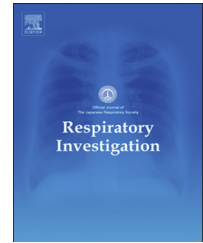




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Low-dose salbutamol suppresses airway responsiveness to histamine but not methacholine in subjects with asthma



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ABSTRACT

Background: Airway hyperresponsiveness is a cardinal feature of asthma. Although the modulation of cholinergic neuroeffector transmission may play a role in airway responsiveness, *in vivo* evidence remains scarce. It is well known that histamine causes bronchoconstriction partly via vagal reflex, whereas methacholine does not. To investigate the significance of modulating neuroeffector transmission, we compared the effect of low-dose salbutamol—a β_2 -adrenoceptor agonist—on airway responsiveness to histamine with that to methacholine.

Methods: We enrolled 12 subjects with stable asthma. After screening confirmed that inhalation of low-dose salbutamol (1 μ g) did not change their basic pulmonary function, subjects underwent measurement of airway responsiveness to inhaled histamine and methacholine with or without pretreatment with low-dose salbutamol, in a randomized, crossover fashion. Airway responsiveness was measured by an astograph by which respiratory conductance (Grs) was assessed by the forced oscillation method during continuous inhalation of histamine or methacholine in stepwise incremental concentrations. Airway responsiveness was calculated as the cumulative dose of bronchoconstrictors that induced a decrease of 35% in Grs.

Results: Inhalation of 1 μ g of salbutamol significantly attenuated airway responsiveness to histamine but not methacholine. This selective attenuation was observed irrespective of disease severity or phenotype, namely atopy or non-atopy.

Conclusion: Low-dose salbutamol suppresses airway responsiveness to histamine but not

Abbreviations: Grs, respiratory conductance; ACh, acetylcholine; PGE₂, prostaglandin E₂; EFS, electrical field stimulation; MCh, methacholine; SABA, short-acting β_2 -adrenoceptor agonist; RAST, radioallergosorbent test; LABA, long-acting β_2 -adrenoceptor agonist; Rrs, respiratory resistance; PD₃₅-Grs, the cumulative dose of bronchoconstrictor that induced a decrease of 35% in Grs; GSEM, geometric standard errors of means

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methacholine in subjects with asthma. The present study may provide a novel insight into the bronchoprotective mechanism of β_2 -adrenoceptor agonist in clinical settings.

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1. Introduction

Airway hyperresponsiveness, a cardinal feature of asthma, is induced by multiple mechanisms including airway inflammation, airway epithelial dysfunction, and disturbance of the autonomic nervous system. Components of autonomic innervation to the human airways are functionally classified as sympathetic, parasympathetic, excitatory, and inhibitory nonadrenergic noncholinergic nerves [1]. The vagus nerve is an anatomically defined component of the parasympathetic nervous system in the airways and is believed to play a role in asthma. Thus, when the vagus nerve is excited, it releases acetylcholine (ACh) from its nerve terminals and causes contraction of smooth muscles in the airway. The release of ACh can be regulated with many endogenous substances; for example, endogenous ACh, prostaglandin E_2 (PGE $_2$), and catecholamine inhibit ACh release by activating M $_2$ -muscarinic autoreceptors, EP $_3$ -prostanoid receptors, and β_2 -adrenoceptors, respectively [1–6]. Given that a variety of bioactive substances are liberated in the process of airway inflammation in asthma, it is important to investigate whether these substances affect the excitability of the vagus nerve and airway hyperresponsiveness.

The release of ACh has been assessed by various *in vitro* methods, such as comparing the smooth muscle contraction evoked by electrical field stimulation (EFS) with that evoked by exogenously applied ACh [2–5,7], measuring EFS-evoked [^{3} H]ACh release [6,8–10], and measuring EFS-evoked ACh release by high-performance liquid chromatography [11]. These methods, however, cannot be applied to *in vivo* human studies of the physiological significance of regulatory mechanisms at the level of neuroeffector transmission.

