



Modulatory effect of anisodamine on airway hyper-reactivity and eosinophilic inflammation in a murine model of allergic asthma

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ABSTRACT

Anisodamine, a peripheral muscarinic receptor antagonist, is a naturally occurring atropine derivative that has been isolated, synthesized and characterized by scientists in China. In the present investigation, we evaluated the modulatory effects of anisodamine on airway hyper-reactivity and inflammation in a murine model of allergic asthma. Asthma model was induced successfully by ovalbumin. The activation of cells, airway eosinophilia, cytokine production, and airway function were examined. Our results collectively show that anisodamine could significantly suppress the accumulation of eosinophils into the airways and dramatically inhibited the histological changes in OVA-induced mice. Additionally, anisodamine could restore the Th1/Th2 balance in BALF by downregulating the level of Th2 cell-associated cytokine IL-4 ($p < 0.01$) and upregulating the level of Th1 cell-associated cytokine IFN- γ ($p < 0.01$). In addition, pretreatment with anisodamine also showed strong suppression of allergen-induced bronchial hyper-reactivity with maximum contraction decreasing from 0.45 ± 0.02 g to 0.28 ± 0.03 g ($p < 0.01$). These results suggested the modulatory effects of anisodamine on Th1/Th2 balance by enhancing Th1-related and suppressing Th2-related parameters, as well as its potential application in airway hyper-reactivity and eosinophilic inflammation.

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1. Introduction

Allergic bronchial asthma represents a major health problem worldwide, characterized by various airway obstruction, airway eosinophilic inflammation and airway hyperresponsiveness [1]. It has been reported that helper T-lymphocyte (Th) cells (particularly Th2 cells) may play a critical part in the development of allergen-induced airway inflammation and remodeling caused by the infiltration of eosinophils [2].

On the basis of the cytokine profiles they synthesize, Th cells can be subdivided into Th1 and Th2. They constitute an immune regulatory loop in response to different immunogenic stimuli and cytokines [2–4]. The overactivity of Th2 cells and increase in the levels of Th2-associated cytokines such as interleukin (IL)-4, IL-5 and IL-13 can induce asthma and related disorders. However, interferon (IFN)- γ , which is secreted by Th1 cells, can control the balance of the development of Th1 and Th2 by inhibiting the production of Th2 cytokines. Imbalance of Th1/Th2 cells plays an important part in the development of asthma [5].

Recent studies have clearly shown that muscarinic receptors are involved in some autoimmune diseases by regulating immune and

inflammatory responses [6,7]. Arecoline can mediate immune responses via the lymphocyte muscarinic acetylcholine receptor [8]. Atropine could inhibit antibody and T-cell proliferative responses [9]. Thus, inhibition of muscarinic receptors may be beneficial to immune/inflammatory responses. Activation of the muscarinic receptor has pro-inflammatory effects in a wide variety of cells implicated in airway inflammation and remodeling [10,11]. Studies in animal models also support the notion that muscarinic antagonists might contribute to the control of inflammatory processes in airway disease [12,13]. The beneficial effects of the long-acting muscarinic antagonist tiotropium bromide have been shown in chronic obstructive pulmonary disease [14].

Anisodamine, a belladonna alkaloid extracted from a Chinese traditional medical herb *Scopolia tangutica* [15], is related to atropine, having a hydroxyl group at asymmetric carbon position six of the tropine radical (Fig. 1). Interestingly enough, anisodamine is a peripheral muscarinic antagonist, but with less toxic and fewer side effects on the central nervous system (CNS) than atropine. It has been employed therapeutically since 1965 in China primarily in the treatment of septic shock and gastrointestinal colic. Recent reports demonstrated that anisodamine could act on cell-mediated immune responses in mice. Chong et al. observed that anisodamine could markedly decrease the mortality induced by lipopolysaccharide (LPS) and significantly reduce the levels of tumor necrosis factor (TNF)- α and IL-1 β [16]. However, studies focusing on its effects on airway hyper-reactivity, airway inflammation and Th1/Th2 balance in a murine model of allergic asthma are lacking.

