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Effects of Muscarinic Receptor Antagonists With or Without M_2 Antagonist Activity on Cholinergic Reflex Bronchoconstriction in Ovalbumin-Sensitized and -Challenged Mice

Hiroyasu Hirose, Jian Jiang, and Masaru Nishikibe*

Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Okubo 3, Tsukuba 300-2611, Japan

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Abstract. To investigate whether the inhibition of muscarinic M_2 receptors results in the enhancement of reflex bronchoconstriction under airway hyperresponsiveness, we evaluated the effects of muscarinic antagonists with or without M_2 antagonist activity on methacholine (MCh)- and SO_2-induced airway responses in ovalbumin (OVA)-sensitized and -challenged mice. In this model, similar airway hyperresponsiveness to MCh (12 mg/ml) was observed on Days 31 and 37 (2.2-fold and 2.7-fold, respectively). However, airway hyperresponsiveness to SO_2 (0.05 l/min) on Day 37 was less than that on Day 31 (4.0- and 2.7-fold on Days 31 and 37), indicating reflex bronchoconstriction was enhanced on Day 31 in comparison to Day 37. Ipratropium (0.03 – 0.3 mg/ml, inhalation) and Compound A (0.1 – 3 mg/kg, p.o.) inhibited MCh-induced responses on Days 31 and 37. Although ipratropium (0.03 – 1 mg/ml) dose-dependently inhibited SO_2-induced responses on Day 31, ipratropium at a dose of 0.1 mg/ml significantly increased SO_2-induced responses on Day 37 (162.2% of the corresponding control). On the other hand, Compound A (0.03 – 0.3 mg/kg, p.o.) inhibited SO_2-induced responses without any increases on Days 31 and 37. These results suggest that two different conditions of reflex bronchoconstriction are presented in this model: 1) SO_2-induced responses are enhanced by dysfunctional M_2 receptors on Day 31; 2) the dysfunctional M_2 receptors are partially restored on Day 37. In addition, the inhibition of the restored M_2 receptors further enhance reflex bronchoconstriction.

Keywords: airway hyperresponsiveness, muscarinic receptor antagonist, compound A, ipratropium, bronchoconstriction

Introduction

The development of airway hyperresponsiveness, which is an important feature of allergic asthma, is implicated in several pathological conditions such as neurogenic abnormalities and airway inflammation. It is suggested that inhaled chemical irritants such as SO_2 stimulate pulmonary reflex through sensory nerve endings, and the reflex pathway via vagus nerve mediates bronchoconstriction (1). Inhaled chemical irritants such as SO_2 induce bronchoconstriction by stimulating afferent vagal sensory nerve endings. Airway hyperresponsiveness to SO_2 has been observed in the asthmatic patients and its animal models (1 – 3). SO_2-induced bronchoconstriction can be partially reversed by administration of the muscarinic antagonists (4, 5), thereby indicating that acetylcholine, which is released from the efferent cholinergic nervous system, is one of the mediators of SO_2-induced reflex bronchoconstriciton. It is known that the released acetylcholine has at least two target sites: 1) postsynaptic muscarinic M_3 receptors on the airway smooth muscle, which induce bronchoconstriction; and 2) presynaptic muscarinic M_2 receptors on the nerve, which inhibit the further release of acetylcholine. Thus, the SO_2-induced reflex airway obstruction is modulated by not only postsynaptic M_3 but also by presynaptic M_2 receptors. Under normal conditions, blockade of muscarinic M_2 receptors causes enhancement of vagally mediated bronchoconstriction by increasing acetylcholine release, so-called "paradoxical bronchoconstriction". Ipratropium, a muscarinic antagonist with M_2

*Corresponding author. FAX: +81-298-77-2028
E-mail: niskbems@banyu.co.jp

antagonistic activity, enhances bronchoconstriction induced by vagus nerve stimulation at low doses (0.01 – 1 μg/kg, i.v.) in normal guinea pigs (6). Cytotoxic factors derived from inflammatory cells, such as major basic proteins (MBP), show allosteric antagonist activity for muscarinic M_2 receptors but not M_3 receptors in binding assays (7). It has been suggested that loss of neuronal M_2 receptors function after antigen challenge is due to the release of eosinophil MBP (8, 9). Therefore, it is considered that reduced M_2 function under pathological conditions can lead to airway hyperresponsiveness to reflex bronchoconstriction. However, it is not clear whether the function of presynaptic M_2 receptors are completely interrupted after antigen challenge, in spite of the changes of the pathological conditions that accompany the development of airway remodeling. Therefore, it has been hypothesized that the severity of the dysfunction of airway presynaptic M_2 receptors influences the release of endogenous acetylcholine via a cholinergic reflex mechanism even after antigen-challenge in asthma or animal models. Thereby, blockade of prejunctional inhibitory M_2 receptors would weaken the functional blockade of postjunctional M_3 receptors in airway smooth muscle.