Histamine is thought to cause ACh release by central reflex via afferent and efferent vagus nerves, because histamine-induced bronchoconstriction is partly inhibited by

atropine or hexamethonium, a ganglionic blocking agent [12,13]. In addition, histamine-induced contraction of an isolated human bronchus was mediated partly by ACh release, via the direct action of histamine on the vagus efferent nerve terminals without the central reflex mechanism [14]. On the other hand, ACh causes bronchoconstriction directly by contracting airway smooth muscles [12,15]. These contrasting properties may be useful for evaluating the effect of bioactive substances on neuroeffector transmission *in vivo*. If administration of some bioactive substance changes the intensity of bronchoconstriction induced by histamine but not by ACh, the substance may act on the sites at least proximal to airway smooth muscle. Indeed, histamine, ACh, and methacholine (MCh)—a synthetic analog of ACh—are commonly used to evaluate airway hyperresponsiveness in clinical research; the basic methods for doing so are already established [16].

Salbutamol is a widely used short-acting β_2 -adrenoceptor agonist (SABA), and SABA was reported to inhibit ACh release from the vagus nerve terminals at a concentration less than that needed to relax airway smooth muscle *in vitro* [2,3]. It is tempting to investigate whether this nerve-selective effect will also occur *in vivo*. Therefore, we evaluated the effect of low-dose salbutamol on cholinergic neuroeffector transmission in subjects with asthma, by evaluating airway responsiveness to histamine versus MCh.

2. Material and methods

2.1. Subjects

The 12 subjects enrolled in the study were non-smokers with mild-to-severe asthma, according to the Global Initiative for Asthma criteria [17], who visited the outpatient clinic at Kyushu University Hospital in Fukuoka, Japan. Table 1

Table 1 – Subject demographics.

Subject	Age (y)	Sex	Phenotype	%FVC	%FEV $_{1.0}$	Steroid	Severity
1	15	M	Non-atopic	107	74	1600	Severe
2	25	F	Non-atopic	118	88	1200	Severe
3	25	M	Non-atopic	112	101	800	Moderate
4	28	M	Atopic	113	103	0	Mild
5	30	M	Atopic	100	83	0	Mild
6	30	M	Non-atopic	99	84	0	Mild
7	35	M	Atopic	101	98	400	Mild
8	47	M	Non-atopic	98	83	400	Mild
9	50	M	Atopic	115	109	1200	Severe
10	55	F	Atopic	85	50	1600	Severe
11	72	F	Non-atopic	98	84	1200	Severe
12	78	M	Atopic	62	47	1600	Severe

Definition of abbreviations: %FVC=baseline forced vital capacity (%predicted); %FEV $_{1.0}$ =baseline forced expiratory volume in 1 s (%predicted) and steroid=daily dose (μ g/day) of inhaled corticosteroids corresponding to dose of beclomethasone dipropionate.

summarizes the demographic data obtained at the time of enrollment. All 12 had been diagnosed with asthma on the basis of clinical history consistent with episodic airway obstruction, diffuse expiratory wheezes on chest auscultation, and the demonstration of an increase of 200 mL and 12.5% or more in forced expiratory volume in 1 s ($FEV_{1.0}$) following the inhalation of 200 μ g of salbutamol (Glaxo-SmithKline; Tokyo, Japan). Airway hyperresponsiveness to MCh was observed in all subjects. Atopy was detected by radioallergosorbent test (RAST): positive results for at least 2 common aeroallergens indicated atopy. Negative RAST results for all common aeroallergens together with a value of total serum IgE within the normal range indicated non-atopy. Patients continued inhaled corticosteroid (400–1600 μ g/day beclomethasone dipropionate) or/and SABA, as needed. None of the patients were treated with oral corticosteroids, long-acting β_2 -adrenoceptor agonist (LABA), slow-releasing theophylline, or leukotriene-receptor antagonist in the last 8 weeks. None of the patients had acute exacerbation of asthma or respiratory tract infection in the last 4 weeks. The study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving human subjects, and all subjects gave informed consent (The study was conducted before an official IRB system was established in our hospital).