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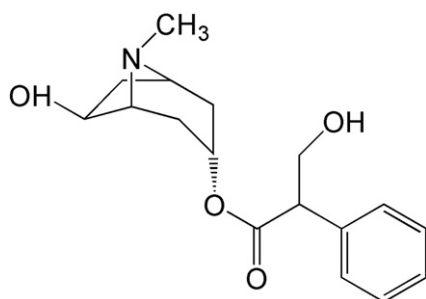


Fig. 1. Chemical structure of anisodamine.

The purpose of this study was to investigate the modulatory effects of anisodamine on airway hyper-reactivity and inflammation in an OVA-sensitized/challenged mice model. OVA-induced activation of cells in bronchoalveolar lavage fluid (BALF), airway eosinophilia, cytokine production, and airway function were investigated. Such detailed information could be valuable for the potential application of anisodamine in the treatment of allergic asthma.

2. Materials and methods

2.1. Animals

BALB/c male mice (age, 10–12 weeks; 18–22 g) were obtained from Shanghai SLAC Laboratory Animal Company, Limited (Shanghai, China). Mice were housed in specific pathogen-free conditions with food and water provided *ad libitum*. Experiments were conducted in accordance with the guidelines for the care and use of laboratory animals set by Shanghai JiaoTong University (Shanghai, China).

2.2. Drugs and reagents

Anisodamine was purchased from Shanghai Number One Pharmaceutical Company (Shanghai, China). Dexamethasone was purchased from Tongyong Pharmaceutical Company (Shanghai, China). OVA (grade III) was purchased from Sigma-Aldrich (St Louis, MO, USA). $\text{Al}(\text{OH})_3$ was obtained from XiTa Chemical Company (Shanghai, China).

2.3. Preparation of an allergic airway inflammation model in mice

BALB/c mice were sensitized on day 1, 8 and 15 by OVA (20 μg , i.p.) together with 2 mg $\text{Al}(\text{OH})_3$ as an adjuvant. After 16 days, mice were placed in an aerosol chamber and challenged with aerosolized 2% OVA in 0.9% saline for 20 min using an ultrasonic nebulizer (Model 4021, Shanghai SILING Medical Apparatus Factory, Shanghai, China) once a day for 10 consecutive days. Twenty-four hours after the final antigen challenge, mice were killed and BALF and lung tissues were collected. A saline control group of mice received the same immunization regimen but inhaled saline aerosol instead of being challenged by OVA.

2.4. Study design

Treatment groups were divided into OVA-sensitized/saline-challenged, OVA-sensitized/OVA-challenged, OVA-sensitized/OVA-challenged/anisodamine-treated, OVA-sensitized/OVA-challenged/atropine-treated, and OVA-sensitized/OVA-challenged/dexamethasone-treated. In anisodamine-treated and dexamethasone-treated groups, mice were pretreated with anisodamine (0.75, 1.5 and 3 mg/kg, i.p.), atropine (1 mg/kg, i.p.) or dexamethasone (1 mg/kg, i.p.) 30 min before OVA challenge. Therapeutic treatment of dexamethasone, atropine and anisodamine were conducted on day 16–25 in this model.

2.5. BALF and cell counts

Mice were killed with an overdose of urethane (25 g/kg, i.p.) 24 h after the final antigen challenge. The trachea was cannulated and the right bronchus tied for histological analyses. BAL was undertaken on the left lung with three lavages of 1 mL phosphate-buffered saline (PBS). Recovered BALF was immediately centrifuged at $300\times g$ for 10 min at 4 °C, and cells resuspended in PBS. The number of cells was counted using a hemocytometer. Aliquots of the cell suspension were centrifuged onto glass slides and stained (Wright-Giemsa) for differential cell counting. The supernatant of BALF was stored at $-25\text{ }^{\circ}\text{C}$ for the determination of cytokines.

