



Expression of M₃ acetylcholine receptor in asthmatic mice and bronchial airway remodeling prediction

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ABSTRACT. This study aimed to investigate the role of M₃ acetylcholine receptor (M₃-AChR) expression in airway remodeling. Additionally, we aimed to evaluate the effects of ipratropium bromide solution inhaled in an early phase of asthma on airway remodeling in ovalbumin (OVA)-sensitized and challenged mice. Thirty BALB/c mice were divided into three groups, namely, control group (saline sensitized/challenged mice), asthma group (OVA sensitized/challenged mice), and treatment group (OVA sensitized/challenged mice treated by ipratropium bromide). Pathological changes were detected by histological staining in the bronchopulmonary tissue of mice. WAt/Pbm (the airway wall area /basement membrane perimeter) ratio of

the asthma group (25.37 ± 4.25) increased significantly ($P < 0.05$) when compared with that of the control (12.89 ± 1.71) and treatment group (15.82 ± 2.91). WAm/Pbm (smooth muscle wall area / basement membrane perimeter) ratio of the asthma group (7.58 ± 2.16) increased significantly ($P < 0.05$) when compared with that of the control (2.55 ± 0.72) and treatment group (3.36 ± 1.69). M_3 -AChR concentration increased in the treatment group (29.24 ± 3.59) and was significantly different ($P < 0.05$) from that of the control group (25.50 ± 1.83). During asthma treatment, SAMA can alleviate airway remodeling in murine model by lessening the thickness of bronchial walls and inhibiting the proliferation of smooth muscle cells. There were no obvious changes in M_3 -AChR density in the murine model of asthma characterized by airway remodeling. However, ipratropium bromide may up-regulate the expression of M_3 -AChR in bronchial walls of asthmatic murine model.

Key words: Asthma; M_3 acetylcholine receptor; Airway remodeling

INTRODUCTION

Bronchial asthma is a chronic obstructive airway disease that was thought to be entirely reversible. Consequently, asthmatic therapeutic measures have mainly focused on symptomatic control, by alleviating the bronchospasm and reducing airway inflammation (Elias et al., 1999). However, there is an increasing recognition that long-standing asthma may lead to chronic persistent airflow obstruction regardless of an optimal and timely therapy. This irreversible airflow obstruction is considered an outcome of airway remodeling, which is characterized by structural changes in the bronchial airways. The pathological correlation between M_3 -AChR and airway remodeling and pathogenic mechanisms of this correlation are not entirely clear. Recent attention has been focused on the structural changes that are mentioned to as the characteristics of airway remodeling. Airway remodeling is thought to be a key pathogenic alteration in the asthmatic airway, when compared with the normal airway. There are many typical pathological characteristics of the remodeling response, for example, mucus hypersecretion and metaplasia, subepithelial fibrosis, thickening of the lamina reticularis, myofibroblast hyperplasia, myocyte hyperplasia and hypertrophy, and epithelial hypertrophy in the lungs (Bergeron et al., 2010). These structural changes are thought to cause irreversible airway obstruction in asthma and airway hyperresponsiveness (Fish and Peters, 1999). Therefore, it is apparent that investigation of the pathogenesis of airway remodeling should contribute to the prevention and therapy of asthma.

Muscarinic acetylcholine receptors (M-AChRs) are representatives of acetylcholine receptors that form G protein-coupled receptor complexes in cell membranes (Eglen, 2006). Five muscarinic receptor subtypes have been determined by using selective radioactively labeled agonists and antagonists, and are referred to as M_1 - M_5 (Caulfield and Birdsall, 1998). M_1 - M_4 receptor subtypes have emerged as crucial drug targets for medical treatment of neurological (Thal et al., 2016), such as schizophrenia and Alzheimer's disease. In human lung tissue, three subtypes of M-AChRs have been identified, including M_1 -AChRs, M_2 -AChRs, and M_3 -AChRs. M_3 -AChRs are located in the airway smooth muscles, submucosal glands, caliciform cells, bronchial epithelial cells, and vascular endothelial cells. M_3 -AChRs help

maintain airway tension, stimulate secretion in submucosal glands, and regulate the function of the cholinergic nerve that is closely related to airway smooth muscle tension. A change in M₃-AChR quantity or function may influence the stability of the lung environment by leading to an imbalance of airway tension and gland secretion.