2.2. Study design

The study was conducted in an open-style, randomized, crossover design. Subjects presented on 5 occasions. At the initial visit, a medical history was taken, and spirometry was performed 5 min before and 15 min after inhalation of low-dose salbutamol (1 μ g), to determine whether this dose of salbutamol affected basic pulmonary function. After this screening, subjects were randomly divided into 2 groups by the envelope method (group A was assigned to MCh followed by histamine, and group B was assigned to histamine followed by MCh). On the next day, subjects underwent measurement of airway responsiveness to either MCh or histamine. A few days later, subjects underwent spirometry 5 min before and 15 min after inhalation of low-dose salbutamol, followed by measurement of airway responsiveness to MCh or histamine, whichever had been used on the previous visit. On the next 2 visits, subjects underwent the same method with the other bronchoconstrictor. Inhalation of other drugs was withheld for 12 h before measurements. Measurements of airway responsiveness were conducted at the same time of day, 3–4 days apart in order to avoid tachyphylaxis induced by histamine inhalation [18–20].

2.3. Inhalation of low-dose salbutamol and spirometry

In our preliminary study, inhalation of 1 μ g of salbutamol appeared not to affect basic pulmonary function, including forced vital capacity (FVC), FEV_1 , $\dot{V}_{50}$, and $\dot{V}_{25}$. Salbutamol sulfate solution (GlaxoSmithKline; Tokyo, Japan) was diluted with physiologic saline to a final concentration of 0.5 μ g/mL. Two milliliters of this solution was inhaled at tidal breathing for a few minutes using a Wright nebulizer. Spirometry data

were recorded by a computed spirometer (D-21FX; Chest; Tokyo, Japan) using the standard technique. Measurements were made in triplicate, and the best values of FVC and $FEV_{1.0}$ were selected for analysis.

2.4. Measurement of airway responsiveness

Airway responsiveness was measured by an astograph (TCK-6100H; Chest; Tokyo, Japan), according to the method of Takishima et al. [21]. Briefly, respiratory resistance (Rrs) and conductance (Grs; the reciprocal of Rrs) were measured by the forced oscillation method (3 Hz) during continuous inhalation of MCh or histamine in stepwise incremental concentrations, until Rrs reached twice the baseline value. MCh chloride (Sigma; St. Louis, USA) or histamine dihydrochloride (Sigma) was diluted in physiologic saline with 2-fold increasing concentrations, from 0.049 to 25 mg/mL or from 0.025 to 12.5 mg/mL, respectively. Each concentration of the bronchoconstrictor solution was inhaled for 1 min. All subjects were examined during quiet breathing in a sitting position. Airway responsiveness was evaluated as the cumulative dose of bronchoconstrictor that induced a decrease of 35% in Grs ($PD_{35}\text{-Grs}$). $PD_{35}\text{-Grs}$ was calculated such that 1 unit equals 1 min inhalation of aerosol solution at 1.0 mg/mL. Thus, a decrease in $PD_{35}\text{-Grs}$ indicates an increase in airway responsiveness, and an increase in $PD_{35}\text{-Grs}$ indicates a decrease in airway responsiveness. This method allows us to measure airway responsiveness without subjecting the participant to forced expiration. Previous studies have reported a significant correlation between airway responsiveness measured by an astograph and airway responsiveness measured by conventional methods, and they have demonstrated the clinical usefulness of the astograph [22–24].

2.5. Statistical methods

Spirogram values are expressed as means and standard errors of means. $PD_{35}\text{-Grs}$ values were log-transformed prior to analysis, and the summary statistics are therefore expressed as geometric means and geometric standard errors of means (GSEM). Statistical analysis was performed with Student paired t test. Correlations between parameters were assessed with Pearson correlation analysis. Values of p less than 0.05 were accepted as statistically significant.