2.6. Histological analyses

Immediately after BAL, the lobes of the right lung were removed, rinsed with PBS, and preserved in 10% phosphate-buffered formalin for 24 h. After fixation, lung lobes were embedded in paraffin, and cut into 4- μm sections. Sections were stained with hematoxylin and eosin (H&E) for histological examination. Eosinophils infiltrating around the airway (between the bronchial epithelium and adventitia) were evaluated using Image-Pro plus 6.0 software (Olympus, Tokyo, Japan), by which cell counts and airway wall area could be calculated. A total of 10 fields per lung were randomly selected and analyzed in each group.

2.7. Cytokine measurements

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used (Rapidbio Lab, CA, USA) for the detection of mouse IFN- γ and IL-4 in BALF according to the manufacturer's protocol. The detection limit of IFN- γ and IL-4 was 2 pg/mL.

2.8. Measurement of airway isometric tension

Immediately after the recovery of BALF, the main bronchus was rapidly removed and dissected free from surrounding connective tissue. Bronchial rings were mounted on two stainless-steel hooks in a conventional organ bath in a modified Krebs solution (composition in mM: NaCl 118; KCl 4.7; MgSO_4 1.2; KH_2PO_4 1.2; NaHCO_3 25; CaCl_2 2.5; glucose 11, $\text{EDTA}\cdot\text{Na}_2$ 0.5), maintained at 37 °C and continuously bubbled with a pre-mixed gas of 95% O_2 and 5% CO_2 . The preparations were connected vertically to a force-displacement transducer under a resting tension of 500 mg. Isometric tension was recorded using a PowerLab 8sp Life Analysis System (Ad Instruments Australia). Preparations were equilibrated for 1 h and washed every 15 min before cumulative concentration-response curves for acetylcholine were constructed. The change in airway isometric tension of each group was expressed as force generated (g); sensitivity to acetylcholine was expressed as pEC_{50} values.

2.9. Statistical analyses

Data are mean $\pm$ SEM. The statistical significance of differences between data was determined by one-way analysis of variance or the Student's *t*-test with Bonferroni correction, as appropriate. Differences were considered statistically significant if $p < 0.05$. All statistical analyses were undertaken using Prism version 5.0 (Graph-Pad Software, San Diego, CA, USA).

3. Results

3.1. Effect of anisodamine on allergen-induced cell infiltration into BALF

Compared with the saline group, OVA-challenged mice exhibited a significant increase in total cell numbers in BALF ($9.10 \pm 0.60 \times 10^6$ vs

Table 1

Effects of anisodamine on the total number of cells and eosinophils in the bronchoalveolar fluid of mice.

Group	Number of cells ($\times 10^6$ cells/mL)		Eosinophils (%)
	Total cells	Eosinophils	
Saline	3.16 ± 0.59	0.02 ± 0.00	0.63 ± 0.30
OVA-challenged	$9.10 \pm 0.60^{##}$	$1.37 \pm 0.12^{##}$	$15.05 \pm 0.58^{##}$
OVA + DEX (1 mg/kg)	$5.45 \pm 0.32^{**}$	$0.48 \pm 0.04^{**}$	$8.80 \pm 0.73^{**}$
Atrop (1 mg/kg)	$6.07 \pm 0.28^{**}$	$0.76 \pm 0.16^{**}$	$12.50 \pm 0.88^*$
OVA + Aniso (0.75 mg/kg)	7.58 ± 0.83	1.12 ± 0.15	14.74 ± 0.52
OVA + Aniso (1.5 mg/kg)	$6.42 \pm 0.39^*$	$0.81 \pm 0.19^*$	$12.08 \pm 0.83^*$
OVA + Aniso (3 mg/kg)	$5.44 \pm 0.63^{**}$	$0.52 \pm 0.06^{**}$	$9.55 \pm 0.74^{**}$

Cell counts were determined in each BALF sample using a hemocytometer and differential cell counts were done on cytospin preparations stained with Wright-Giemsa stain. Eosinophil counts are expressed as a percentage of total cells. Values are mean $\pm$ SEM ($n=10$ per group). DEX: dexamethasone, Atrop: atropine, Aniso: anisodamine.