In 2001, Shen performed radioligand binding assay in lung tissue specimens from patients with chronic obstructive pulmonary disease and found that the number of muscarinic cholinergic receptors was decreased in COPD patients; subtype M₁ and M₃ ratios were increased and subtype M₂ ratio was decreased (Shen et al., 2001). In 1995, Chang found that M-AChR density was regionally increased in asthmatic guinea pigs, and M-AChR grains in the bronchial/bronchiolar smooth muscle and bronchial mucosal epithelium were significantly more numerous than in those of the control group (Chang, 1995). Kistemaker demonstrated that acetylcholine regulates inflammation via M₃ receptors on structural cells (Kistemaker et al., 2015), and Zhang suggested that bencycloquidium bromide (BCQB; a novel M₃ receptor antagonist) might be used in a potential therapy for inflammation in cigarette smoke-induced pulmonary diseases (Zhang et al., 2015). Other authors found that M₂-AChR functions were reduced in asthmatic guinea pigs, mice, rats, and human patients. Although asthmatic patients are known to be hypersensitive to cholinergic agonists, currently no evidence supports the hypothesis that airway smooth muscle contraction mediated by M₃-AChR is affected by asthma. The aim of this study was to investigate the role of M₃-AChR expression in the airway and airway remodeling, and to investigate the effect of muscarinic antagonist ipratropium bromide inhaled in an early phase of asthma on allergen-induced airway remodeling in BALB/c mice. The mice were sensitized and challenged with ovalbumin (OVA), and the airway was tested for M₃-AChR expression.

MATERIAL AND METHODS

Animal models

Thirty 8-week-old female BALB/c mice were procured for the study. The mice (18-22 g) were housed in a conventional room at The Second Hospital of Jilin University, where the research center provided tap water and standard rodent chow diet.

OVA-induced murine asthma model

Thirty female BALB/c mice were divided into three groups (10 per group): control group (group DZ), asthma group (group MX), and treatment group (group YB). The method was modified, based on published literature (Temelkovski et al., 1998; Shen and Wang, 2005; Huang and Qi, 2007).

Asthma group (MX)

Mice were injected intraperitoneally with 0.2 g/mL OVA solution on days 0, 7, and 14, and challenged by ultrasonic aerosol inhalation of 2.5 g/mL OVA (A5378, Sigma, USA; freshly prepared in saline) solution for 30 min daily from day 15 to 19. They were then challenged every other day from day 20 to 54 with 2.5 g/mL OVA solution to establish the murine model of asthma. As a positive control group, saline was given to the mice by ultrasonic atomization inhalation before challenging.

Treatment group (YB)

Mice were injected intraperitoneally with 0.2 g/mL OVA solution on days 0, 7, and 14, and challenged by ultrasonic aerosol inhalation of 2.5 g/mL OVA solution for 30 min daily from day 15 to 19. They were then challenged every other day from day 20 to 54 with 2.5 g/mL OVA solution. An appropriate dose of ipratropium bromide (Batch No. 709566, Boehringer Ingelheim, Germany) was given to YB mice 20 min before the challenge (100 µg/20 x 25 x 10 cm in volume) to determine whether ipratropium bromide inhibited airway remodeling.

Control group (DZ)

As a negative control group, mice were sensitized and exposed to saline in a similar manner as control. Animals were sacrificed on day 55 after the last challenge.

Histopathology

Lung tissues were fixed in 10% formalin, dehydrated in a graded alcohol series (20, 30, 50, 70, 95%), cleared with methyl benzoate, embedded in paraffin, and sectioned at 4-5 µm. Slides were stained with hematoxylin and eosin (H&E; Batch No. C-0021, Bioss, China) to detect airway remodeling. Airway remodeling was evaluated by H&E staining of small bronchial wall thickening.