Recently, we obtained Compound A, (2*R*)-*N*-[1-(6-aminopyridin-2-ylmethyl)piperidin-4-yl]-2-[(1*R*)-3,3-difluorocyclopentyl]-2-hydroxy-2-phenylacetamide, which has been identified as an orally available muscarinic receptor antagonist without M_2 antagonistic activity (10, 11). Compound A is considered to be a powerfully pharmacological tool that will elucidate the physiological function of the muscarinic M_2 receptor subtype.

In the present study, we investigated whether the inhibition of M_2 receptors further enhances reflex bronchoconstriction via the cholinergic nerve under airway hyperresponsiveness. We evaluated 1) airway responsiveness between methacholine (MCh) and SO_2 in ovalbumin (OVA)-sensitized and -challenged mice and 2) the effects of muscarinic antagonists with or without M_2 antagonist activity on MCh- and SO_2-induced airway obstruction, using ipratropium and Compound A, respectively.

Materials and Methods

All experiments complied with the Guidelines for Biological and Pharmacological Experiments approved by Tsukuba Research Institute of Banyu Pharmaceutical Co., Ltd. and the Guiding principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Sensitization and airway challenge

Female BALB/c mice (36 – 49 g, 3 – 8 mice/group) were sensitized by intraperitoneal injection of 20 μg ovalbumin (OVA) (Sigma, St. Louis, MO, USA) emulsified in 2 mg aluminum hydroxide (Sigma) in a total volume of 100 μl on Days 1 and 14. Mice were challenged via the airway with 1% OVA-saline solution for 20 min on Days 28, 29, and 30 by ultrasonic nebulization (5500D; Devilbis, Sommerset, PA, USA). This procedure was described by Hamelmann et al. (12). All of the sensitized and challenged mice developed allergen-specific immediate cutaneous responsiveness to intradermal injections of OVA.

Determination of airway responsiveness

Airway responsiveness was measured in unrestrained animals by barometric plethysmography using whole body plethysmography (WBP) (model PLY3211; Buxco, Troy, NY, USA). Pulmonary airway obstruction was expressed as 'Penh' using the following formula (12):

$$Penh = [(Te/RT) - 1] \times PEF/PIF$$

Penh is a dimentionless value that has been shown to correlate with the changes in airway resistance that occur during bronchial challenge with MCh. Time of expiration (Te) is defined as the time from the end of inspiration to the start of the next inspiration. Maximum box pressure signal occurring during one breath in a negative or positive direction is defined as peak inspiratory flow (PIF, ml/s) or peak expiratory flow (PEF, ml/s), respectively. Relaxation time (RT) is defined as the time of pressure decay to 36% of the total expiratory pressure signal (area under the box pressure signal curve in expiration). The pressure signal was measured with a transducer (model TRD5100, Buxco) that was connected to preamplifier modules (model MAX2270) and analyzed by system XA software (model SFT1810, Buxco). Before readings were obtained, the box was calibrated with a rapid injection of 150 μl air into the main chamber. The main chambers were ventilated through the inlet and outlet of the main chamber by bias flow regulator (model PLY1030, Buxco) at an air flow of 1.5 l/min.

Mice were placed in the main chamber, and baseline readings were taken and averaged for a 1 min-period. MCh (3, 6, and 12 mg/ml) was aerosolized (5500D; DeVilbiss Health Care, Sommerset, PA, USA) and mixed at a flow rate of 0.01 l/min into the air flow (1.5 l/min) through an inlet of the main chamber for 10 min. Similarly, 1% SO_2 gas (N_2 balance) was mixed at a flow rate of 0.01, 0.02, and 0.05 l/min into the air flow. The concentration ranges of SO_2 in the chamber were 60 – 62 PPM at 0.01 l/min, 127 – 132 PPM at

0.02 l/min, and 228 – 245 PPM at 0.05 l/min, respectively (DY-106; Dyrec, Ibaraki). Airway obstruction was expressed as the area under the curve of increase in Penh for 30 min after the start of MCh- or SO_2-provocation.

Treatment of mice with Compound A or ipratropium

After stable Penh was obtained at least for 30 min, the test drugs were administered. One hour before each measurement of airway responsiveness on Days 28 without OVA challenge and Days 31 and 37 with OVA challenge, mice were treated with Compound A or ipratropium. Compound A was orally administered at a dose of 0.03 – 3 mg/kg. Ipratropium was administered by aerosol inhalation for 10 min at a dose of 0.03 – 1 mg/ml using the DeVilbiss ultrasonic nebulizer. The effects of Compound A or ipratropium were evaluated as the inhibition for MCh (12 mg/ml)- or SO_2 (0.05 l/min)-induced airway response, which was estimated by the area under the curve of increase in Penh for 30 min after the start of MCh- or SO_2-provocation.