$^{##}$ $P<0.01$ vs saline group.

* $P<0.05$.

** $P<0.01$ vs OVA-challenged group.

$3.16 \pm 0.59 \times 10^6$ cells/mL, $p<0.01$). Differential cell counts revealed that OVA-challenged mice also exhibited a marked increase in the number of eosinophils in BALF to $1.37 \pm 0.12 \times 10^6$ cells/mL ($0.02 \pm 0.00 \times 10^6$ cells/mL in the saline group, $p<0.01$), which represented 15.05% of the total number of BALF cells (0.63% in saline group, $p<0.01$). The numbers of neutrophils, lymphocytes or macrophages were moderately increased in OVA-challenged mice, but there was no statistical significance (data not shown). Treatment with dexamethasone caused a significant reduction in the numbers of total cells and eosinophils in BALF as compared with OVA-challenged mice ($p<0.01$). Atropine and anisodamine also possessed the inhibitory effect. Intraperitoneal administration of atropine could inhibit the influx of total cells into BALF by 51%, and the influx of eosinophils into BALF by 45%. Anisodamine treatment at 0.75, 1.5, and 3 mg/kg could inhibit

the influx of total cells into BALF by 26%, 45% and 62%, respectively, and the influx of eosinophils into BALF by 18%, 41%, and 62%, respectively (Table 1).

3.2. Effect of anisodamine on allergen-induced histological changes

Airway epithelial hyperplasia, thickening of the basement membrane, and peribronchial and perivascular infiltration of inflammatory cells were observed in the lungs of OVA-challenged mice (Fig. 2B) compared with saline-challenged control mice (Fig. 2A). Predominant infiltration of eosinophils was observed around the airways of OVA-challenged mice compared with saline controls ($p<0.05$, Fig. 3). Administration of anisodamine (3 mg/kg, i.p.) or dexamethasone (1 mg/kg, i.p.) dramatically inhibited these histological changes in OVA-challenged mice (Fig. 2C and D), and strongly suppressed the influx of eosinophils into the airway after OVA challenge to 1.40 ± 0.17 ($p<0.05$) and 1.53 ± 0.19 , respectively (Fig. 3) compared with OVA-challenged mice.

3.3. Effect of anisodamine on allergen-induced cytokine release

Compared with the saline control group, IL-4 concentration was significantly increased to 0.22 ± 0.02 ng/mL ($p<0.01$, increased by 122%), whereas that of IFN- γ was significantly decreased to 0.36 ± 0.04 ng/mL ($p<0.01$, decreased by 37%) in the BALF of OVA-challenged mice (Fig. 4A). The ratio of IFN- γ /IL-4 in the OVA-challenged group was much lower than that in the saline control group ($p<0.01$, decreased by 72%) (Fig. 4B). Administration of atropine (1 mg/kg, i.p.) resulted in a significant reduction of IL-4 level (0.14 ± 0.02 ng/mL, $p<0.05$) and increase in the IFN- γ level (0.48 ± 0.04 ng/mL, $p<0.05$) in OVA-challenged mice. Administration of anisodamine (3 mg/kg, i.p.) also resulted in a significant reduction (83%) of IL-4 level (0.12 ± 0.05 ng/mL, $p<0.01$) and increase (42%) in the IFN- γ level (0.51 ± 0.03 ng/mL, $p<0.01$) in OVA-challenged mice.

