fat milk for 1 h and incubated overnight at 4°C with primary antibodies: β-actin (1:1000, Cat# ab8226, Abcam, Cambridge, MA, USA), TRPM8 (1:1000, Cat# ab3243, Abcam, Cambridge, MA, USA), NF-κB p65 (1:1000, Cat# ab288751, Abcam, Cambridge, MA, USA), and TRPM8 (1:1000, Cat# ab3243, Abcam, Cambridge, MA, USA). The membrane was then washed and incubated for 1 h at 37°C with horseradish

peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2000, Cat# ab288151, Abcam, Cambridge, MA, USA). After washing, the membrane was immersed in enhanced chemiluminescent solution (P0018S, Beyotime, Shanghai, China) for measuring. The target bands were analyzed using a gel imaging system (Molecular Devices, LLC., San Jose, CA, USA), and the target proteins were quantified using β-actin as the internal reference (Image J, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Quantitative data were presented as mean ± standard deviation. To compare multiple groups, one-way ANOVA was employed, and Tukey's test was used for post-hoc analysis. $P<0.05$ was deemed significant.

Results

Effect of CA intervention in HNECs on IL-25, IL-33, and TSLP

We treated HNECs with different concentrations of CA for various durations. After 24 h of treatment with 2 mM CA, the mRNA levels of pro-inflammatory cytokines (*IL-25*, *IL-33*, and *TSLP*) were significantly reduced (**Figure 1A**). We then performed ELISA measurements of IL-25, IL-33, and TSLP in the cell supernatants. The levels of IL-25, IL-33, and TSLP were significantly decreased after 24 h of CA treatment (**Figure 1B**). These findings suggest that CA intervention in HNECs can reduce the expression and secretion of IL-25, IL-33, and TSLP in a time- and dose-dependent manner.

Effect of CA intervention on TRPM8 in HNECs

The optimal condition for CA intervention in HNECs was 2 mM for 24 h. Therefore, we investigated the effects of CA on TRPM8 expression levels in HNECs under this condition. TRPM8 expression levels were significantly reduced following CA treatment (**Figure 2**), suggesting that CA intervention in HNECs led to lower TRPM8 expression, and this may serve as the molecular mechanism for its anti-inflammatory effects.

We investigated the effects of CA intervention on NF-κB p65 expression levels in HNECs by using four experimental groups: control group, CA group (optimal intervention condition), prostratin group, and prostratin+CA group. Prostratin is an NF-κB activator used to increase NF-κB p65 expression levels in HNECs. Under CA intervention, the protein expression levels of NF-κB p65 in HNECs were significantly reduced (**Figure 3**). The Prostratin+CA group also exhibited significantly decreased NF-κB p65 protein levels than the Prostratin group (**Figure 3**). This finding confirms the ability of CA intervention to reduce NF-κB p65 expression in HNECs.

Effect of CA intervention in the CRS mouse model

This study further investigated the role of CA intervention in the TRPM8 pathway within the pathogenesis of CRS by using an animal model. Symptom scores of mice were counted on the 2nd, 4th, and 8th weeks after intranasal administration. As shown in **Figure 4**, on the 2nd, 4th, and 8th weeks, the frequency of sneezing (3.33±0.58 times) and nose scratching (4.33± 0.58 times) in the model group were higher than those of the CA group (1.67±0.58 times and 2.67±0.58 times, respectively). This finding indicates that intranasal administration of CA can alleviate symptoms in CRS mice.

Effect of CA intervention on IL-25, IL-33, and TSLP expression in CRS mice

ELISA was employed to measure the expression levels of the pro-inflammatory cytokines (IL-25, IL-33, and TSLP) in the nasal lavage fluid of mice. The levels of IL-25, IL-33, and TSLP were significantly elevated in the nasal mucosa and nasal lavage fluid of the CRS mice, while CA effectively reduced these levels (**Figure 5A**). We also collected nasal mucosal tissues from the mice and measured the mRNA levels of IL-25, IL-33, and TSLP, and found results consistent with the ELISA findings (**Figure 5B**). These data further confirm that CA intervention can reduce the expression of IL-25, IL-33, and TSLP in CRS mice.