Histological analysis

The lungs were obtained from antigen-sensitized mice after they had been killed on Day 28, 31, 37, or 44. After pulmonary infiltration with 0.5 ml of air via tracheal insertion, the animals underwent in situ irrigation with 10% neutral formalin via the cervic vein. Five to ten minutes later, the lung and trachea were removed as a whole and further fixed in the same fixative for 15 to 18 h. Left lungs were harvested for histopathologic assessment. The lungs were cut coronally and embedded in paraffin wax. The tissue blocks were mounted onto a microtome and sectioned into pieces 4 μm in thickness. Hematoxylin & Eosin (H.E.) and Periodic acid Schiff (PAS) stainings were used for general histologic observation and detection of mucus cells. Measurements of PAS-positive cells were carried out using an optical microscope (OPTIPHOT-2; Nikon, Tokyo). The circumference of the segmental bronchus was measured at a magnification of ×160. PAS-positive cells were counted and expressed as the ratios of positive cells over the bronchial circumference. Four different areas were randomly selected to obtain the mean value for each animal.

Expression of results

Values are expressed as the mean ± S.E.M. unless otherwise noted. Statistical analyses for data of airway responses (Penh) were performed by Dunnett's test after an analysis of variance between groups or performed by the paired Student's *t*-test within a group. Wilcoxon's rank test for histological counts of mucus cells were conducted.

Drugs and chemicals

Compound A was synthesized at the Tsukuba Research Institute of Banyu Pharmaceutical Co., Ltd. as described in a published patent application (WO 9805641). SO_2 gas was purchased from Takachiho Kagaku Kogyo (Tokyo). All other reagents were purchased from Sigma Chemical Co.

Results

Airway responsiveness for MCh- or SO_2-provocation

Airway responses to MCh (3 – 12 mg/ml)- or SO_2 (0.01 – 0.05 l/min)-inhalation were dose-dependently induced in OVA-sensitized mice without OVA challenge on Day 28 (Fig. 1). The increases in Penh peaked within 5 – 10 min during the provocation with either MCh-aerosol or SO_2-gas, and Penh returned to basal values approximately 10 min after the provocation. The area under the curve of the increase in Penh (ΔPenh) for MCh-provocation at doses of 3, 6, and 12 mg/ml were 5.1 ± 0.6, 9.4 ± 1.3, and 20.8 ± 3.4, respectively. ΔPenh for SO_2-provocation at doses of 0.01, 0.02, and 0.05 l/min were 7.2 ± 1.9, 8.4 ± 2.8, and 25.3 ± 2.5, respectively.

In OVA-sensitized and -challenged mice, increases in Penh in response to MCh (6 and 12 mg/ml) were observed on Days 31 and 37 (Fig. 1). The ΔPenh in response to MCh at a dose of 6 mg/ml was 2.1-fold (P<0.05) and 2.5-fold (P<0.01) higher on Days 31 and 37, respectively, than on Day 28; and the ΔPenh in response to MCh at 12 mg/ml were 2.2-fold (P<0.01) and 2.7-fold (P<0.01) higher on Days 31 and 37, respectively, than on Day 28.

Increases in ΔPenh in response to SO_2 (0.02 and 0.05 l/min) were also observed on Days 31 and 37 in OVA-challenged mice. However, the airway responsiveness to SO_2 at 0.02 l/min on Day 37 was significantly decreased compared with that on Day 31 (P<0.05, Fig. 1). The airway responsiveness to SO_2 at 0.05 l/min on Day 37 tended to be decreased compared with that on Day 31, although the decrease was not significant. ΔPenh in response to SO_2 at a dose of 0.02 l/min was 4.8-fold (P<0.01) and 2.0-fold (P<0.05) higher on Days 31 and 37, respectively, than on Day 28; and ΔPenh in response to SO_2 at a dose of 0.05 l/min was 4.0-fold (P<0.01) and 2.7-fold (P<0.01) higher on Days 31 and 37, respectively, than on Day 28.

Airway hyperresponsiveness to both MCh and SO_2 in OVA-sensitized and -challenged mice was not observed on Day 44.