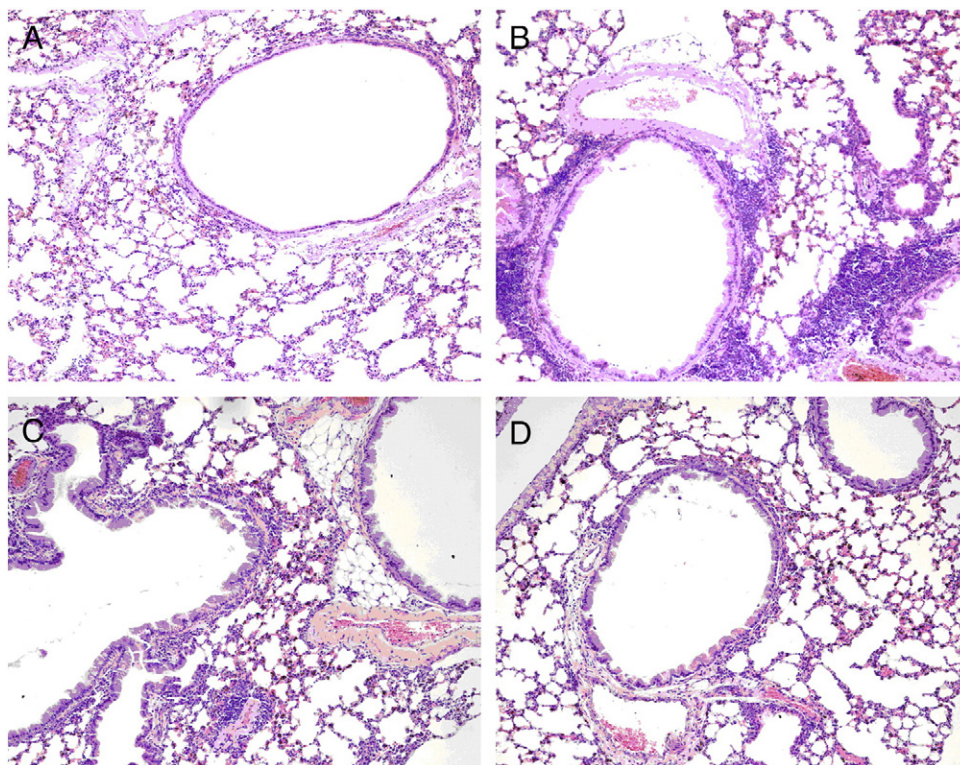


Fig. 2. Effect of anisodamine on allergen-induced airway inflammation. Lung sections were obtained from sensitized BALB/c mice 24 h after final antigen challenge (H&E stain, magnification $\times 100$). A: Saline-challenged mice, B: OVA-challenged mice, C: DEX (dexamethasone, 1 mg/kg)-treated mice, and D: Aniso (anisodamine, 3 mg/kg)-treated mice.

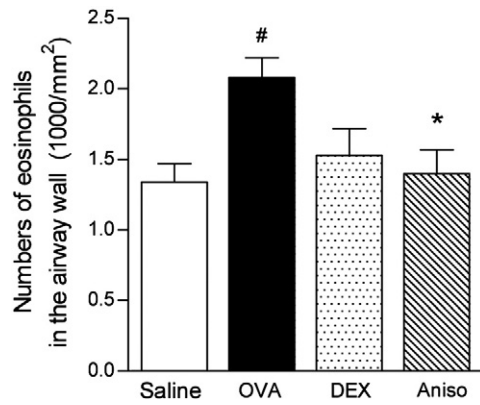


Fig. 3. Effects of anisodamine on eosinophil infiltration into airway walls after antigen challenge. Values are the number of eosinophils per mm² ± SEM ($n=6$ per group). DEX: dexamethasone (1 mg/kg, i.p.), Aniso: anisodamine (3 mg/kg, i.p.). [#] $p<0.05$ vs saline group, ^{*} $p<0.05$ vs OVA-challenged group.

The ratio of IFN- γ /IL-4 in the BALF of OVA-challenged group was significantly restored by anisodamine at 1.5 and 3 mg/kg ($p<0.01$, increased by 49% and 71%, respectively). A similar effect was found in the atropine group ($p<0.05$, increased by 66%) and dexamethasone group ($p<0.01$, increased by 85%).

