



Effect of tiotropium bromide on airway remodeling in a chronic asthma model

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ABSTRACT

Background: Recent evidence suggests that acetylcholine acting through muscarinic receptors may play an inhibitory role in the mechanisms that drive the structural changes in the airways called airway remodeling. The novel anticholinergic drug tiotropium bromide, which selectively antagonizes muscarinic receptors, especially the M3 subtype, and is long acting, could be beneficial in attenuating airway remodeling in chronic asthma.

Objective: To investigate the effect of tiotropium bromide on parameters of airway remodeling, including smooth muscle hypertrophy and peribronchial thickening, in a mouse model of chronic asthma.

Methods: To develop the murine models of acute and chronic asthma, BALB/c mice were sensitized and challenged to ovalbumin for 1 and 3 months, respectively. The effect of tiotropium bromide (0.1mM in 50 μ L of phosphate-buffered saline) on pulmonary inflammation and remodeling was evaluated. The expression of muscarinic receptors M2 and M3 was analyzed.

Results: In the chronic asthma model, the tiotropium-treated group significantly decreased smooth muscle thickening and peribronchial collagen deposition. As for pulmonary inflammation, the chronic asthma model had a reduction of inflammatory cells and T_H2 cytokines by tiotropium bromide, but the effects in the asthma acute model were reversed. In the chronic asthma model, expression of the M3 receptor was inhibited and that of the M2 receptor was elevated by the administration of tiotropium bromide.

Conclusion: This study suggests that tiotropium bromide might have an inhibitory effect on airway remodeling in a murine model of chronic asthma. Differential effects on muscarinic receptor subtypes may explain why tiotropium bromide has different effects on acute and chronic asthma.

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Introduction

Acetylcholine is the primary parasympathetic neurotransmitter in the airways, and its role in the respiratory system is related to airway smooth muscle contraction and mucous secretions.¹ Acetylcholine is also produced by nonneuronal cells, mainly airway inflammatory cells, and modulates their behavior. This is termed the *nonneuronal cholinergic system*.² The receptors for acetylcholine are classified into muscarinic and nicotinic receptors, and the respiratory effects are mediated by muscarinic receptors. Muscarinic receptors exist as subtypes 1 through 5, and the M3 subtype is known to be the most important effector in obstructive airway diseases, such as asthma and chronic obstructive pulmonary disease (COPD),³ because expression of this subtype occurs mostly in

smooth muscle cells and submucosal glands. It is thought that parasympathetic activity is increased in these chronic airway diseases,¹ and, particularly in COPD, exaggerated vagal tone appears to be the only reversible component of airflow limitation.⁴ In asthma, cholinergic activity is thought to be increased by (1) altered afferent stimulation by inflammation,⁵ (2) aberrant muscarinic receptor expression, (3) increased release of acetylcholine from cholinergic nerve endings, and (4) decreased levels of neuromodulators that attenuate cholinergic neurotransmission.^{5–9}

Tiotropium bromide is a long-acting anticholinergic that has characteristics distinct from other anticholinergics, including its relative functional selectivity and higher affinity for particular muscarinic receptor subtypes,³ which results in slow dissociation from the M3 receptor and fast dissociation from the M2 autoinhibitory receptor. Clinical studies in COPD show that tiotropium bromide reduces rates of acute exacerbation, improves symptoms, and slows the progressive decline in pulmonary function.^{10,11} The last observation implies that the cholinergic system is associated with long-term structural changes in COPD. However, in asthma, the role of anticholinergics, especially tiotropium bromide, on airway remodeling remains to be elucidated.

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The objective of the present study was to investigate whether tiotropium bromide has an effect not only on pulmonary inflammation but also on airway remodeling in a murine model of chronic asthma. To further explore the underlying mechanisms, we compared the effect of tiotropium bromide in mouse models of acute and chronic asthma and assessed the expression of M2 and M3 subtype of muscarinic receptors in both models.

Materials and Methods

Sensitization and antigen challenge protocol

Female BALB/c mice (The Dae-Han Experimental Animal Center, Daejeon, Korea), 8 to 10 weeks of age, were used in the experiments and divided into 3 groups ($n = 8$ per group): control, ovalbumin, and ovalbumin with tiotropium bromide (Boehringer Ingelheim, Ingelheim, Germany) treatment. We processed the experiments in 2 steps to give the models of acute and chronic asthma. In the acute asthma model, the mice were immunized on days 0 and 7 by subcutaneous injection of 25 μg of ovalbumin (grade V; Sigma-Aldrich, St Louis, Missouri), which was adsorbed to 1 mg of aluminum hydroxide (Aldrich, Milwaukee, Wisconsin), in 200 μL of normal saline. Intranasal ovalbumin challenges (20 ng/50 μL in phosphate-buffered saline [PBS]) were administered on days 21, 23, 25, and 27 with the mice under isoflurane (Vedco, St. Joseph, Missouri) anesthesia. In the chronic asthma model, the mice were immunized once a week for 1 month, and then intranasal OVA challenges were performed starting on day 31 and repeated twice a week for 3 months, based on a previously reported protocol of inducing chronic asthma.^{12–14} Mice were killed 24 hours after the final ovalbumin challenge. Bronchoalveolar lavage (BAL) fluid and lung tissues were analyzed. All animal experimental protocols were approved by the Animal Subjects Committees of the Catholic University of Korea.