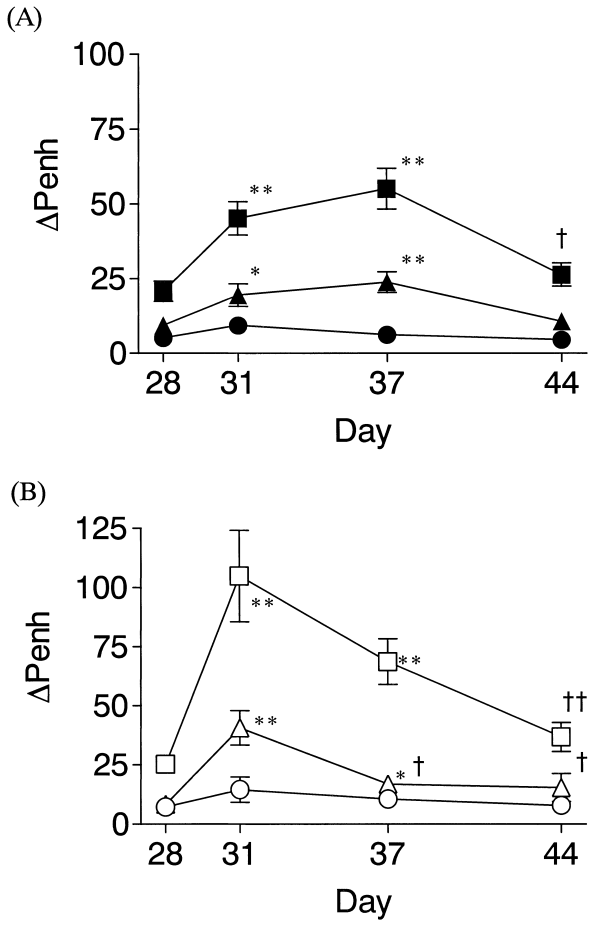

Fig. 1. Time course of increases in Penh with methacholine (A)- and SO_2 (B)-airway provocation on Day 28 without ovalbumin (OVA) challenge and on Days 31, 37, and 44 with OVA challenge in OVA-sensitized mice. Aerosolized methacholine (3 (closed circle), 6 (closed triangle), and 12 (closed square) mg/ml) was mixed at a flow rate of 0.01 l/min into the air flow (1.5 l/min) through an inlet of the main chamber for 10 min. One percent SO_2 gas (0.01 (open circle), 0.02 (open triangle), and 0.05 (open square) mg/ml) was mixed at flow rate of 0.01, 0.02, and 0.05 l/min into the air flow. Airway obstruction was expressed as the area under the curve of the increase in Penh for 30 min after the start of the airway provocation. Data are presented as the mean ± S.E.M. of 4 – 8 animals. *P<0.05 and **P<0.01 versus data on Day 28. $^{\dagger}P$<0.05 and $^{\dagger\dagger}P$<0.01 versus data on Day 31.

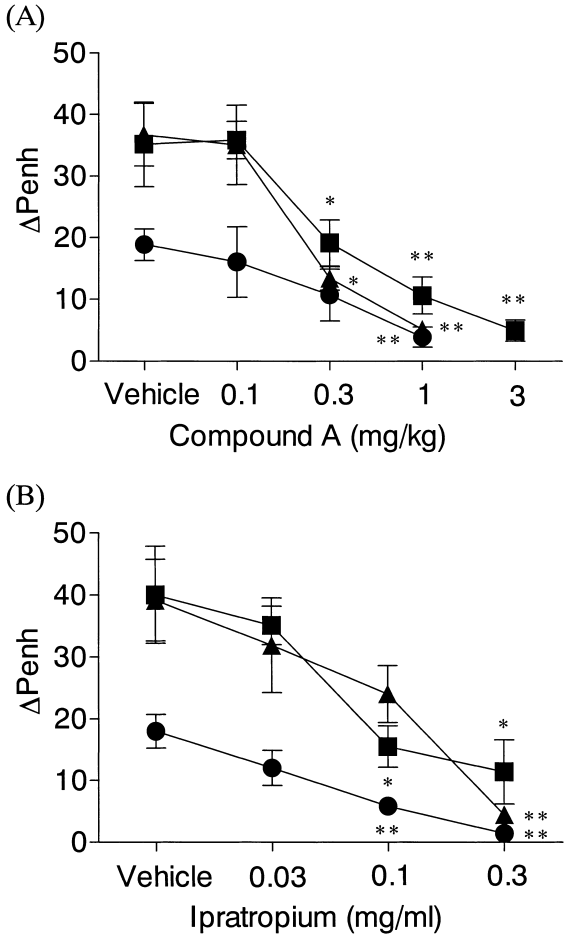

Fig. 2. Effects of Compound A (A) and ipratropium (B) on methacholine-induced increases in Penh on Day 28 without ovalbumin (OVA) challenge (closed circle) and on Days 31 (closed triangle) and 37 (closed square) with OVA challenge in OVA-sensitized mice. Compound A (0.1, 0.3, 1, and 3 mg/kg, p.o.) or ipratropium (0.03, 0.1, and 0.3 mg/ml, inhalation) was administered 1 h before methacholine (12 mg/ml, 10 min)-induced airway provocation was measured. The airway obstruction was expressed as the area under the curve of the increase in Penh for 30 min after the start of the airway provocation. Data are presented as the mean ± S.E.M. of 5 – 7 animals. *P<0.05 and **P<0.01 versus vehicle.

Effects of Compound A and ipratropium on MCh-induced airway obstruction

Without OVA challenge on Day 28, both Compound A and ipratropium dose-dependently inhibited MCh (12 mg/ml)-induced increases in ΔPenh in OVA-sensitized mice (Fig. 2). Percent control values of Compound A at doses of 0.1, 0.3, and 1 mg/kg (p.o.) were 85.1%, 56.7%, and 20.7% (P<0.01), respectively. The % control values of ipratropium at inhaled-doses of 0.03, 0.1, and 0.3 mg/ml were 67.1%, 32.4% (P<0.01), and 9.1% (P<0.01), respectively (Fig. 2).