3.4. Effect of anisodamine on allergen-induced airway hyper-reactivity

The responses of each group to the accumulating concentration of acetylcholine are shown in Fig. 5. The concentration–response

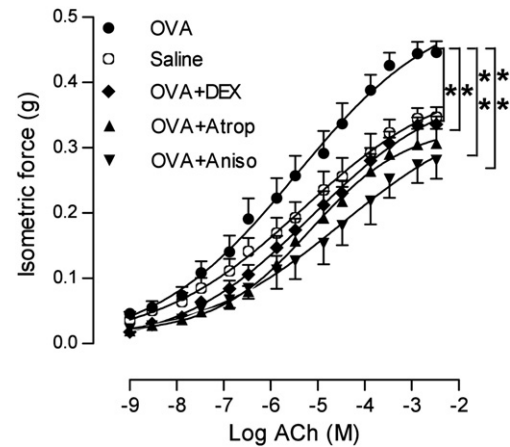


Fig. 5. Effects of anisodamine on the bronchial response to cumulative addition of acetylcholine. Each point represents mean ± SEM of 5–6 mice. DEX: dexamethasone (1 mg/kg, i.p.), Atrop: atropine (1 mg/kg, i.p.), Aniso: anisodamine (3 mg/kg, i.p.). ^{*} $p<0.05$, ^{**} $p<0.01$ vs OVA-challenged group.

curve of bronchial preparations from the OVA-challenge group was markedly shifted upward compared with that from the saline group ($p<0.05$). The maximum contraction of saline-challenged group was maintained at 0.35 ± 0.02 g, whereas that of the OVA-challenged group reached 0.45 ± 0.02 g. The pEC₅₀ values of acetylcholine were 5.31 ± 0.43 and 5.46 ± 0.33 for saline- and OVA-challenged groups, respectively. Dexamethasone pretreatment completely prevented the increase in contractility induced by repeated OVA challenge with the maximum response reduced to 0.34 ± 0.01 g ($p<0.05$) and pEC₅₀ values of 5.12 ± 0.27 . Pretreatment with atropine also prevented the increase in contractility induced by repeated OVA challenge with the maximum response reduced to 0.31 ± 0.02 g ($p<0.01$) and pEC₅₀ values of 5.14 ± 0.20 . Similar results were obtained in the anisodamine-treated group; the maximum contraction of the acetylcholine concentration–effect curve was 0.28 ± 0.03 g (decreased 33% of the OVA-challenge group, $p<0.01$) and pEC₅₀ value was 4.88 ± 1.07 . Although the initial resting tension of smooth muscle tone presented a slight change in dexamethasone- and anisodamine-treated groups as compared with OVA challenge, this change was not statistically significant. The OVA-sensitized/saline-challenged/anisodamine-treated group was also assessed, but there was no obvious difference in maximum contraction (0.32 ± 0.01 g) and pEC₅₀ value (5.42 ± 0.31) compared with the saline control group. There were no differences in pEC₅₀ values among all groups.

4. Discussion

The present study clearly showed that the muscarinic antagonist, anisodamine could reverse several aspects of allergen-induced airway inflammation. These included peribronchial and perivascular infiltration of inflammatory cells (mainly eosinophils), and infiltration of eosinophils in BALF, in addition to the anti-remodeling effects of attenuating airway epithelial hyperplasia, thickening of the basement membrane and bronchial hyper-reactivity, which were similar to those of dexamethasone and atropine. This is the first evidence *in vivo* to demonstrate an important regulatory role for anisodamine in OVA-induced airway inflammatory responses.