Effect of CA intervention on TRPM8 in the nasal cavity of CRS mice

We also investigated changes in TRPM8 expression in the nasal mucosal tissues of CRS mice following CA intervention. The CA group had lower protein and mRNA levels of TRPM8 than

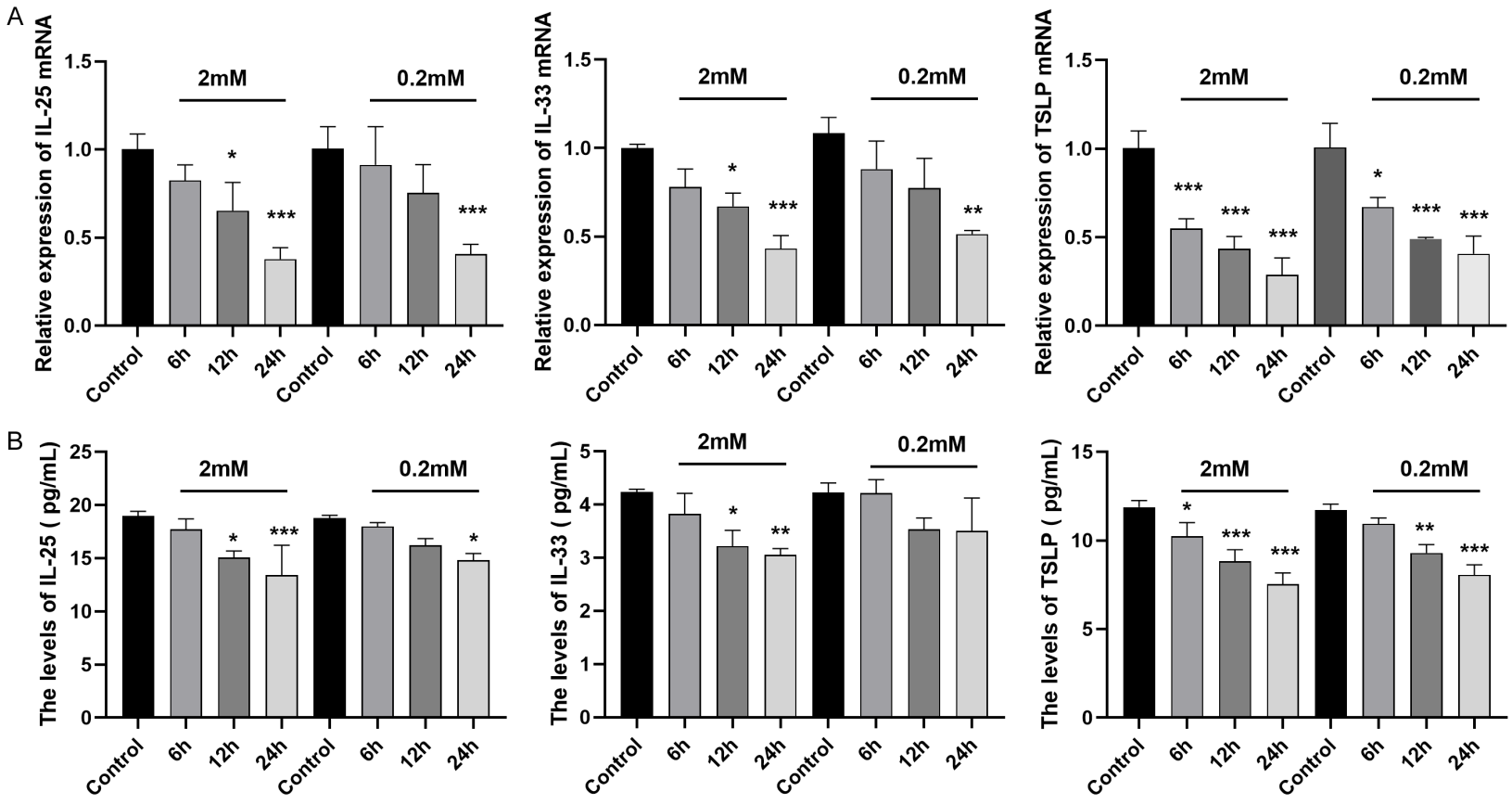

Figure 1. The mRNA expression (A) and secretion (B) of IL-25, IL-33, and TSLP after CA intervention in HNECs. Note: compared to the control group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