On Days 31 and 37, both Compound A (0.1, 0.3, 1, and 3 mg/kg, p.o.) and ipratropium (0.03, 0.1, and 0.3 mg/ml, inhalation) also dose-dependently inhibited of MCh-induced increases in ΔPenh in OVA-sensitized and -challenged mice (Fig. 2).

Effects of Compound A and ipratropium on SO_2-induced airway obstruction

SO_2 (0.05 l/min)-induced increases in ΔPenh were inhibited by Compound A on Days 28, 31, and 37 in a dose-dependent manner. Percent control values of Compound A at doses (p.o.) of 0.03, 0.1, and 0.3 mg/kg were 88.6%, 76.3%, and 66.6%, respectively, on

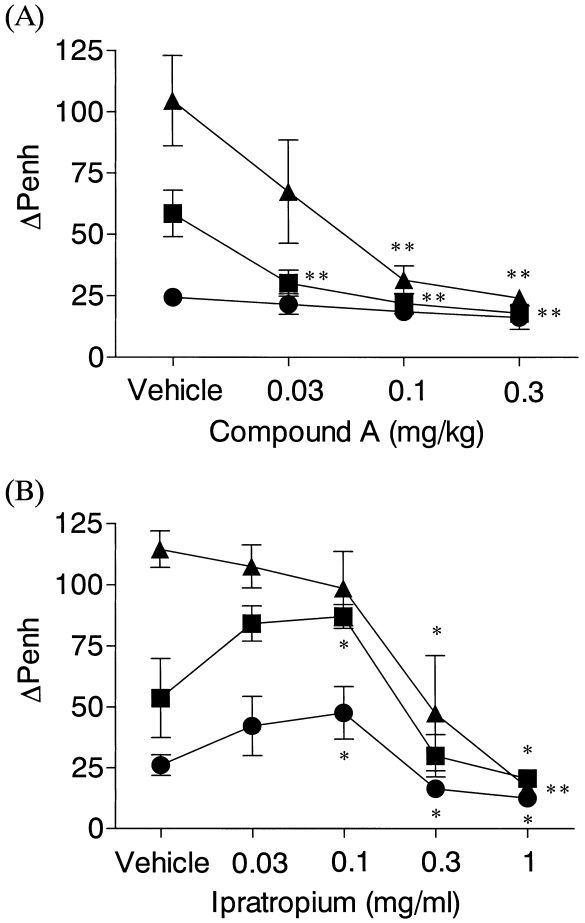

Fig. 3. Effects of Compound A (A) and ipratropium (B) on SO_2-induced increases in Penh on Day 28 without ovalbumin (OVA) challenge (closed circle) and on Days 31 (closed triangle) and 37 (closed square) with OVA challenge in OVA-sensitized mice. Compound A (0.03, 0.1, and 0.3 mg/kg, p.o.) or ipratropium (0.03, 0.1, 0.3, and 1 mg/ml, inhalation) was administered 1 h before 1% SO_2 (0.05 ml/min, 10 min)-induced airway provocation was measured. The airway obstruction was expressed as the area under the curve of the increase in Penh for 30 min after the start of the airway provocation. Data are presented as the mean ± S.E.M. of 5 – 7 animals. **P*<0.05 and ***P*<0.01 versus vehicle.

Day 28. The inhibition of Compound A at the same doses was enhanced on Days 31 and 37; and % control values for Compound A at the maximal dose (0.3 mg /kg) were 18.3% on Day 31 (*P*<0.01) and 30.7% on Day 37 (*P*<0.01). In addition, the minimum effective dose of Compound A was 0.1 mg/kg on Day 31 (30.1% of control) and 0.03 mg/kg on Day 37 (51.5% of control).

In contrast, ipratropium at low doses (0.03 and 0.1 mg/ml) enhanced SO_2-induced increases in *Δ*Penh on Days 28 and 37; however, higher doses of ipratropium (0.3 and 1 mg/ml) inhibited the increases in *Δ*Penh (Fig. 3). On Day 28, % control values for ipratropium at inhalation doses of 0.03, 0.1, 0.3, and 1 mg/ml

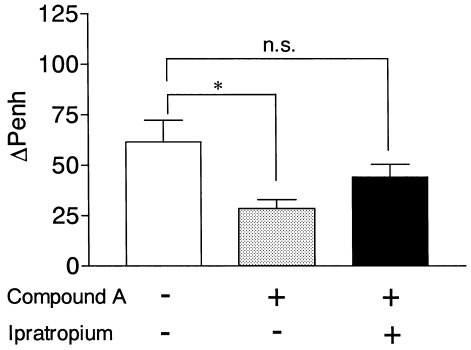

Fig. 4. Effects of the combination treatment with Compound A and ipratropium on SO_2-induced increases in Penh on Day 37 with OVA challenge in OVA-sensitized mice. Compound A (0.1 mg/kg, p.o.) or ipratropium (0.1 mg/ml, inhalation) was administered 1 h before 1% SO_2 (0.05 ml/min, 10 min)-induced airway provocation was measured. The airway obstruction was expressed as the area under the curve of the increase in Penh for 30 min after the start of the airway provocation. Data are presented as the mean ± S.E.M. of 3 or 4 animals. n.s., not significant. **P*<0.05 versus vehicle alone.