Administration of tiotropium bromide

Tiotropium bromide was generously donated by Boehringer Ingelheim Co Ltd. In the acute asthma model, on days 21 to 27, 0.1mM tiotropium bromide in 50 μL of PBS was administered via the intranasal route 0.5 hour before the ovalbumin challenge with saline or ovalbumin. In the chronic asthma model, from day 38, tiotropium bromide was administered twice a week for 3 months by the same manner used in the acute asthma model. Control and ovalbumin mice were treated in the same way with PBS.

Bronchoalveolar lavage

Mice were subjected to thoracotomy under anesthesia induced by intraperitoneal injection of ketamine and lidocaine. The trachea was exposed and cannulated with silicone tubing attached to a 23-gauge needle on an 800- μL tuberculin syringe. The left main bronchus was tied, and after instillation of 1.2 mL of sterile PBS through the trachea into the right lung, the BAL fluid was withdrawn. Recovered BAL fluids were cytospun (7 minutes at 353 g at 4°C), and the supernatants were stored at -70°C . The total cell counts in the BAL fluids were counted using a hemocytometer. After adjusting the cell number to $1 \times 10^6/\text{mL}$, 50- μL aliquots of BAL fluid were centrifuged for 5 minutes at 43 g onto microscope slides and stained with Diff-Quick (Sysmax, Kobe, Japan). The percentages of macrophages, eosinophils, lymphocytes, and neutrophils in the BAL fluids were obtained by counting 500 leukocytes in randomly selected fields of the slide under a light microscope.

Enzyme-linked immunosorbent assay

The levels of interleukin (IL) 4, IL-5, and IL-13 in the supernatants of BAL fluids were measured by enzyme-linked immunosor-

bent assay (R&D Systems, Minneapolis, Minnesota, and BioSource International, Camarillo, California). The samples were assayed according to the manufacturers' recommendations.

Immunohistochemistry

Sections (4 μm thick) cut from lung tissues embedded in paraffin blocks were deparaffinized with xylene and hydrated in ethanol. The presence of α -smooth muscle actin and collagens I and III was confirmed by immunohistochemistry. For immunohistochemical detection of α -smooth muscle actin, the lung sections were incubated overnight at 4°C with either a primary monoclonal antibody directed against α -smooth muscle actin (Sigma-Aldrich) or normal mouse serum as a negative control. Immunoreactivity was detected by sequential incubations of the lung sections with a biotinylated secondary antibody followed by incubation with peroxidase reagent and 3-amino-9-ethylcarbazole chromogen. Similar methods were used for the immunohistochemical detection of collagens I and III. The primary monoclonal antibodies against mouse collagens I and III were obtained from Sigma-Aldrich.

Assay for hydroxyproline

Measurements of total collagen content in the peripheral portions of the lung were based on estimations of hydroxyproline content. A saline solution (1.5 mL) was added to approximately 30 mg of tissue sample collected from the peripheral part of the left lower lobe, then the sample was homogenized on ice and the hydroxyproline content was measured by the spectrophotometrical method.

Measurement of smooth muscle area

The immunostained area corresponding to α -smooth muscle actin in each paraffin-embedded lung was outlined and quantified using a light microscope attached to an image analysis system (BX50; Olympus, Tokyo, Japan). The results are expressed as the area of immunostaining per micrometer length of basement membrane of the bronchioles (internal diameter, 150–200 μm). At least 10 bronchioles were counted in each slide.

Western blot of M2 and M3 muscarinic receptors

Protein samples from lung tissue were quantified using the Bradford assay with bovine serum albumin as standard and electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide gels. The samples were transferred to nitrocellulose membrane through a transfer system (X cell II Module). Membranes were blocked with 5% milk in Tris-buffered saline containing 0.05% Tween 20 for 1 hour at 25°C and then incubated with the primary antibodies, polyclonal rabbit anti-mAChR M2 and mAChR M3 (Santa Cruz Biotechnology, Santa Cruz, California), in 5% milk in Tris-buffered saline containing 0.05% Tween 20 buffer overnight at 4°C, followed by treatment with secondary anti-rabbit antibodies for 1 hour at 25°C. The proteins were detected by the enhanced chemiluminescence system by exposure to x-ray film (PRO-MG; DaeSung Company, Seoul, Korea).

Statistical analysis

The results for each group were compared by the nonparametric Kruskal-Wallis test, followed by the post hoc Dunn multiple comparison. All results are presented as mean $\pm$ standard error of the mean. $P < .05$ was considered statistically significant.