Airway allergen challenge causes cellular inflammation in the airway in humans and mice, which is dominated by eosinophils. It has been suggested that eosinophils contribute to several of the features of allergic asthma: tissue damage, vascular leakage, mucus secretion, and airway hyper-responsiveness [17]. We observed increasing numbers of eosinophils and the increased contractility of isolated bronchi in OVA-challenged mice, whereby the increase in the number

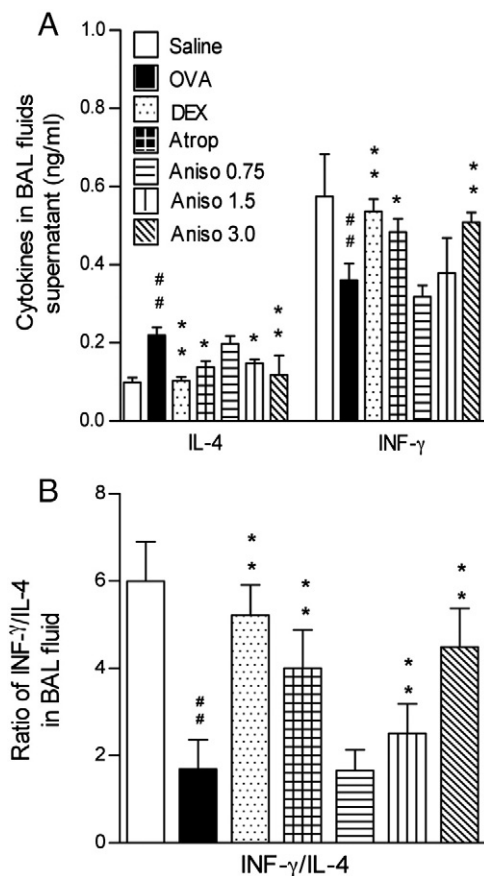


Fig. 4. Effects of anisodamine on cytokine release in BALF supernatant obtained from sensitized BALB/c mice 24 h after final antigen challenge. Values are mean ± SEM ($n=10$ per group). DEX: dexamethasone (1 mg/kg), Atrop: atropine (1 mg/kg, i.p.), Aniso: anisodamine (0.75, 1.5 or 3 mg/kg). ^{##} $p<0.01$ vs saline group, ^{*} $p<0.05$, ^{**} $p<0.01$ vs OVA-challenged group.

of eosinophils showed a positive correlation to contractility (data not shown). Similar results have been reported by other investigators using different immunization protocols [18]. Glucocorticoids could attenuate airway eosinophilia by inducing eosinophil apoptosis and inhibiting IL-5- and granulocyte macrophage-colony-stimulating factor (GM-CSF)-induced eosinophil survival, which might be important in the treatment of allergic and other eosinophilic disorders [19,20]. High doses of anisodamine showed similar effects to those of atropine or dexamethasone in allergen-challenged mice to prevent airway eosinophilia with the reduction of eosinophilis in BALF and histological sections of lung tissue. This suggested the involvement of muscarinic receptors in airway inflammation caused by eosinophil infiltration.

It was observed in mice bronchi that: isometric force was markedly shifted upward after challenge by OVA; the increase in the contractile response to acetylcholine was significantly inhibited by anisodamine or dexamethasone; and the sensitivity to acetylcholine was not altered, besides, anisodamine attenuated airway epithelial hyperplasia, and thickening of the basement membrane in our present study. These effects demonstrated that anisodamine has protective effects on the airway hyper-reactivity as well as airway remodeling. The protective effects of anisodamine are not related to residual blockade of the muscarinic receptor because residual effects of the receptor would not be shown after exposure to muscarinic receptor antagonists for 24 h. This speculation was supported by Urso et al. who showed a similar inhibitory effect with the short-acting anticholinergic atropine [21], and by Gosens et al. who showed no residual effect on methacholine-induced guinea pig tracheal contraction 24 h after *in-vivo* exposure [10]. In the present study, anisodamine was effective only in the prevention of bronchial contractile responses in mice challenged with OVA. This indicated that acetylcholine affects airway smooth muscle in inflamed, but not healthy, airways. These findings could be explained by augmented endogenous acetylcholine release after allergen challenge. The possible action of endogenous acetylcholine in inflammation is thought to promote the release of chemokines and cytokines from structural cells in the airway such as epithelial cells and smooth muscle cells. The released chemokines and cytokines attract inflammatory cells to the airway and induce direct remodeling on structural cells. Recent findings of Pieper et al. supported the above perspective that the proliferation of fibroblasts and myofibroblasts could be induced by acetylcholine and inhibited by tiotropium bromide [11]. Despite not studying the smooth-muscle thickness of the bronchus, the airway epithelial hyperplasia, thickening of the basement membrane and bronchial hyper-reactivity presented here support the relationship between the increase in contractility and structural changes in the airway. The lower efficiency of dexamethasone on the contractile response of isolated bronchi to acetylcholine indicated that smooth muscle cells may not be the specific target of dexamethasone.