For each slide that was observed under an optical microscope (400X magnification; OLYMPUS BX50, Japan), we selected three whole cross sections of small bronchi with an average diameter of 50-200 µm. We then measured and calculated the basement membrane perimeter (Pbm), airway wall area (WAt), and smooth muscle wall area (WAm) using an image analysis system (Xu et al., 2003; Image-pro plus 6.0, Media Cybernetics, USA).

Immunohistochemical staining

Slides were baked in an electric constant-temperature drying oven for 3 h at 80°C. After a series of procedures, including xylene de-waxing, gradient ethanol hydration, and tap water washing, slides were incubated in 3% hydrogen peroxide solution for 10 min at ambient temperature, and blocked in 10% animal serum for 1-2 h. These slides were incubated with rabbit anti-mouse M₃ acetylcholine receptor antibodies (Batch No. bs-1289R, Bioss, P.R. China) at 1:100 dilution, at 4°C overnight, washed three times with phosphate buffer saline, and then incubated with sheep anti-rabbit polyclonal antibodies (Batch No. KIT-9720, Maixin-Bio, P.R. China) at 1:1000 dilution, at 37°C, for 10 min. Color reactions were developed by incubating with diaminobenzidine and counterstaining with hematoxylin. Controls were obtained by replacing the primary antibodies with PBS.

For each slide that was observed (400X magnification) under the optical microscope, we selected three whole cross sections of small bronchi with an average diameter of 50-200 µm. We then evaluated M₃-AChR density in the bronchopulmonary tissue and measured the integral optical density (IOD) values by using an image analysis system (Image-pro plus 6.0, Media Cybernetics, USA).

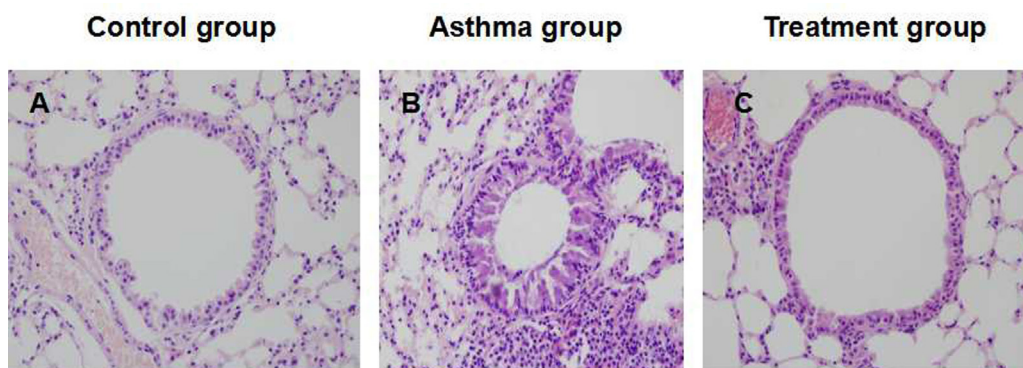
Statistical analysis

Data are reported as means \pm SD. Comparisons of different groups were performed using one-way ANOVA, and SNK *q* was used for comparisons between two groups, with SPSS 13.0 data analysis and graphing software. $P < 0.05$ was considered significant.

RESULTS

Histological changes in the lungs of asthmatic mice

Morbid lung tissues undergoing airway remodeling, from asthmatic mice, were histologically examined using H&E staining. The analysis revealed that the small bronchial airways were thickened due to hyperplasia and hypertrophy of smooth muscle cells. Normal lung tissues from the control group (DZ) served as controls (Figure 1A). Many obvious cellular infiltrates of inflammatory macrophages, neutrophils, and lymphocytes were seen in the interstitium, along with epithelium damage and mucus metaplasia (Figure 1B). Histological changes of lung tissues in the treatment group were considerably reduced (Figure 1C) compared with the asthma group.



(H&E staining; 400 \times)

Figure 1. Histopathological features of airway remodeling. Compared with the lungs of the control group (A), most sections of the small bronchial wall (smooth muscle, non-smooth muscle connective tissue, mucus glands, and bronchial epithelium) were increased in the lungs of the asthma group with airway remodeling (B) but not the treatment group (C) (H&E staining; 400X).