were 161.5%, 181.9% (*P*<0.05), 63.0%, and 48.3% (*P*<0.05), respectively. On Day 37, the corresponding % control values for ipratropium (0.03, 0.1, 0.3, and 1 mg/ml, inhalation) were 156.9%, 162.2% (*P*<0.05), 55.9%, and 38.4% (*P*<0.05), respectively. On Day 31, however, ipratropium (0.03, 0.1, 0.3, and 1 mg/ml, inhalation) dose-dependently inhibited SO_2-induced increases in *Δ*Penh without any enhancing effects (Fig. 3). Percent control values for ipratropium at doses of 0.03, 0.1, 0.3, and 1 mg/ml were 93.9%, 86.0%, 41.5% (*P*<0.05), and 14.9% (*P*<0.01), respectively.

In addition, the combination treatment with Compound A (0.1 mg/kg, p.o.) and ipratropium (0.1 mg/ml, inhalation) did not affect SO_2-induced increases in Penh on Day 37 with OVA challenge in OVA-sensitized mice, although Compound A alone (0.1 mg/kg, p.o.) showed a significant inhibition of SO_2-induced increases in *Δ*Penh (Fig. 4). Percent control values for combination treatment and Compound A alone were 71.6% and 46.4% (*P*<0.05), respectively.

Histological analysis of the airway in OVA-sensitized and -challenged mice

After OVA-challenge, thickening of airway wall and an increase in airway epithelial mucus-containing cells were observed. Maximal changes in airway wall thickening and increases in epithelial mucus-containing cells were observed on Day 37 (Fig. 5). A semiquantitative analysis of mucus-containing cells showed a gradual increase after OVA challenge, and a maximal score for mucus-containing cells was observed on Day 37 (0.380 ± 0.027, *P*<0.001, Table 1). Thereafter, the increases in