3. Results

During the initial screening, no subject demonstrated airway reversibility greater than 12.5% of baseline $FEV_{1.0}$ after inhaling low-dose salbutamol (data not shown). All subjects completed the study protocol without incident.

During the MCh study, there was no significant change in FVC, $FEV_{1.0}$, $\dot{V}_{50}$, or $\dot{V}_{25}$ after inhalation of low-dose salbutamol. $PD_{35}\text{-Grs}$ for MCh did not change after inhalation of low-dose salbutamol (Fig. 1). FVC, $FEV_{1.0}$, $\dot{V}_{50}$, and $\dot{V}_{25}$ were also unaffected by inhalation of low-dose salbutamol during the histamine study. However, $PD_{35}\text{-Grs}$ for histamine was significantly increased by inhalation of low-dose salbutamol,

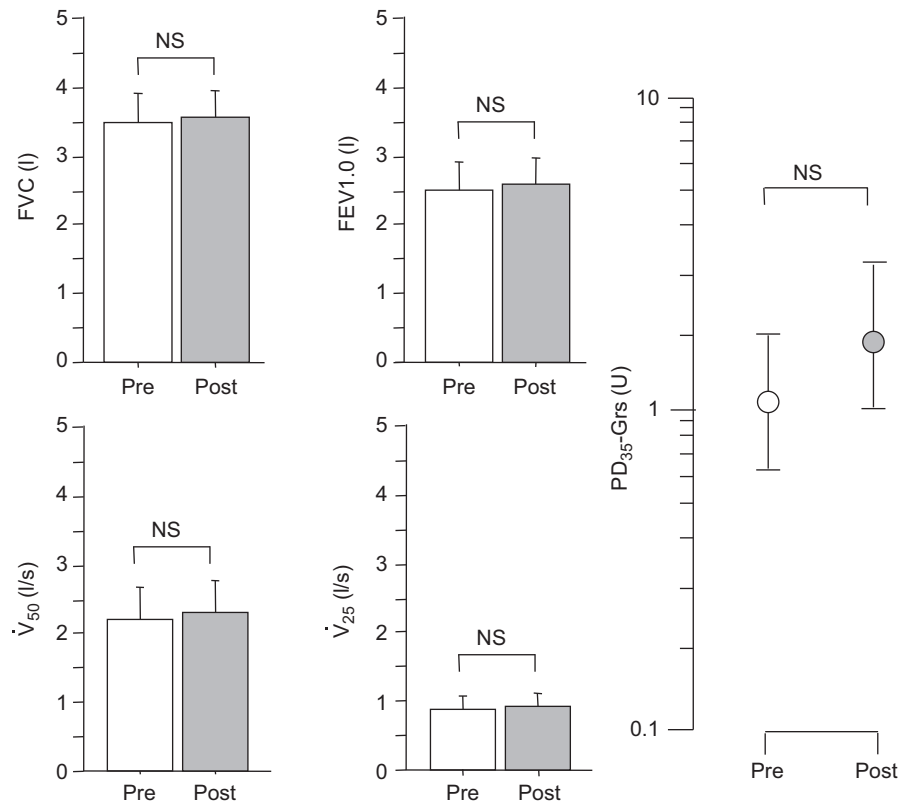


Fig. 1 – Basic pulmonary function and airway responsiveness to MCh before and after inhalation of low-dose salbutamol (1 μ g) in subjects with asthma ($N=12$). Values of FVC, FEV_{1.0}, $\dot{V}_{50}$, and $\dot{V}_{25}$ are expressed as means and standard errors of means. PD₃₅-Grs is expressed as the geometric mean and geometric standard error of the mean. Abbreviations: FEV_{1.0}, forced expiratory volume in 1 s; FVC, forced vital capacity; GSEM, geometric standard error of the mean; MCh, methacholine; PD₃₅-Grs, the cumulative dose of bronchoconstrictor that induced a decrease of 35% in respiratory conductance.

suggesting a decrease in airway responsiveness to histamine (Fig. 2). There was significant correlation between PD₃₅-Grs for MCh and PD₃₅-Grs for histamine, before ($r=0.701$, $p<0.01$) and after ($r=0.667$, $p<0.02$) inhalation of low-dose salbutamol.