An imbalance of Th1/Th2 cytokines is thought to induce different immune responses and immunologically dysregulated states [22]. Th2 cytokines and chemokines are regarded to regulate the infiltration of eosinophils into, and hyper-responsiveness, of the airway [23,24]. The increase in eosinophil number has been attributed to a shift in the balance between Th1 cells and Th2 cells [25,26]. Th1 cytokines are known to inhibit the allergic response. Dexamethasone can alter the cytokine network responsible for the adaptive immune response by inhibiting the production of Th1 cytokines but favoring the production of Th2 cytokines [27,28]. Interestingly, atropine or high-dose anisodamine (a dose similar to that used in humans as a spasmolytic drug) also presented similar effects in the present study to modulate an imbalance of Th1/Th2, and thereby suppress Th2 lymphocyte-mediated immune reactions and enhance Th1 lymphocyte-mediated immune responses. These effects were associated with inhibited airway eosinophilia in BALF and attenuated eosinophilic airway inflammation, suggesting that endogenous acetylcholine might be involved in immune inflammatory responses. It is unclear if the acetylcholine involved in

the allergen-induced imbalance of Th1/Th2 is neuronal or non-neuronal in origin. However, endogenous acetylcholine acting on muscarinic receptors might have a wide role in this appearance. It was recently reported that the cholinergic receptor agonist arecoline could interfere with the immune system by targeting the muscarinic acetylcholine receptors of the non-neuronal cholinergic system [8]. In addition, Nakagawa et al. demonstrated that anisodamine could directly act on human peripheral blood T-lymphocytes and significantly reduce proinflammatory cytokine production in serum [29]. According to the fact that an independent non-neuronal cholinergic system including nicotinic and muscarinic receptors on T-lymphocytes [30] and a cholinergic system is involved in immune/inflammatory responses [9,12,31,32] in which muscarinic receptors can regulate the functions of Th1 and Th2 lymphocytes [33], it could be speculated that the regulatory effects on the function of T-lymphocytes was relevant to the cholinergic system of T-lymphocytes. Moreover, studies by Ohta et al. indicated that the long-acting muscarinic receptor inhibitor tiotropium bromide can inhibit production of Th2 cytokines and airway inflammation, which is consistent with the present study [13]. Cholinoceptors are widely distributed in the lung and airways. They would receive neuronal or non-neuronal acetylcholine to break the balance of Th1 and Th2 lymphocytes and favor the development of inflammation. Therefore, the immunomodulatory effects of anisodamine might be mediated by muscarinic receptors.

In conclusion, we have provided the first evidence that anisodamine, the peripheral muscarinic antagonist, can inhibit eosinophilic airway inflammation and attenuate airway hypersensitivity associated with restoring the Th1/Th2 balance by downregulating IL-4 levels and upregulating IFN- γ levels in OVA-challenged mice. These results suggested that anisodamine had modulatory effects on the immune system and therapeutic roles in eosinophilic airway inflammation. Further studies are required to investigate these putative mechanisms for a better understanding of anisodamine which may pave the way for potential new therapeutic strategies in asthma.

Conflict of interest

All authors declared that there is no actual or potential conflict of interest with other people or organizations within three years of beginning the submitted work that could inappropriately influence this work.

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Submission declaration

All authors declared that the work described has not been published previously, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that it will not be published elsewhere in the same form, in English or in any other language.

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