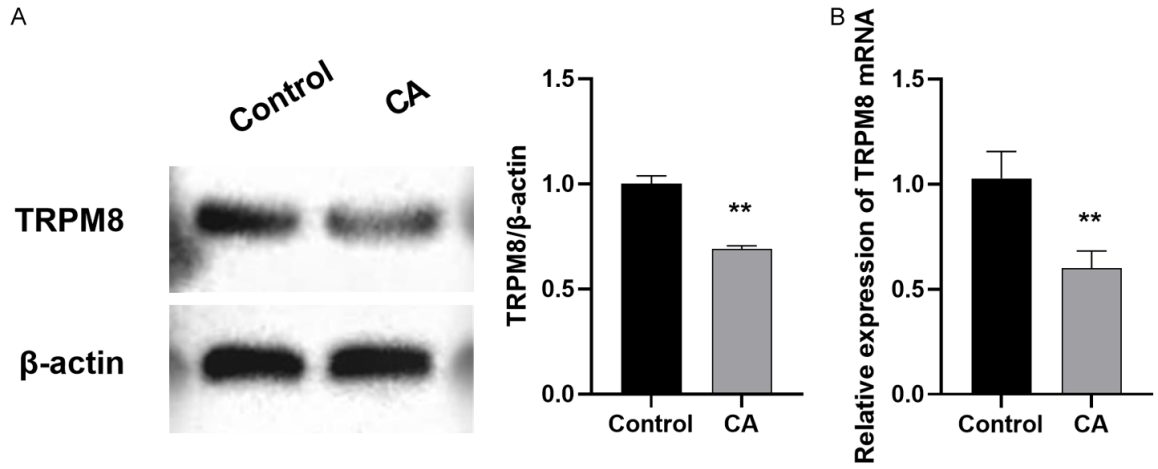

Figure 2. Protein (A) and mRNA (B) expression levels of TRPM8 in HNECs. Note: compared to the control group, $^{**}P<0.01$.