Results

Effects of tiotropium bromide on airway inflammation and cytokine levels in BAL fluids in acute and chronic asthma models

To investigate the airway inflammation, we analyzed cell counts and concentrations of $\text{T}_\text{H}2$ cytokines in the BAL fluids. In

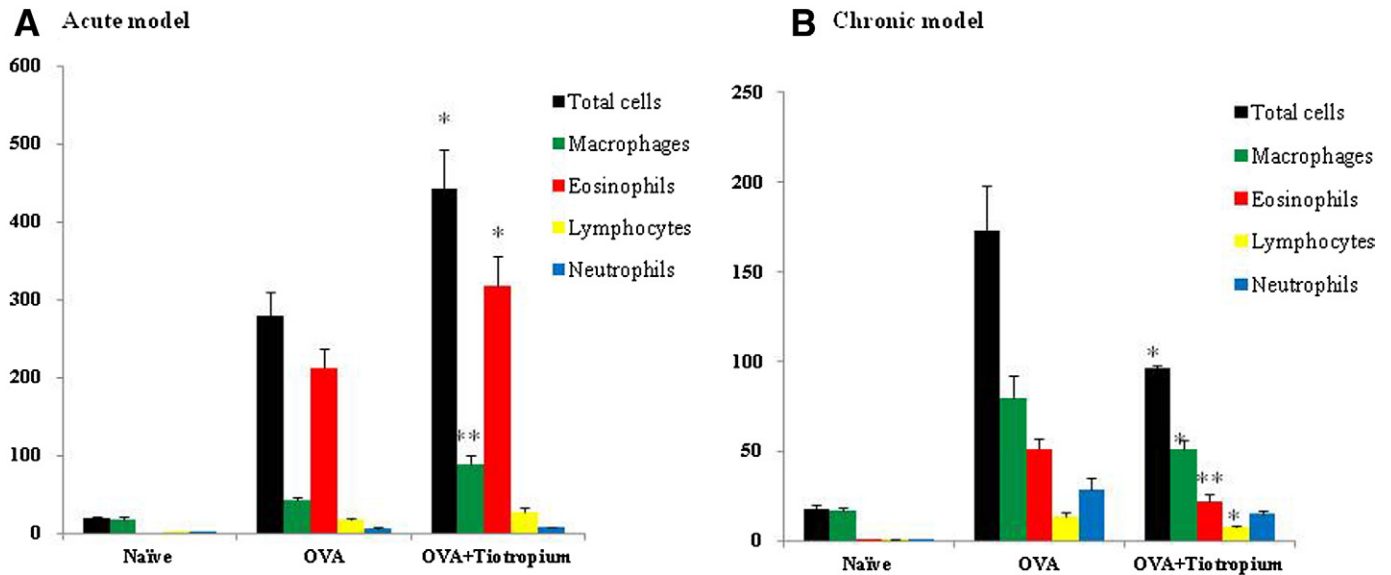


Fig. 1. Effects of tiotropium bromide on total and differential cell counts in bronchoalveolar lavage fluids in acute (A) and chronic (B) models of asthma. Values are given as mean $\pm$ SEM (n = 8 per group). * $P < .05$ and ** $P < .01$ compared with the ovalbumin group.

the acute model of asthma, the numbers of total cells, macrophages, and eosinophils in the BAL fluids were significantly increased in the tiotropium treatment group compared with the ovalbumin group (Fig 1A). In contrast, in the chronic asthma model, the tiotropium group had significantly lower counts in total cells, macrophages, eosinophils, and lymphocytes compared with the ovalbumin group (Fig 1B). For the cytokine profiles, in the acute

asthma model, the tiotropium group showed increased levels of IL-4, IL-5, and IL-13 compared with the ovalbumin group (Fig 2A). In the chronic asthma model, the concentration of IL-5 in BAL fluids was markedly lower after the administration of tiotropium bromide, whereas the concentrations of IL-4 and IL-13 showed a trend toward a decrease in the tiotropium group compared with the ovalbumin group (Fig 2B).