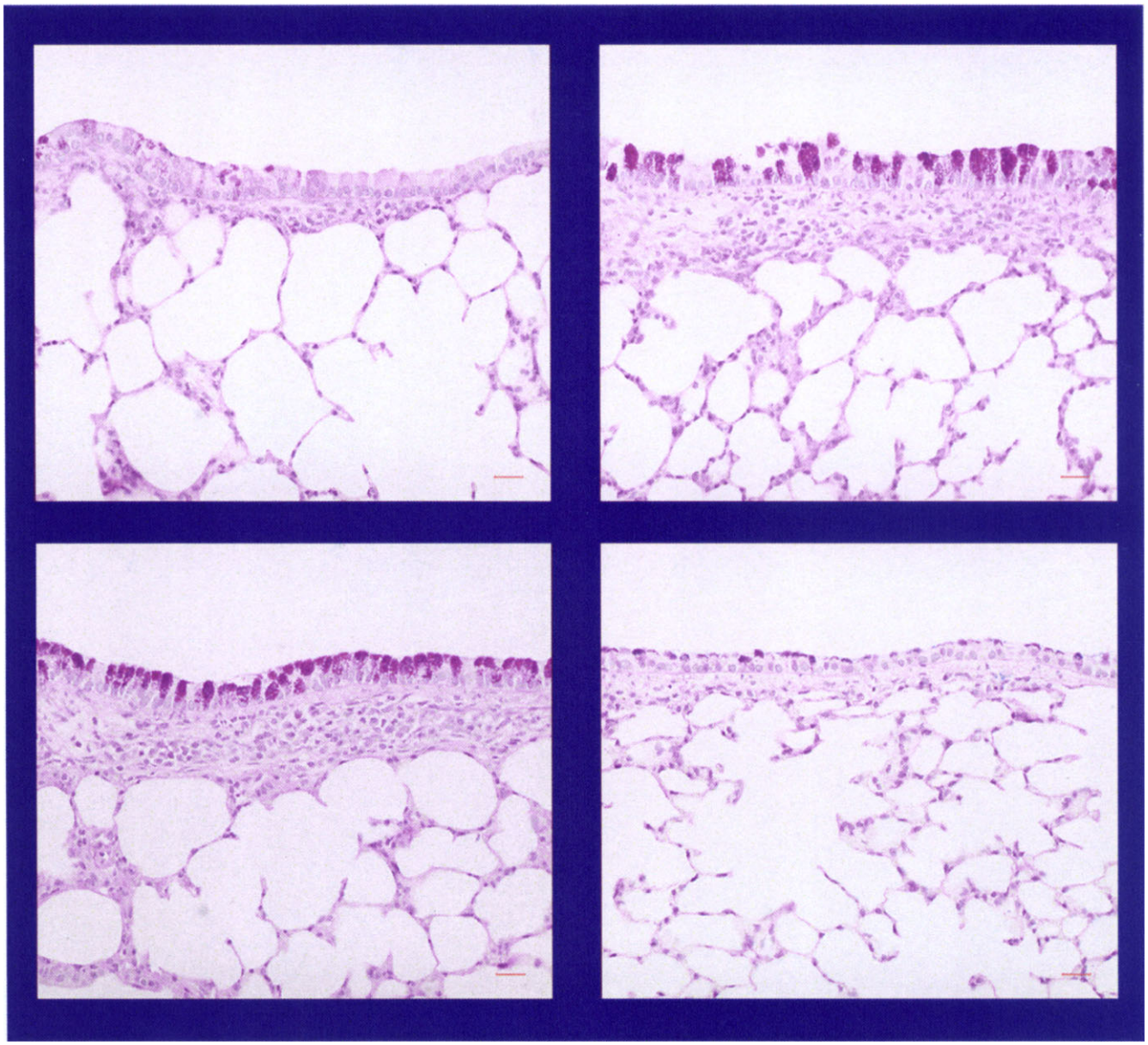

Fig. 5. Periodic acid Schiff (PAS) staining of mucus cells in the segmental bronchi on Days 31 (left-upper), 35 (left-lower), 37 (right-upper), and 44 (right-lower) with ovalbumin (OVA) challenge in OVA-sensitized mice. The color of the cellular mucus is red. Original magnification: ×160. Bar: 20 μm.

Table 1. Time-course of mucus-containing cell numbers in bronchus in ovalbumin (OVA)-sensitized mice on Day 28 without OVA challenge and on Days 31, 35, 37, and 44 with OVA challenge

	Day 28	Day 31	Day 35	Day 37	Day 44
Score	0.006 ± 0.002	0.162 ± 0.019 [b]	0.288 ± 0.013 [b]	0.380 ± 0.027 [b]	0.123 ± 0.051 [a]

Mucus-containing cells, which are Periodic acid Schiff (PAS)-positive, were counted and expressed as the ratios of positive cells over the bronchial circumference. Data are presented as the mean ± S.E.M. of 5 – 7 animals. [a]P<0.01 and [b]P<0.001 versus data on Day 28.

this score were reduced up to Day 44 (0.123 ± 0.051, $P<0.01$).

Discussion

In this study, we demonstrated that airway hyperresponsiveness occurs in OVA-sensitized and -challenged mice. Both on Day 31 (1 day after OVA challenge) and Day 37 (7 days after OVA challenge), airway hyperresponsiveness to both SO_2 and MCh was observed. Generally, cytotoxic mediators are released from inflammatory cells after OVA challenge, and these cytotoxic mediators induce damage to the epithelium barrier. Therefore, it is likely that airway hyperreactivity is due to penetration of an inhaled muscarinic agonist or a chemical irritant into target sites such as airway smooth muscle or sensory nerve ending.

In our functional in vivo study, we found different time-course changes in the severity of airway hyperresponsiveness to MCh and SO_2 in OVA-sensitized and -challenged mice. Although airway hyperresponsiveness to MCh on Day 37 was almost identical to that on Day 31, airway hyperresponsiveness to SO_2 on Day 37 was less than that on Day 31. These results suggest that the SO_2-induced reflex bronchoconstriction is enhanced on Day 31 and partially reduced on Day 37, despite the similar airway hyperresponsiveness to MCh both on Days 31 and 37. In our muscarinic antagonists study, a low dose of ipratropium (0.1 mg/ml, inhalation) significantly enhanced SO_2-induced airway obstruction both on Day 28 (before OVA challenge) and Day 37 (181.9% and 162.2% of the corresponding control, respectively), so ipratropium showed paradoxical bronchoconstriction. Compound A (0.03 – 0.3 mg/kg, p.o.) did not produce any paradoxical bronchoconstriction on Days 28 and 37. Additionally, the antagonistic action of Compound A (0.1 mg/kg, p.o.) on Day 37 tends to be inhibited by adding ipratropium (0.1 mg/ml, inhalation). In contrast, Compound A or ipratropium produced a dose-dependent inhibition without showing any paradoxical bronchoconstrictions on Day 31. Therefore, these results suggest that the effect of ipratropium on Day 37 might be due to its inhibition of presynaptic M_2 receptors and that the effect of ipratropium on Day 31 might be due to the dysfunctional presynaptic M_2 receptors after antigen challenges. On the other hand, in a preliminary assay using normal animals, lung levels of inhaled ipratropium at a dose of 0.3 mg/ml and Compound A at a dose of 1 mg/kg were 68 ng/g (0.2 nmol/g, almost equal to 200 nM) and 202 ng/g (0.45 nmol/g, almost equal to 450 nM) 2 h after dosing, respectively (data not shown). Our previous reports have indicated that the K_i values (nM) of ipratropium for m1, m2, and m3 are 0.49, 1.5, and 0.51, respectively, and the K_i values (nM) of Compound A for m1, m2, and m3 are 1.5, 540, and 2.8, respectively (10, 11). Therefore, these lung levels roughly support that the in vivo activity of both ipratropium and Compound A on SO_2-induced airway obstruction are nearly compatible with their in vitro muscarinic receptor antagonistic potency. However, there are no available data to know whether ipratropium has an identical binding affinity for muscarinic receptors in lung with developed airway hyperresponsiveness. Exogenously administered MCh directly stimulates postsynaptic M_3 receptors on airway smooth muscles or mucus cells. However, inhaled SO_2 stimulates afferent vagal sensory nerve ending and induces endogenous acetylcholine release from the efferent cholinergic nerve. Subsequently, the SO_2-induced reflex airway obstruction is modulated not only by postsynaptic M_3 but also by presynaptic M_2 receptors. Taken together, our results suggest that two different conditions of reflex bronchoconstriction are presented in this model: 1) SO_2-induced responses are enhanced by dysfunctional M_2 receptors on Day 31; 2) the dysfunctional M_2 receptors are partially restored on Day 37. Histological observation revealed a thickening of the airway wall and an increase in the number of airway epithelial mucus-containing cells on Days 35 – 37 when compared with those on Day 31. These histological features with airway remodeling obtained on Day 37 are closer to those observed in human asthma (13 – 15). Therefore, we hypothesize that airway remodeling is involved in the restoration of presynaptic M_2 receptor dysfunction in this model. Further histological analysis is needed to characterize our model.