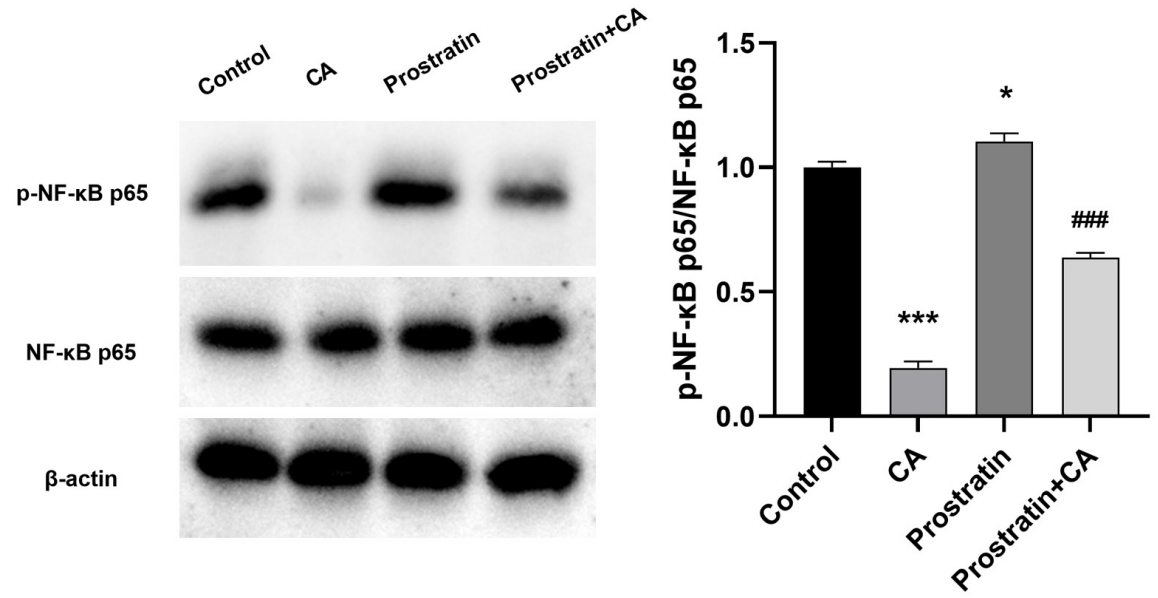

Figure 3. Protein expression levels of NF-κB p65 after CA intervention in HNECs. Note: compared to the control group, $^{*}P<0.05$, $^{***}P<0.001$. Compared to the CA group, $^{###}P<0.001$.

the model group (**Figure 6**). These findings suggest that the application of CA can effectively inhibit TRPM8 expression in the nasal mucosal tissues, alleviate mucosal inflammation, and improve CRS symptoms in CRS mice.

Effect of CA intervention on nasal pathology in CRS mice

Following model establishment, we conducted H&E staining on nasal-sinus tissues of mice. Mice in the model group exhibited increased submucosal infiltration of inflammatory cells, disordered arrangement of columnar epithelial cells, and incomplete epithelial cilia compared to the control group (**Figure 7**). By contrast, the CA group showed alleviated inflammation compared to the model group (**Figure 7**). This finding suggests that the inhibition of TRPM8 by CA can mitigate nasal mucosal inflammatory responses in CRS mice.

Discussion

CRS is a highly prevalent disease that not only imposes a significant economic burden on

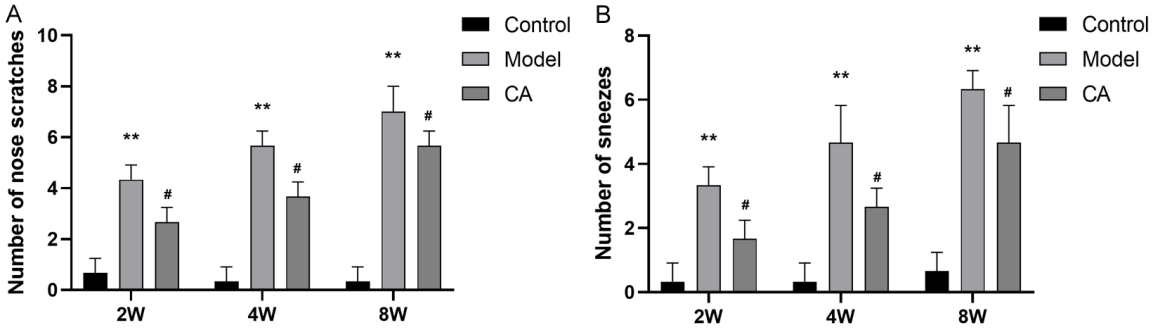

Figure 4. Performance of the symptomatology in CRS mice. A. Number of nose scratches. B. Number of sneezes. Note: compared to the control group, $^{**}P<0.01$. Compared to the model group, $^{\#}P<0.05$.