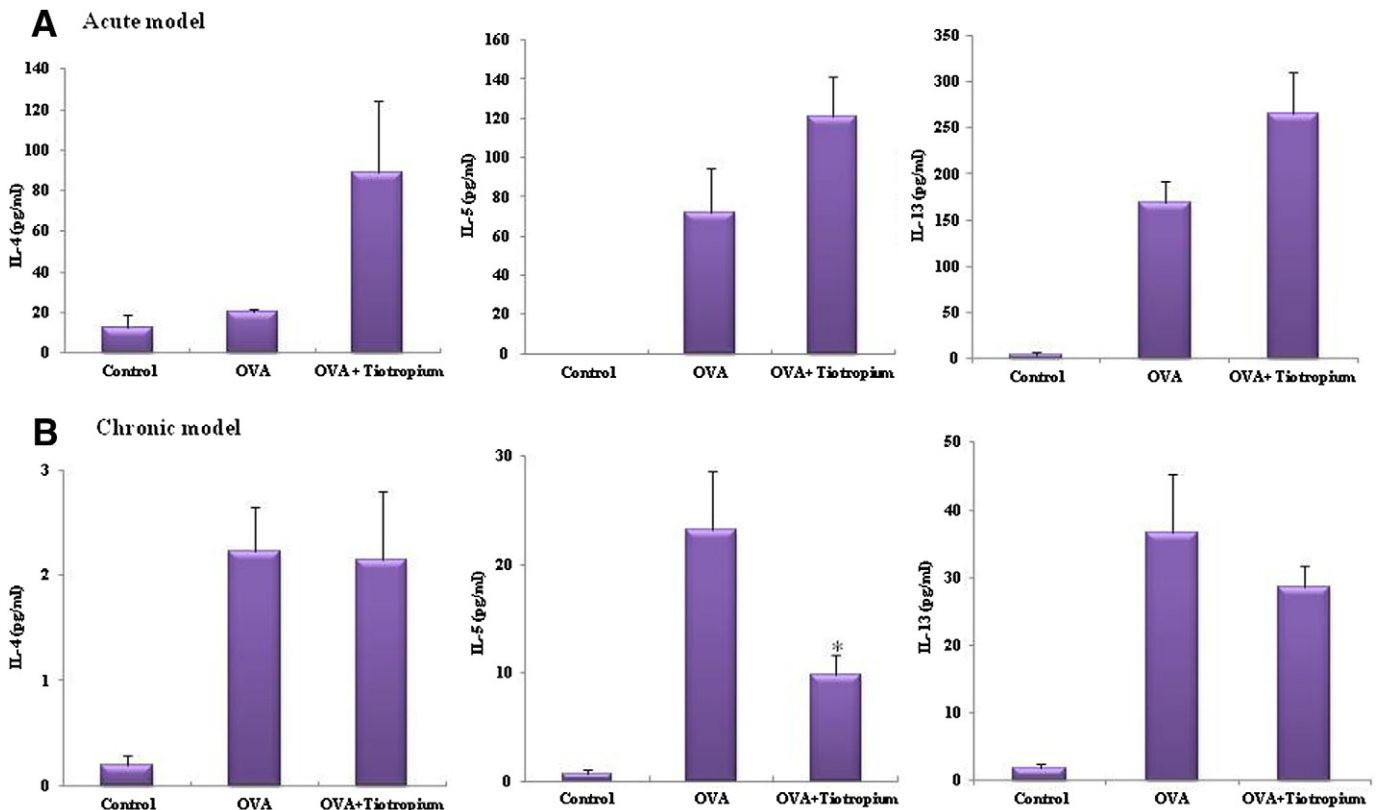


Fig. 2. Effects of tiotropium bromide on the levels of Th2 cytokines (interleukins 4, 5, and 13) in bronchoalveolar lavage fluid in acute (A) and chronic (B) models of asthma. Values are given as mean $\pm$ SEM (n = 8 per group). * $P < .05$ compared with the ovalbumin group.

Effects of tiotropium bromide on airway remodeling in the chronic asthma model

We analyzed peribronchial fibrosis and smooth muscle hypertrophy, which are important structural changes involved in airway remodeling in asthma, in the lung tissues. Peribronchial fibrosis was verified by immunohistochemical staining for collagens I and III and quantified by hydroxyproline assay (Fig 3). The mice in the tiotropium group had a significantly smaller area of collagens I and III expression than those in the ovalbumin group. The hydroxyproline content yielded a trend toward a decrease in the tiotropium group compared with the ovalbumin group. For smooth muscle hyperplasia, the area immunostained for peribronchial α -smooth muscle actin in mice repeatedly challenged with ovalbumin during 3 months was significantly greater than in control mice. The administration of tiotropium bromide showed a significantly reduced area of α -smooth muscle actin immunostaining compared with the corresponding area in the ovalbumin group (Fig 4).

Effect of tiotropium bromide on expression of muscarinic receptors in the acute and chronic asthma models

In the acute model of asthma, the M3 subtype of muscarinic receptors was more highly expressed in the ovalbumin group than in the control group, and the tiotropium group had a level similar to the ovalbumin group. However, in the chronic asthma model, although the ovalbumin group had a higher expression of M3 receptor than the control group, the treatment with tiotropium bromide significantly reduced the expression of the M3 receptor (Fig 5A). As for the M2 receptor, the ovalbumin group had a lower expression than the control group in the acute and chronic models of asthma, and the change was seen remarkably in the chronic asthma model. In addition, the administration of tiotropium bromide for 3 months demonstrated a significantly higher expression of the M2 receptor than in the ovalbumin group (Fig 5B).

Discussion

The current study demonstrates that tiotropium bromide treatment for 3 months in a mouse model of chronic asthma has a protective effect against parameters of airway remodeling, including peribronchial fibrosis and smooth muscle thickening, by differential regulation of expression of the M2 and M3 muscarinic receptors. Regarding airway inflammation, tiotropium bromide has different effects in acute and chronic asthma, suggesting that tiotropium bromide might not have a beneficial role in acute asthma.