To investigate whether the selective effect of low-dose salbutamol on airway responsiveness to histamine was associated with disease severity or asthma phenotype, we divided subjects into the following groups, according to demographic data (Fig. 3). When we divided subjects into a group with mild-to-moderate disease ($n=6$) and a group with severe disease ($n=6$), we found that PD₃₅-Grs for MCh and PD₃₅-Grs for histamine before low-dose salbutamol were significantly lower in the latter group. There was no difference between groups with respect to the distribution of atopy. In both groups, low-dose salbutamol had no significant effect on PD₃₅-Grs for MCh but significantly increased PD₃₅-Grs for histamine (Fig. 4).

Next, we defined groups according to phenotype. Subjects with atopy ($n=6$) did not differ from those without atopy ($n=6$) with respect to basic pulmonary function, PD₃₅-Grs for MCh before low-dose salbutamol, PD₃₅-Grs for histamine before low-dose salbutamol, or variance of disease severity. Low-dose salbutamol had no significant effect on PD₃₅-Grs for MCh but significantly increased PD₃₅-Grs for histamine in both groups.

4. Discussion

Inhalation of 1 μ g of salbutamol significantly attenuated airway responsiveness to histamine in subjects with asthma. The dose of salbutamol used in this study, which was 1/100 of the dose used in clinical settings, did not affect basic pulmonary function or airway responsiveness to MCh. Asthma is regarded as a heterogenous disease entity encompassing ranges of severity and etiological phenotype and is classified as atopic or non-atopic. To understand whether these differences might be related to the selective effect of low-dose salbutamol on airway responsiveness to histamine [13], we performed 2 additional analyses: we divided subjects into groups based on (1) disease severity (mild-to-moderate asthma versus severe asthma) or (2) phenotype (atopy versus non-atopy). These analyses show that low-dose salbutamol significantly attenuated airway responsiveness to histamine but not MCh, irrespective of severity or phenotype.

Inhalation of cholinergic agonists directly causes bronchoconstriction through M₃ muscarinic receptors on airway smooth muscle [25,26]. Thus, MCh-induced bronchoconstriction was not inhibited by hexamethonium, but it was markedly inhibited by atropine or ipratropium bromide [12,15,27]. On the other hand, histamine-induced bronchoconstriction was inhibited not only by H₁ histaminic receptor antagonists but also by pretreatment with hexamethonium, atropine, or