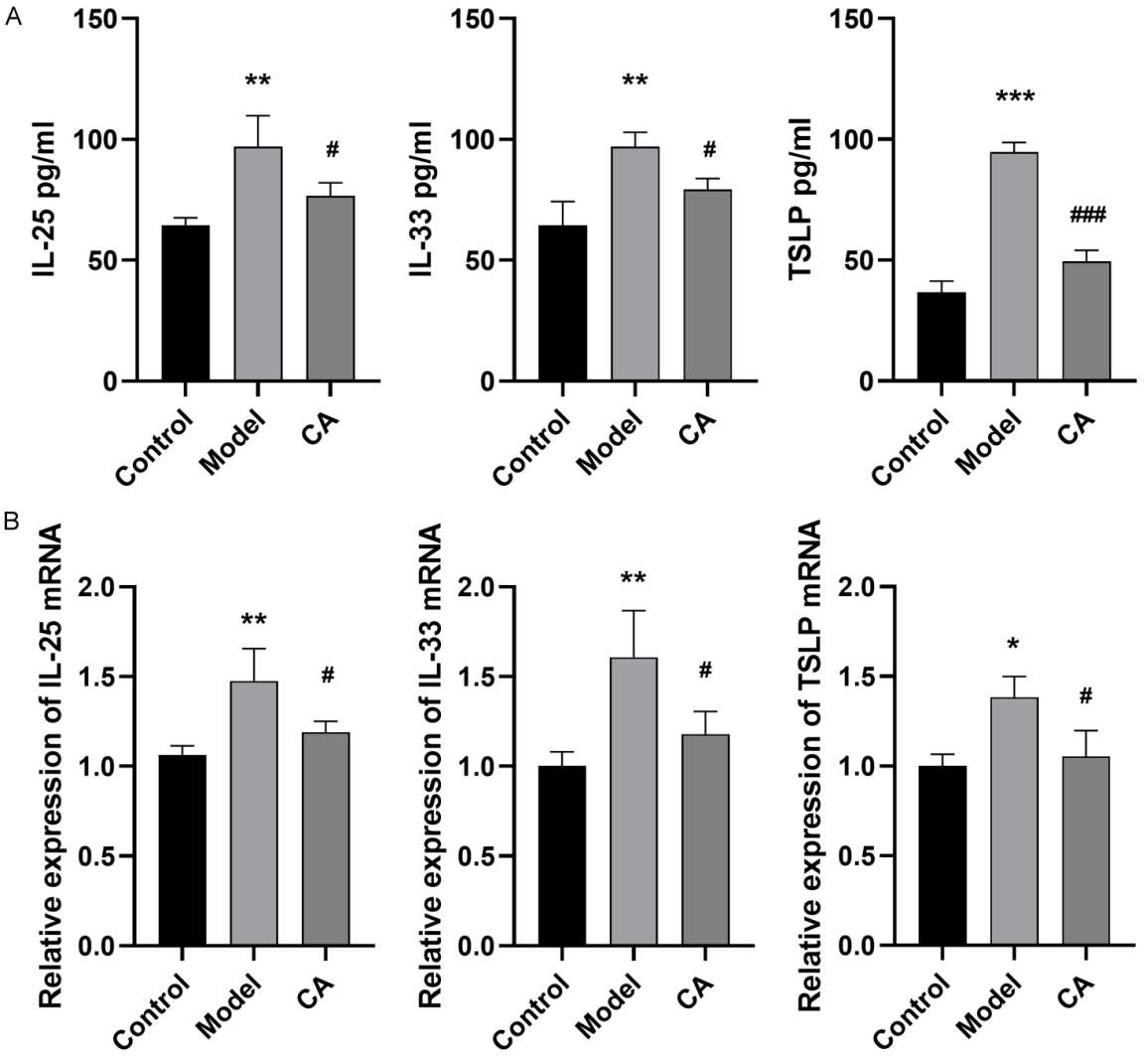

Figure 5. Secretion (A) and mRNA expression (B) of IL-25, IL-33 and TSLP after CA intervention in mice. Note: compared to the control group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Compared to the model group, $^{\#}P<0.05$, $^{\#\#\#}P<0.001$.

healthcare systems but also severely worsens patients' quality of life [15]. Therefore, new and effective treatment strategies should be developed to improve the current management of