Assessment of airway remodeling in asthmatic mice

WAt/Pbm ratio

Compared with the control (12.89 ± 1.71) and treatment group (15.82 ± 2.91), WAt/Pbm ratio of the asthma group (25.37 ± 4.25) was significantly increased ($P < 0.05$). No significant differences were observed ($P > 0.05$) between the treatment group and the control group (Table 1).

Table 1. The values of WAt and WAm in small bronchi of mice.

Group	N	WAt/Pbm ($\mu\text{m}^2/\mu\text{m}$)	WAm/Pbm ($\mu\text{m}^2/\mu\text{m}$)
Control group	10	12.89 \pm 1.71	2.55 \pm 0.72
Asthma group	10	25.37 \pm 4.25*	7.58 \pm 2.16*
Treatment group	10	15.82 \pm 2.91 [†]	3.36 \pm 1.69 [†]

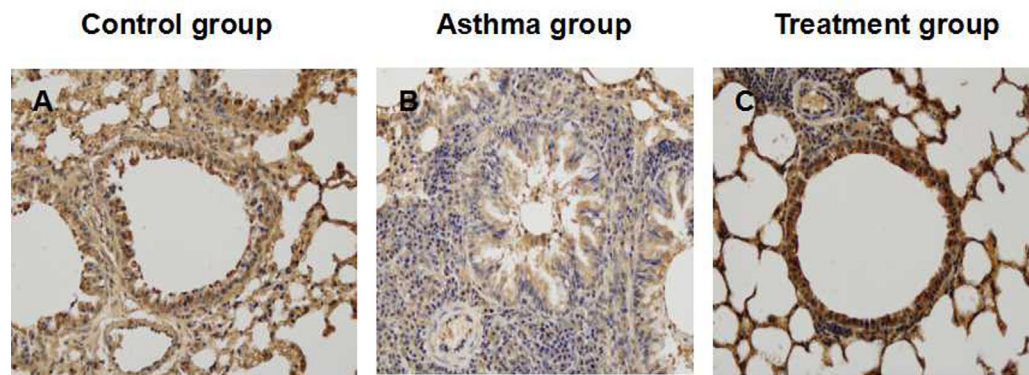
*P < 0.05 vs control group; [†]P < 0.05 vs asthma group.

WAm/Pbm ratio

WAm/Pbm ratio of the asthma group (7.58 \pm 2.16) significantly increased (P < 0.05) when compared with that of the control (2.55 \pm 0.72) and treatment group (3.36 \pm 1.69). No significant differences were apparent (P > 0.05) between the treatment group and the control group (Table 1).

M₃-AChR expression in small bronchi of mice

Lung tissues from the control (Figure 2A) and asthma group (Figure 2B) were histologically examined using immunohistochemical staining. Tissues stained positive for M₃-AChR in the bronchial epithelium, bronchial smooth muscle cells, submucosal glands, and goblet cells. M₃-AChR expression in the small bronchial wall was higher in the treatment group (Figure 2C) than in the negative control group (Figure 2A).



(Immunohistochemical staining for M₃-AChR; 400 \times magnification)

Figure 2. Assessment of M₃-AChR expression in small bronchi of mice. Immunohistochemical staining of M₃-AChR in the lung tissue of the control (A) and asthma group (B), with detectable positive staining in the bronchial epithelium, bronchial smooth muscle cells, submucosal glands, and goblet cells. M₃-AChR expression in the small bronchial wall was higher in the treatment group (C) than in the control group (A) (Immunohistochemical staining for M₃-AChR; 400X).

Quantitation of M₃-AChR expression in small bronchi of mice

No significant differences (P > 0.05) were noted for M₃-AChR IOD values in bronchopulmonary tissues between the asthma group (24.72 \pm 3.87) and the control group

(25.50 ± 1.83). However, M₃-AChR density increased in the treatment group (29.24 ± 3.59) and was significantly different (P < 0.05) from that in the control group (25.50 ± 1.83; Table 2).

Table 2. The IOD values of M₃-AChR in small bronchi of mice.

Group	N	IOD (x 10 ³)
Control group	10	25.50 ± 1.83
Asthma group	10	24.72 ± 3.87 [†]
Treatment group	10	29.24 ± 3.59*

*P < 0.05; [†]P > 0.05 vs control group.