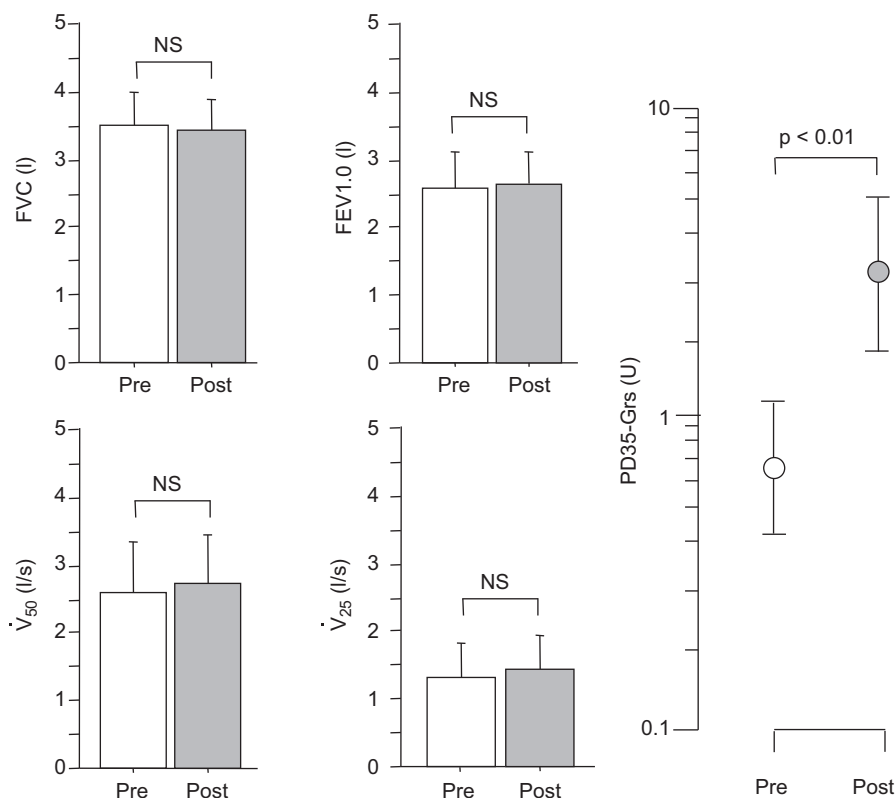


Fig. 2 – Basic pulmonary function and airway responsiveness to histamine before and after inhalation of low-dose salbutamol (1 μ g) in subjects with asthma ($N=12$). Values of FVC, FEV_{1.0}, $\dot{V}_{50}$, and $\dot{V}_{25}$ are expressed as means and standard errors of means. PD₃₅-Grs is expressed as the geometric mean and geometric standard error of the mean (GSEM). Abbreviations: FEV_{1.0}, forced expiratory volume in 1 s; FVC, forced vital capacity; GSEM, geometric standard error of the mean; PD₃₅-Grs, the cumulative dose of bronchoconstrictor that induced a decrease of 35% in respiratory conductance.

ipratropium bromide [12,13,15,27–29], although contradictory data have been reported [30]. We observed that inhalation of oxitropium bromide, a currently available short-acting cholinergic antagonist, partly attenuated airway responsiveness to histamine in subjects with mild-to-moderate asthma (unpublished observation). Accumulating evidence suggests that histamine causes bronchoconstriction both directly, by stimulating H₁ receptors on airway smooth muscle, and indirectly, through vagal reflex. If inhalation of salbutamol directly reduces the contractility of airway smooth muscle, airway responsiveness to both MCh and histamine should be attenuated, at least by a common mechanism downstream of M₃-receptor-mediated and H₁-receptor-mediated pathways. The dose of salbutamol used in this study did not alter basic pulmonary function or airway responsiveness to MCh, but it did alter airway responsiveness to histamine. Another possibility is that salbutamol suppresses the activation of H₁-receptor-coupled Gs proteins without affecting activation of M₃-receptor-coupled Gs proteins, although we have not found any reports of investigations supporting this possibility. In this context, the present study may be in accordance with the hypothesis that inhalation of low-dose salbutamol attenuates airway responsiveness to histamine, presumably by suppressing cholinergic neuroeffector transmission.

The present study may provide a novel insight into the bronchoprotective mechanism of β_2 -adrenoceptor agonists in clinical settings. There are difficulties in applying the

results obtained from experimental studies *in vitro* directly for the findings of clinical efficacy. A majority of data regarding the relaxant effects of salbutamol on human airway smooth muscle preparations show that the duration of relaxant action (e.g., $T_{1/2}$) was within 10 min [31,32], while the bronchodilatory effects of the same drug on subjects with asthma, evaluated by increases in FEV_{0.75} and FVC from baseline values, continued for more than 210 min [33]. Human airway smooth muscle tissues show intrinsic tone that is maintained by various chemical mediators and neurotransmitters, including ACh. The muscle tone is markedly augmented by the increase in local concentrations of those spasmogens in symptomatic asthma, which may account for the improvement in spirometric measurements after inhalation of a clinical dose of salbutamol. It is rational to suppose that a high concentration of salbutamol in the airway tissues has a direct relaxant effect on airway smooth muscles. However, given the rapid decline in drug concentration, it is not plausible that the subsequent relaxant effect is due to direct action on airway smooth muscles [33]. This efficacy is, at least partly, explained by the indirect bronchoprotective action of low-dose salbutamol.