Other investigators have reported that inhaled gallamine, which is believed to be an M_2-receptor antagonist, enhances the increase in bronchoconstriction induced by nerve stimulation 1 and 4 days after OVA challenge in OVA-sensitized guinea pigs, but does not enhance bronchoconstriction 6 h after OVA challenge (16, 17). The restoration period of dysfuncting M_2 receptors, which is demonstrated by gallamine-induced enhancement, differs from our results. These investigators employed a single OVA provocation in OVA-sensitized guinea pigs, whereas we employed 3 OVA challenges over 3 consecutive days. Since it has been reported that airway hyperresponsiveness is much greater following repeated challenges than after a single challenge (18), it has been considered that the differences in the restoration period of dysfunctional M_2 receptors are affected by repeated antigen challenges.

It is of interest that the inhibition potency of Compound A in SO_2-induced airway obstruction is higher than that of Compound A in MCh-induced airway

obstruction. Although higher doses of Compound A (0.3 mg/kg and more) inhibited MCh-induced airway obstruction, a lower dose of Compound A (0.1 mg/kg) inhibited SO_2-induced airway obstruction in our OVA-sensitized and -challenged mice. Our previous reports have indicated that the muscarinic m1 binding affinity of Compound A is slightly higher than m3 (10, 11). The K_i values (nM) of Compound A for m1, m2, and m3 are 1.5, 540, and 2.8, respectively. Since presynaptic M_1 receptors facilitate acetylcholine release from cholinergic nerve endings in the airway (19, 20), it is believed that the inhibition of both presynaptic M_1 and postsynaptic M_3 receptors without M_2 antagonism participates in the potent inhibition of SO_2-induced airway obstruction, when a cholinergic reflex mechanism is activated.

Acute exacerbation of asthma appears to respond more favorably when muscarinic antagonists such as ipratropium are added to β-agonists than when β-agonists are used alone. Inhaled ipratropium has been shown to decrease hospitalization rates in patients with acute asthma attacks (21 – 23). However, the clinical use of muscarinic antagonists such as ipratropium in chronic stable asthma has fallen into disfavor (24). Also, paradoxical bronchoconstriction in response to ipratropium has been reported in humans (25, 26). Since it is likely that the restoration of M_2 dysfunction increases the feedback inhibition of the release of acetylcholine in reflex bronchoconstriction, the clinical efficacy of anticholinergics with M_2 antagonistic activity may be reduced by their antagonistic activity against airway presynaptic M_2 receptors. However, clinically available muscarinic antagonists have limited selectivity between M_2 and M_3 subtypes. It remains to be elucidated whether muscarinic antagonists without M_2 antagonistic activity reduce the risk of paradoxical bronchoconstriction due to the blockade of presynaptic M_2 receptors.

In conclusion, we show that despite the similar airway hyperresponsiveness to MCh, two different conditions of reflex bronchoconstriction are present in OVA-sensitized and -challenged mice: 1) cholinergic reflex bronchoconstriction is enhanced by dysfunctional M_2 receptors on Day 31; 2) the dysfunctional M_2 receptors are partially restored on Day 37. Furthermore, it is suggested that the inhibition of restored M_2 receptors result in further risk enhancement of reflex bronchoconstriction under airway hyperresponsiveness conditions.

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