DISCUSSION

Our study found no obvious differences in M₃-AChR levels in the small bronchial walls of asthmatic mice and the negative control group mice, which was consistent with the literature. We also found that ipratropium bromide could prevent airway remodeling in asthmatic mice. A large number of clinical and laboratory data on asthma have reported that M₂-AChR dysfunction might have contributed to increased acetylcholine release from parasympathetic nerve endings in the airways (Mirotti et al., 2010). The enhanced release of ACh due to M₂-AChR dysfunction led to increased mucus secretion and contraction of airway smooth muscles via M₃-AChR present in glandular epithelia and airway smooth muscle cells (Rogers, 2001), leading to induced persistent airway hyperresponsiveness. Ipratropium bromide blocks muscarinic acetylcholine receptors irrespective of their subtypes, resulting in decreased contractility of smooth muscle in the lungs, inhibited mucus secretion and bronchoconstriction, ultimately preventing airway remodeling. Brown found that cholinergic antagonism of muscarinic receptors in human and rat small airways inhibits airway contraction by direct inhibition of contraction through M₃-AChR, and by M₂-AChR-mediated inhibition of relaxation (Brown et al., 2013). Consequently, M₃-AChR plays an important role in the development of asthmatic airway remodeling. Down-regulation of M₃-AChR and up-regulation of M₂-AChR in the airway should prevent the development of airway remodeling in patients with asthma.

Currently, the most common types of acetylcholine receptor antagonists are ipratropium bromide and tiotropium bromide. Ipratropium bromide is a quaternary ammonium derivative of atropine that has been found to exert a remarkable bronchodilating effect on asthmatics and chronic bronchitis. It blocks AChRs irrespective of the subtype, and accelerates degradation of cyclic guanosine monophosphate (cGMP), leading to a decrease of intracellular cGMP levels. This is most probably caused by the effect of cGMP on intracellular calcium, resulting in decreased contractility of smooth muscle in the lungs, suppressing mucus secretion and bronchoconstriction. Similarly to ipratropium, tiotropium bromide is a synthetic quaternary anticholinergic drug, rendering it appropriate for once-daily therapy. The pharmacology and clinical safety of tiotropium have been recently reviewed (Gross, 2004).

Recently, other studies have indicated that long-term use of acetylcholine receptor antagonists affects the number and function of M-AChRs. In 2002, Billington reported that M₃-AChR levels were increased in the bronchial airways of patients who inhaled ipratropium bromide as a long-term treatment, which increased bronchial hyperresponsiveness (Billington and Penn, 2002). Similarly, we observed that small bronchial wall M₃-AChR expression was higher in mice treated with ipratropium bromide than in the negative control group. Kang et al. (2013) found that

the expression of M₂-AChR and M₃-AChR with age tended to decrease and increase, respectively. Therefore, it is necessary to regulate the dose of acetylcholine receptor antagonists used taking into consideration the number and function of M-AChRs in the lung tissue.

Our data demonstrate that acetylcholine contributes to allergen-induced smooth muscle mass and airway remodeling via M₃-AChR, and that the level of M₃-AChR expression in a murine model of chronic asthma can be used to predict bronchial airway remodeling. In conclusion, it is necessary to characterize the expression, functional state, and ratio of the three subtypes of muscarinic receptors in the lung tissue of healthy individuals and asthmatic patients, which would be beneficial for a more informed and effective therapy of respiratory disorders.