To our knowledge, there are no publications examining the effect of salbutamol on histamine-induced ACh release in human airway smooth muscle preparations. Bai et al. reported that the concentration of fenoterol (165 nM) required to suppress EFS-evoked contraction of human tracheal

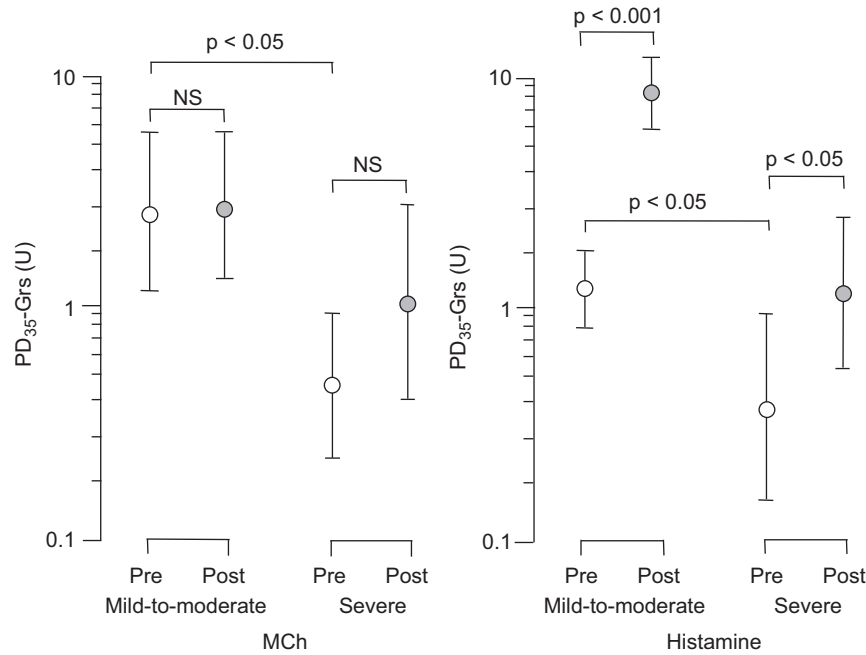


Fig. 3 – Airway responsiveness to MCh and histamine before and after inhalation of low-dose salbutamol (1 μ g) in subjects with mild-to-moderate asthma ($n=6$) and those with severe asthma ($n=6$). Values of PD₃₅-Grs are expressed as geometric means and geometric standard errors of means (GSEM). Abbreviations: MCh, methacholine; PD₃₅-Grs, the cumulative dose of bronchoconstrictor that induced a decrease of 35% in respiratory conductance.