Asthma is a chronic inflammatory disease characterized by reversible bronchoconstriction, hyperresponsiveness, and airway remodeling. These structural changes in the airways have characteristic histologic features, including epithelial denudation, goblet cell metaplasia, subepithelial fibrosis, smooth muscle hypertrophy and hyperplasia, increased angiogenesis, and alterations to extracellular matrix components.¹⁵ From a clinical viewpoint, airway remodeling has important implications because the changes in asthma are considered to be linked to persistent increases in airway responsiveness, variable or fixed airflow limitation, and possibly declines in pulmonary function.¹⁶ In this study, administration of tiotropium bromide significantly reduced peribronchial fibrosis, as assessed by measuring the hydroxyproline content of lung tissue and immunohistochemical staining for collagen I and III deposits. In addition, in the chronic asthma model, the tiotropium bromide-treated mice had decreased thickness of the smooth muscle layer compared with the ovalbumin-challenged mice. These findings verify antiremodeling effects in chronic asthma of this long-acting muscarinic receptor antagonist, consistent with the results of several animal studies.^{17–19} Some clinical trials^{20,21} have demonstrated that adding tiotropium bromide to conventional treatment is helpful for severe or uncontrolled asthma, an effect that could be related to airway remodeling. Bos et al¹⁸ investigated the effect of tiotropium on airway remodeling and compared it with budesonide, which is a standard medicine in asthma management. They showed

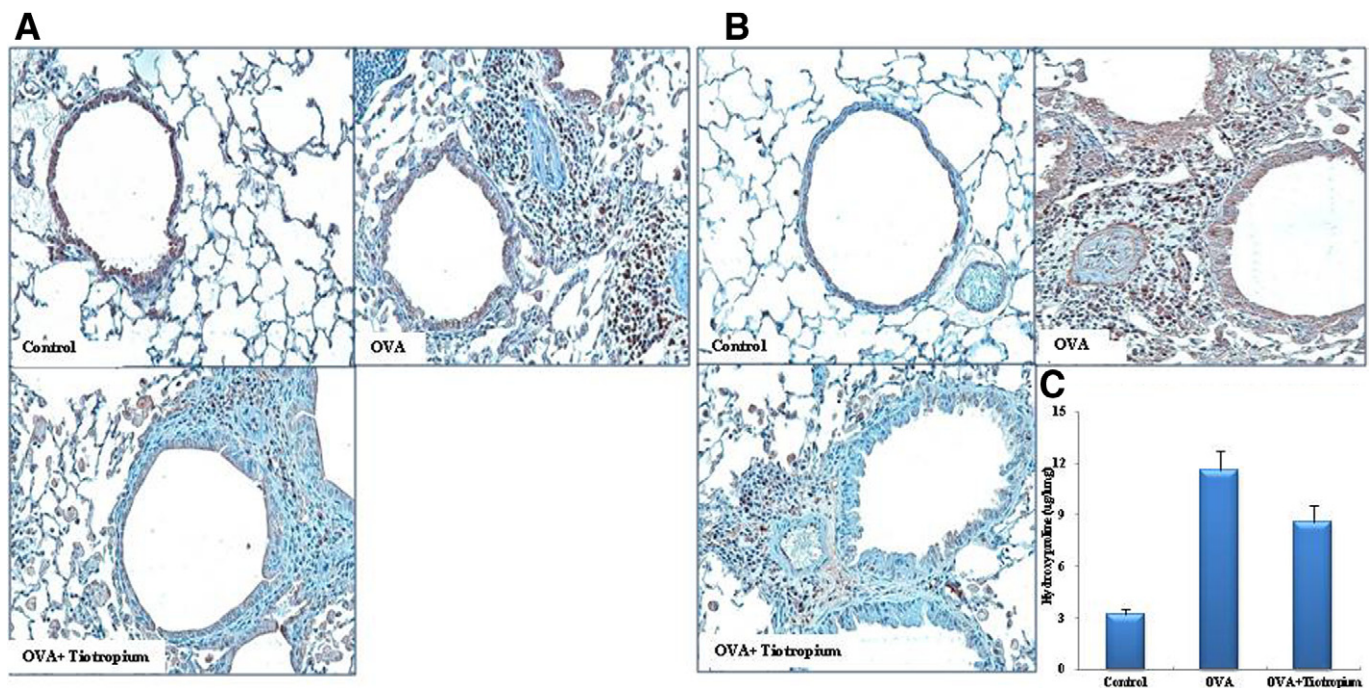


Fig. 3. Effect of tiotropium bromide on collagen expression in the lung in the chronic asthma model. Representative photomicrographs of lung sections stained for collagen I (A) and III (B) (original magnification $\times 200$) and hydroxyproline levels in the lung tissues (C). Values are given as mean $\pm$ SEM ($n = 8$ per group).