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REFERENCES

- Bergeron C, Tulic MK and Hamid Q (2010). Airway remodelling in asthma: from benchside to clinical practice. *Can. Respir. J.* 17: e85-e93. <http://dx.doi.org/10.1155/2010/318029>
- Billington CK and Penn RB (2002). m3 muscarinic acetylcholine receptor regulation in the airway. *Am. J. Respir. Cell Mol. Biol.* 26: 269-272. <http://dx.doi.org/10.1165/ajrcmb.26.3.f232>
- Brown SM, Koarai A, Sturton RG, Nicholson AG, et al. (2013). A role for M(2) and M(3) muscarinic receptors in the contraction of rat and human small airways. *Eur. J. Pharmacol.* 702: 109-115. <http://dx.doi.org/10.1016/j.ejphar.2013.01.054>
- Caulfield MP and Birdsall NJ (1998). International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.* 50: 279-290.
- Chang Q (1995). Characterization of M-cholinergic receptors by autoradiography in guinea pig lung tissue and their changes in asthmatic onset. *Chin. J. Pathophysiol.* 11: 11-15.
- Elias JA, Zhu Z, Chupp G and Homer RJ (1999). Airway remodeling in asthma. *J. Clin. Invest.* 104: 1001-1006. <http://dx.doi.org/10.1172/JCI8124>
- Eglen RM (2006). Muscarinic receptor subtypes in neuronal and non-neuronal cholinergic function. *Auton. Autacoid Pharmacol.* 26: 219-233. <http://dx.doi.org/10.1111/j.1474-8673.2006.00368.x>
- Fish JE and Peters SP (1999). Airway remodeling and persistent airway obstruction in asthma. *J. Allergy Clin. Immunol.* 104: 509-516. [http://dx.doi.org/10.1016/S0091-6749\(99\)70315-5](http://dx.doi.org/10.1016/S0091-6749(99)70315-5)
- Gross NJ (2004). Tiotropium bromide. *Chest* 126: 1946-1953. <http://dx.doi.org/10.1378/chest.126.6.1946>
- Huang MY and Qi HW (2007). Effect of fluticasone propionate on airway remodeling and TGFβ₁ expression in murine models of asthma. *J. Fourth Mil. Med. Univ.* 28: 1088-1090.
- Kang JY, Lee SY, Rhee CK, Kim SJ, et al. (2013). Effect of aging on airway remodeling and muscarinic receptors in a murine acute asthma model. *Clin. Interv. Aging* 8: 1393-1403. <http://dx.doi.org/10.2147/CIA.S50496>
- Kistemaker LE, van Os RP, Dethmers-Ausema A, Bos IS, et al. (2015). Muscarinic M3 receptors on structural cells regulate cigarette smoke-induced neutrophilic airway inflammation in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 308: L96-L103. <http://dx.doi.org/10.1152/ajplung.00259.2014>
- Mirotti L, Castro J, Costa-Pinto FA and Russo M (2010). Neural pathways in allergic inflammation. *J. Allergy* 2010: 1-11. <http://dx.doi.org/10.1155/2010/491928>
- Rogers DF (2001). Motor control of airway goblet cells and glands. *Respir. Physiol.* 125: 129-144. [http://dx.doi.org/10.1016/S0034-5687\(00\)00209-7](http://dx.doi.org/10.1016/S0034-5687(00)00209-7)
- Shen HH and Wang SB (2005). Effects of budesonide on airway inflammation and airway remodeling in the ovalbumin sensitized and challenged mice. *Zhonghua Jie He He Hu Xi Za Zhi* 28: 154-159.
- Shen N, Yao W and Zhu H (2001). Study on the muscarinic receptor and its subtypes in patients with chronic obstructive pulmonary disease. *Zhonghua Jie He He Hu Xi Za Zhi* 24: 212-214.

- Temelkovski J, Hogan SP, Shepherd DP, Foster PS, et al. (1998). An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. *Thorax* 53: 849-856. <http://dx.doi.org/10.1136/thx.53.10.849>
- Thal DM, Sun B, Feng D, Nawaratne V, et al. (2016). Crystal structures of the M1 and M4 muscarinic acetylcholine receptors. *Nature* 531: 335-340. <http://dx.doi.org/10.1038/nature17188>
- Xu SY, Xu YJ, Zhang ZX, Ni W, et al. (2003). A study of protein kinase C signal pathway in regulating airway smooth muscle cell proliferation in asthmatic rats. *Zhonghua Jie He He Hu Xi Za Zhi* 26: 756-760.
- Zhang SJ, Jiang JX, Ren QQ, Xie QM, et al. (2015). Effects of the inhalation of the m3 receptor antagonist bencycloquidium bromide in a mouse cigarette smoke-induced airway inflammation model. *Drug Dev. Res.* 76: 123-131. <http://dx.doi.org/10.1002/ddr.21248>