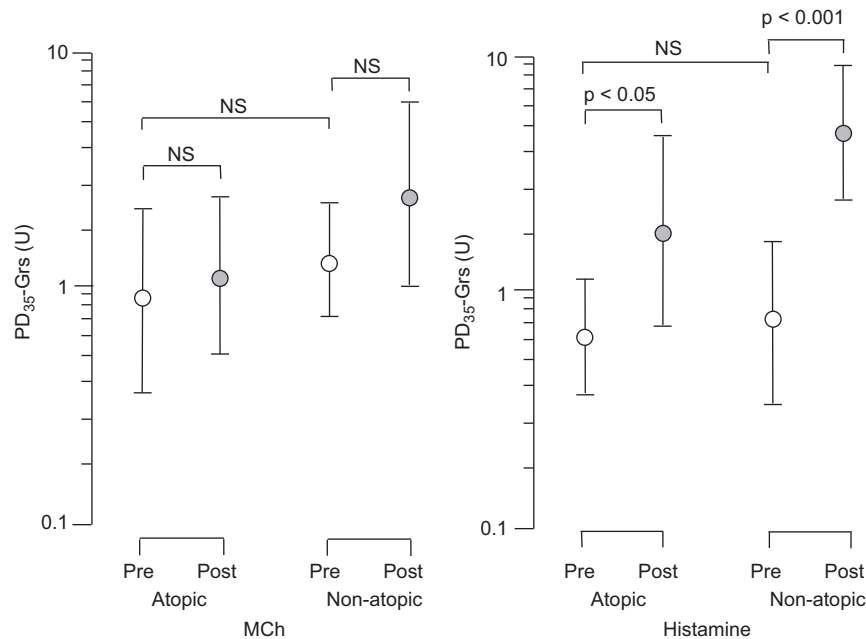


Fig. 4 – Airway responsiveness to MCh and histamine before and after inhalation of low-dose salbutamol (1 μ g) in the atopic group ($n=6$) and non-atopic group ($n=6$). Values of PD₃₅-Grs are expressed as geometric means and geometric standard errors of means (GSEM). Abbreviations: MCh, methacholine; PD₃₅-Grs, the cumulative dose of bronchoconstrictor that induced a decrease of 35% in respiratory conductance.

smooth muscles by 50% (IC₅₀) was significantly less than that required to suppress closely matched contraction induced by exogenously applied ACh to a comparable degree (302 nM) [3]. Given that the relaxant potency of fenoterol (EC₅₀) is 10 times greater than that of salbutamol [31,32], the minimum concentration of salbutamol required to suppress

ACh release from vagal nerve terminals is estimated to be 10⁻⁶ M. It remains uncertain whether this range of concentration could be attained in the airway tissues by our experimental protocol. We determined the dose of salbutamol on the basis of our previous *in vitro* study [2], in which procaterol (10⁻¹⁰–10⁻⁷ M) suppressed EFS-evoked contraction

of isolated human bronchus in a dose-dependent manner, while procaterol (10^{-10} – 10^{-9} M) had no effect on ACh-induced contraction. Although the data were obtained with procaterol but not salbutamol, this may provide a rationale for using 1/100 of the clinical dose of β_2 -adrenoceptor-agonists to test our hypothesis.

Concerns have risen that disease progression may be associated with regular or frequent use of SABA without corticosteroid medication in patients with moderate to severe asthma, partly via possible impairment of β_2 -adrenoceptor function [17,34,35]. Although the majority of patients with severe asthma may be treated with optimal inhaled corticosteroids in addition to other drugs including SABA or/and LABA, there is a possibility that continuous use of β_2 -adrenoceptor agonists might selectively induce the impairment of β_2 -adrenoceptor function in cholinergic neuroeffector transmission. In the present study, all subjects with severe asthma had been treated with high-dose inhaled corticosteroids but had a considerable history of topical SABA use. However, significant protection of histamine-induced bronchoconstriction by low-dose salbutamol was still observed in subjects with severe asthma, as in those with mild to moderate asthma, suggesting that the function of β_2 -adrenoceptor in cholinergic neuroeffector transmission might be well preserved even in severe asthma.

In conclusion, inhalation of low-dose salbutamol suppressed histamine-induced bronchoconstriction without affecting MCh-induced bronchoconstriction, irrespective of asthma severity or phenotype. This property may partly explain bronchoprotection attained with a substandard dose of β_2 -adrenoceptor agonists in clinical practice.

Conflict of interest

Koichiro Matsumoto received subsidies or donations from AstraZeneca.

Hiromasa Inoue received lecture fees from GlaxoSmith-Kline and Boehringer Ingelheim; research funding from Eisai; and subsidies or donations from AstraZeneca, GlaxoSmith Kline, Boehringer Ingelheim/Pfizer, and Novartis.

Hisamichi Aizawa, Satoru Fukuyama, Makoto Yoshida, Masashi Komori, Syohei Takata, and Hiroshi Koto have no potential conflicts of interest to declare.

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