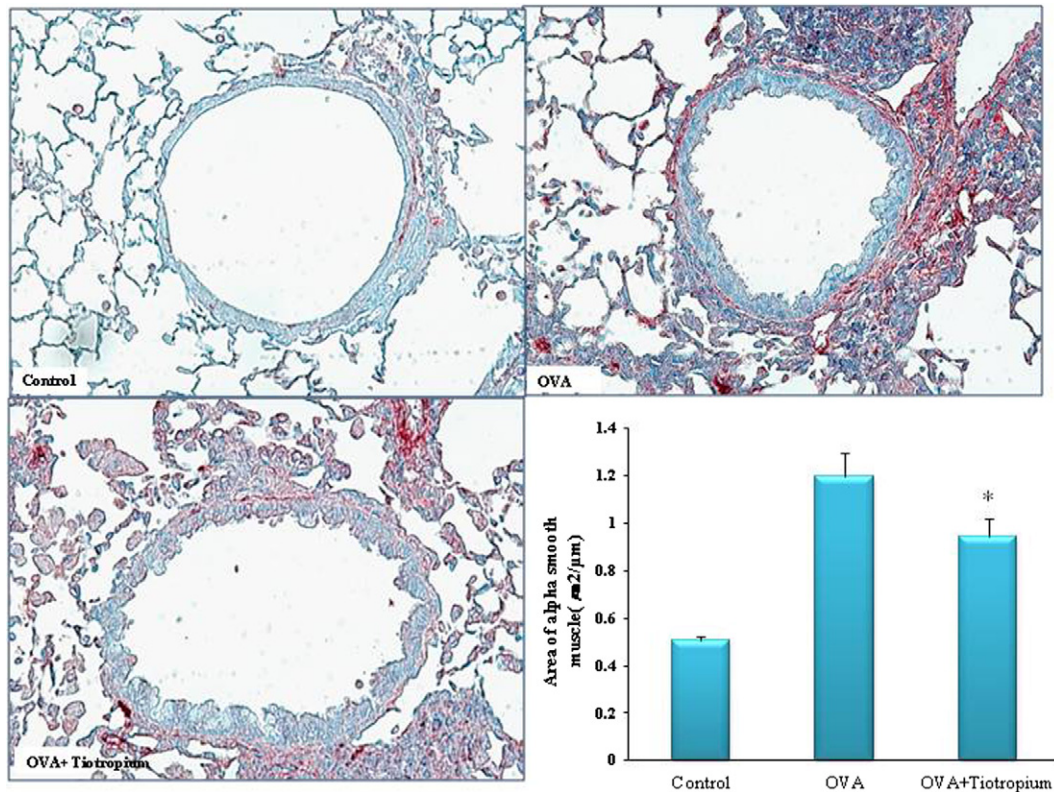


Fig. 4. Effect of tiotropium bromide on airway smooth muscle area in the chronic asthma model. Representative photomicrographs of smooth muscle area-stained lung sections (original magnification $\times 200$) and smooth muscle areas of immunostaining per micrometer length of basement membrane of the bronchioles, as assessed using an image analyzer. Values are given as mean $\pm$ SEM ($n = 8$ per group). * $P < .05$ compared with the ovalbumin group.

similar effects between the 2 drugs on inhibiting several aspects of airway remodeling.

In the present study, we observed that in the chronic asthma model, tiotropium bromide treatment reduced inflammatory cells and T_H2 cytokines. However, in an acute asthma model, the administration of tiotropium resulted in significantly higher levels of pulmonary inflammatory cells and T_H2 cytokines compared with the ovalbumin group. This result is in contrast to that of Ohta et al,¹⁹ who found that in a mouse model of asthma, tiotropium bromide inhibited T_H2 cytokine production and airway inflammation in the acute and chronic asthma models. This contradictory finding might be associated with different protocols used in the studies. In the current study, 2 different schedules were used to make acute and chronic asthma model, unlike with that in the study by Ohta et al. The rationale we applied to the different protocol is to make more representative and effective models of asthma. Indeed, using the 2 protocols induced the characteristic features of chronic human asthma in a chronic model and the verification of the effect of tiotropium bromide in an acute asthma model. However, we did not conduct an underlying mechanism of an aggravation of inflammatory cells and cytokines in an acute asthma model. Therefore, the definite function of acetylcholine in pulmonary inflammation in asthma remains to be resolved,² and further investigation is needed in the future. However, it seems clear that acetylcholine may have unexpected effects on inflammation in asthma.

Some *in vitro* studies^{22–24} demonstrated that M2 and M3 muscarinic receptors mediate mitogenesis of fibroblasts or myofibroblasts and airway smooth muscle cells, respectively, both of which are essential cell types for the induction of structural changes in the asthmatic airway. Thus, it might be expected that the long-acting muscarinic antagonist tiotropium bromide modulates airway remodeling via antiproliferative effects. To explore the underlying

mechanism of the antiremodeling effect of tiotropium bromide in chronic asthma, we measured for the first time, to our knowledge, in an animal study the expression of the pivotal M2 and M3 muscarinic receptors in the lung tissue. Intriguingly, whereas in acute asthma the expression of both receptors was similar in the ovalbumin group and the tiotropium group, in chronic asthma, administration of tiotropium bromide decreased the expression of the M3 receptor and increased that of the M2 receptor compared with the ovalbumin group. This finding suggests that tiotropium bromide not only binds to the muscarinic receptors but also modulates their expression. We speculate that this different regulation of muscarinic receptors might induce an improvement in airway remodeling in chronic asthma. In addition, the difference in the expression of the receptors, depending on the phase of asthma, suggests that an extended duration of treatment with tiotropium bromide is required for a beneficial effect.