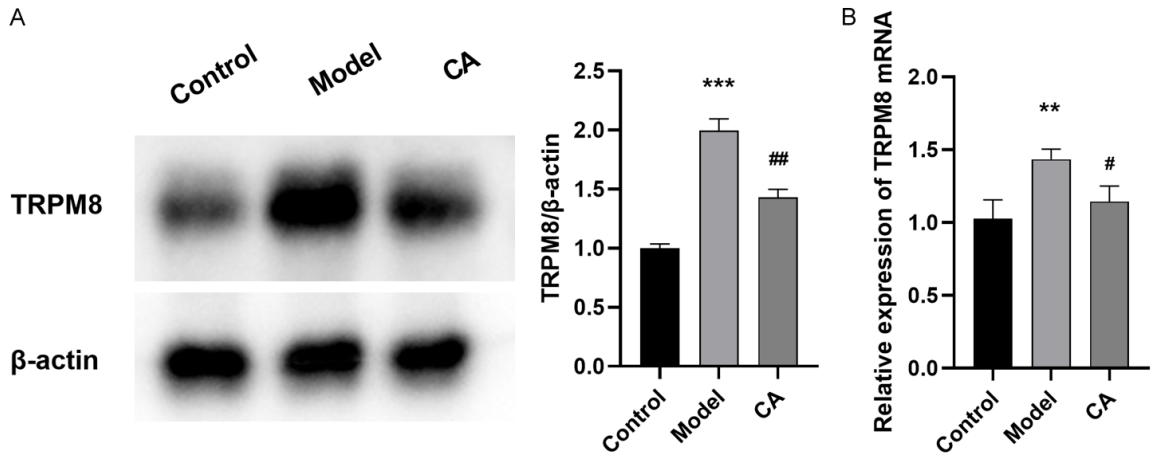

Figure 6. Protein (A) and mRNA (B) expression levels of TRPM8 in the nasal mucosa of CRS mice. Note: compared to the control group, $^{**}P<0.01$, $^{***}P<0.001$. Compared to the model group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

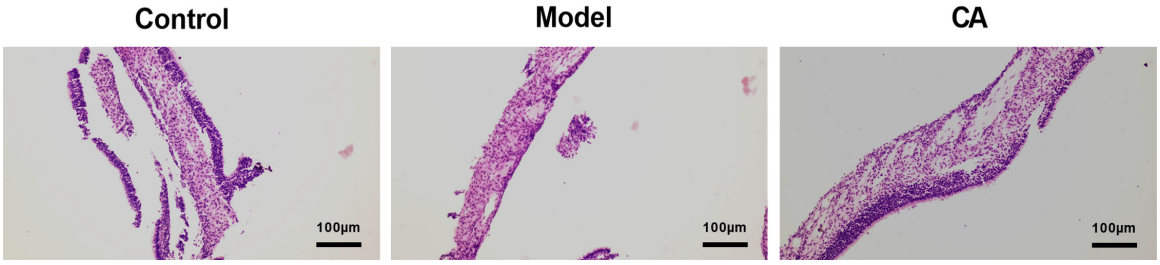

Figure 7. H&E staining of the mouse nasal tissues (100×).

CRS. The present study aims to investigate the role of CA in the development and progression of CRS by conducting *in vitro* experiments on HNECs and establishing a CRS mouse model.

The airway epithelium collaborates with immune cells to defend against environmental changes and various pathogenic stimuli, such as temperature variations, chemicals, and inhaled allergens [16], thereby regulating nasal inflammation and immune responses. IL-25 is a widely discussed cytokine that could enhance the response of other epithelial cytokines during inflammation [17]. IL-25 plays a crucial role in delaying eosinophil apoptosis and maintaining eosinophil activity in allergic airway inflammation [18]. IL-33, an epithelial cell-derived cytokine, induces innate and adaptive Th2 inflammatory responses in asthma, CRS, and allergic rhinitis (AR) [19]. TSLP has been implicated in the pathogenesis of inflammatory airway diseases, including AR, CRS, asthma, and chronic obstructive pulmonary disease [20, 21]. CRS is associated with significant immune dysfunction, with TSLP as an important cytokine that influences its progression [22]. After CA intervention in HNECs, the levels of the epithelial-derived cytokines IL-25, IL-33, and TSLP decreased, suggesting that CA may alleviate inflammation in HNECs by reducing the expression and secretion of IL-25, IL-33, and TSLP.

TRPM8 is a channel directly gated by cold and chemical agonists, and its stimulation can exacerbate inflammation through bronchial epithelial cells [23, 24]. Cold and heat are the crucial aspects of the Eight Principles in TCM. The theory of cold and heat is also an important part of the theory of medicinal properties in TCM. CA, a component of hot-natured medicinal herbs, can significantly downregulate the function of the TRPM8 channel protein [25]. After CA intervention in HNECs, the expression of TRPM8 was decreased, suggesting that CA may regulate TRPM8 due to its hot-natured properties, possibly serving as a molecular mechanism for its anti-inflammatory effects. Current research suggests that most TRP fami-

ly members ultimately mediate the activation of the NF-κB pathway, participating in a series of cellular functional changes [26]. The expression levels of IL-25, TSLP, and IL-33 are also regulated by the NF-κB pathway [27]. Our study found that CA intervention can reduce the expression level of phosphorylated p65 protein in the NF-κB pathway in HNECs. This effect persists even with the presence of an NF-κB activator. Furthermore, the use of CA reduces the transcription levels of TRPM8, IL-33, and TSLP in HNECs. Hence, CA intervention in HNECs may alleviate inflammatory responses in HNECs by inhibiting the NF-κB pathway.

This study further investigated the role of CA intervention in the TRPM8 pathway within the pathogenesis of CRS by using an animal model. Comparison of the symptomatic behavior of mice among different groups showed that CA intervention effectively alleviated symptoms, such as nose scratching and sneezing. A chronic inflammatory response is a primary characteristic of CRS pathogenesis, and regulating the expression of inflammatory cytokines to inhibit inflammation is one of the effective treatment strategies for CRS [28]. IL-25 has been less studied in nasal diseases, but a high expression of IL-25 has been observed in nasal mucosal epithelial cells in AR [29]. IL-33 can induce Th2 immune inflammatory responses, such as those seen in AR, asthma, atopic dermatitis, and allergic purpura [30]. Inhibition of TSLP can improve airway inflammation and reduce epithelial cell damage [31]. TSLP levels significantly decreased before and after treatment in children with asthma, indicating that reduced TSLP can help control the disease [32]. In mouse models, TSLP mainly promotes type 2 airway inflammation by activating dendritic cells [33]. The results of this study showed that the levels of IL-25, IL-33, and TSLP were significantly elevated in the nasal mucosa and nasal lavage fluid of CRS mice, and these levels were effectively reduced by CA. These findings suggest that CA can alleviate inflammatory responses in CRS by regulating the expression of pro-inflammatory cytokines such as IL-25, IL-33, and TSLP.

Asthma-related research has shown that knocking out TRPM8 can inhibit the MAPK and NF-κB pathways, suggesting that TRPM8 may be involved in the pathogenesis of asthma, including airway inflammation and remodeling [34]. A previous genome-wide association study indicated that TRPM8 was significantly associated with the phenotype of toluene diisocyanate-induced occupational asthma [35]. In this experiment, CA intervention reduced the expression of TRPM8 in the nasal mucosa of CRS mice. Overall, CA may exert its anti-inflammatory effects by regulating TRPM8 expression, thereby alleviating the symptoms of CRS.

This study has some limitations, including the need for further exploration of specific signaling pathways involved in TRPM8 modulation. Future research should elucidate the molecular mechanisms underlying the effects of CA on CRS and investigate its interactions with other inflammatory mediators.

Conclusion

In this study, we found that CA intervention significantly reduced the expression and secretion of pro-inflammatory cytokines (IL-25, IL-33, and TSLP) in a concentration- and time-dependent manner in HNECs. Additionally, CA regulated the NF-κB pathway, inhibited the expression of TPRM8, and alleviated inflammatory responses in HNECs. The results from *in vivo* mouse experiments indicated that CA intervention significantly ameliorated the symptoms in an OVA-induced CRS mouse model; that is, it reduced nose scratching and sneezing symptoms and decreased the expression of pro-inflammatory cytokines and TPRM8 in nasal lavage fluid. Our findings provide new insight into the prevention and treatment of CRS.

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Disclosure of conflict of interest

None.

Address correspondence to: Jiang Bian, Department of Otolaryngology-Head and Neck Surgery, Jinhua People's Hospital, No. 267 Danxi East Road, Jinhua 321000, Zhejiang, China. Tel: +86-13586988280; E-mail: 13586988282@163.com

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