Recently, Graingae et al²⁵ reported in a study of 48 asthmatic patients that bronchoconstriction alone, in the absence of inflammation, could induce airway remodeling. They showed that subepithelial collagen deposition and mucous gland hyperplasia were increased to a similar degree in the allergen- and methacholine-treated groups compared with the control group. However, aggravated eosinophilic infiltration was observed only in the allergen group. In clinical studies of severe asthma,^{5,20,21} treatment with tiotropium bromide produced an improvement of lung function. The current study also revealed that enhanced pause (Penh) value in response to methacholine was significantly lower in the tiotropium group compared with the ovalbumin group in the acute and chronic models of asthma (data not shown). In this context, the antiremodeling effect of tiotropium bromide in chronic asthma might be related to continuous inhibition of bronchoconstriction

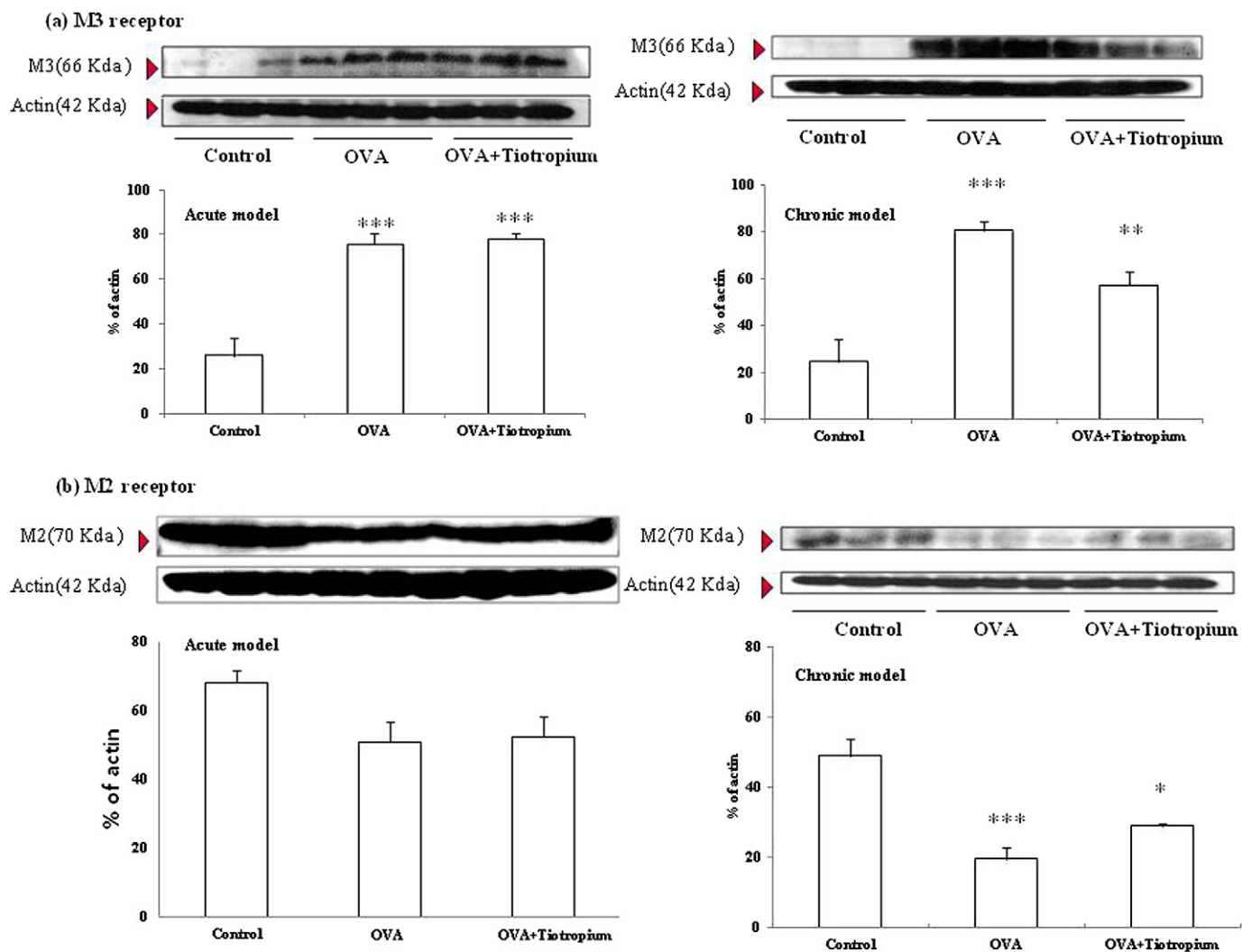


Fig. 5. Effect of tiotropium bromide on the expression of M3 (A) and M2 (B) muscarinic receptors in the lungs in acute and chronic models of asthma. Values are given as mean $\pm$ SEM (n = 8 per group). * $P < .05$ compared with the ovalbumin group, ** $P < .01$ compared with the ovalbumin group, and *** $P < .05$ compared with the control group.

because in the acute asthma model tiotropium treatment did not attenuate pulmonary inflammation.

In conclusion, this study provides evidence that tiotropium bromide, a long-acting muscarinic receptor antagonist, ameliorates airway remodeling in chronic asthma. Considering that it is frequently difficult to control asthma if structural alterations in the airway occur, this result suggests that tiotropium bromide could be an additional effective option for treatment not only to relieve bronchoconstriction in the present but also to prevent airway remodeling in the future.

Acknowledgments

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References

- [1] Gosens R, Zaagsma J, Meurs H, Halayko AJ. Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respir Res.* 2006;7:73.
- [2] Gwilt CR, Donnelly LE, Rogers DF. The non-neuronal cholinergic system in the airways: an unappreciated regulatory role in pulmonary inflammation? *Pharmacol Ther.* 2007;115:208–222.
- [3] Restrepo RD. Use of inhaled anticholinergic agents in obstructive airway disease. *Respir Care.* 2007;52:833–851.
- [4] Gross NJ, Skorodin MS. Role of the parasympathetic system in airway obstruction due to emphysema. *N Engl J Med.* 1984;311:421–425.
- [5] Park HW, Yang MS, Park CS, et al. Additive role of tiotropium in severe asthmatics and Arg16Gly in ADRB2 as a potential marker to predict response. *Allergy.* 2009;64:778–783.
- [6] Molino NA, Slutsky AS, Julià-Serdà G, et al. Assessment of airway tone in asthma: comparison between double lung transplant patients and healthy subjects. *Am Rev Respir Dis.* 1993;148:1238–1243.
- [7] Ayala LE, Ahmed T. Is there loss of protective muscarinic receptor mechanism in asthma? *Chest.* 1989;96:1285–1291.
- [8] Barnes PJ. Modulation of neurotransmission in airways. *Physiol Rev.* 1992;72:699–729.
- [9] Kanazawa H, Kawaguchi T, Shoji S, et al. Synergistic effect of nitric oxide and vasoactive intestinal peptide on bronchoprotection against histamine in anesthetized guinea pigs. *Am J Respir Crit Care Med.* 1997;155:747–750.
- [10] Vincken W, van Noord JA, Greefhorst AP, et al. Improved health outcomes in patients with COPD during 1 yr's treatment with tiotropium. *Eur Respir J.* 2002;19:209–216.
- [11] Anzueto A, Tashkin D, Menjoge S, Kesten S. One-year analysis of longitudinal changes in spirometry in patients with COPD receiving tiotropium. *Pulm Pharmacol Ther.* 2005;18:75–81.
- [12] Cho JY, Miller M, Baek KJ, et al. Immunostimulatory DNA inhibits transforming growth factor-beta expression and airway remodeling. *Am J Respir Cell Mol Biol.* 2004;30:651–661.
- [13] Cho JY, Miller M, Baek KJ, et al. Inhibition of airway remodeling in IL-5-deficient mice. *J Clin Invest.* 2004;113:551–560.
- [14] Rhee CK, Lee SY, Kang JY. Effect of peroxisome proliferator-activated receptor-gamma on airway smooth muscle thickening in a murine model of chronic asthma. *Int Arch Allergy Immunol.* 2009;148:289–296.
- [15] Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma: from bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med.* 2000;161:1720–1745.

- [16] Boulet LP, Sterk PJ. Airway remodeling: the future. *Eur Respir J*. 2007; 30:831–834.
- [17] Gosens R, Bos IS, Zaagsma J, Meurs H. Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling. *Am J Respir Crit Care Med*. 2005;171:1096–1102.
- [18] Bos IS, Gosens R, Zuidhof AB, et al. Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison. *Eur Respir J*. 2007; 30:653–661.
- [19] Ohta S, Oda N, Yokoe T, et al. Effect of tiotropium bromide on airway inflammation and remodelling in a mouse model of asthma. *Clin Exp Allergy*. 2010; 40:1266–1275.
- [20] Wamoto H, Yokoyama A, Shiota N, et al. Tiotropium bromide is effective for severe asthma with noneosinophilic phenotype. *Eur Respir J*. 2008;31:1379–1380.
- [21] Kerstjens HA, Disse B, Schröder-Babo W, et al. Tiotropium improves lung function in patients with severe uncontrolled asthma: a randomized controlled trial. *J Allergy Clin Immunol*. 2011;128:308–314.
- [22] Pieper MP, Chaudhary NI, Park JE. Acetylcholine-induced proliferation of fibroblasts and myofibroblasts in vitro is inhibited by tiotropium bromide. *Life Sci*. 2007;80:2270–2273.
- [23] Matthiesen S, Bahulayan A, Kempkens S, et al. Muscarinic receptors mediate stimulation of human lung fibroblast proliferation. *Am J Respir Cell Mol Biol*. 2006;35:621–627.
- [24] Gosens R, Nelemans SA, Grootte Bromhaar MM, et al. Muscarinic M3-receptors mediate cholinergic synergism of mitogenesis in airway smooth muscle. *Am J Respir Cell Mol Biol*. 2003;28:257–262.
- [25] Grainge CL, Lau LC, Ward JA, et al. Effect of bronchoconstriction on airway remodeling in asthma. *N Engl J Med*. 2011;364:2006–2015.