Airway Responsiveness to Acetylcholine in Congenitally Bronchial-Hypersensitive (BHS) and Bronchial-Hyposensitive (BHR) Guinea Pigs in Vivo and in Vitro

Yukihiro YAGI¹⁾, Masayoshi KUWAHARA¹⁾, Masashi MAEDA¹⁾, Hiromi KADOTA¹⁾,
Shizue SAEGUSA¹⁾, Jun-ichi BIRUMACHI¹⁾, Shigeru SUGANO¹⁾,
Ryoji NISHIBATA²⁾, Hiroki MIKAMI²⁾, and Hirokazu TSUBONE¹⁾

¹⁾Department of Comparative Pathophysiology, Graduate School of Agriculture and Life Sciences,
The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, and ²⁾Institute of
Bio-Active Science, Nippon Zoki Pharmaceutical, Hyogo 673-1461, Japan

Abstract: The characteristics of airway responsiveness to acetylcholine (ACh) in congenitally bronchial-hypersensitive (BHS) and bronchial-hyposensitive (BHR) guinea pigs were clarified in vivo and in vitro. We measured the change in ventilatory mechanics in response to ACh inhalation by means of the bodyplethysmograph and the contractile responses of isolated trachea to ACh and carbachol (CCh). Further, muscarinic receptor subtypes involved these responses were identified. The basal values for ventilatory mechanics in BHS were not significantly different from those in BHR. Respiratory resistance to ACh was progressively increased in a time- and dose-dependent manner in BHS. The contractile responses of tracheal smooth muscle to ACh in BHS were significantly greater than those in BHR, but CCh-induced responses in BHS and BHR were similar. ACh- and CCh-induced contractions were mediated via M_3 receptors. These results suggested that the falling-down of BHS in response to ACh inhalation was caused by the strong constriction of the airway and the reduction in ventilation. Moreover, the airway hyperresponsiveness to ACh in BHS might be partly dependent on the change in acetylcholinesterase activity.

Key words: acetylcholine, acetylcholinesterase, airway smooth muscle, carbachol, muscarinic receptor

Introduction

Airway hyperresponsiveness (AHR) is associated with numerous respiratory diseases including asthma

and chronic obstructive pulmonary disease [3, 13, 29]. Clarification of the cause of AHR is important for the clinical treatment of these disease and the development of drugs, but the physiological changes that account for

(Received 5 February 1998 / Accepted 27 March 1998)

Address corresponding: Y. Yagi, Department of Comparative Pathophysiology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

This study was supported by Grant-in-Aid from the Scientific Research Fund of the Ministry of Education, Science, Sports and Culture, Japan.

increases in responsiveness are poorly understood. To advance research on AHR, we have developed two Hartley-derived lines of guinea pigs which have different bronchial reactivity to acetylcholine (ACh) exposure through selective breeding techniques based on the onset time of falling-down due to bronchoconstriction caused by inhalation of ACh (0.08%) [20]. One (bronchial-hypersensitive; BHS) has a more sensitive bronchial reaction to ACh than the other (bronchial-hyposensitive; BHR). Moreover, we have demonstrated recently that acetylcholinesterase (AChE)-positive nerves around bronchi are morphometrically more BHR than BHS [30], but the characteristics of respiratory function and contractile response of airway smooth muscle in BHS and BHR remain uncertain. Therefore, the purpose of this study was to (1) clarify the airway responsiveness to ACh inhalation *in vivo*, (2) evaluate the role of AChE activity in the expression of bronchial hypersensitivity *in vitro* by comparison with the bronchial contractile response to ACh and carbachol (CCh; totally resistant to hydrolysis by either AChE or non-specific cholinesterase) in BHS and BHR, and (3) characterize the muscarinic receptor subtypes involved in the ACh-induced bronchial contractile response of airway smooth muscle in BHS and BHR.

Materials and Methods

Animals

Thirty four male BHS and 35 male BHR guinea pigs (400–700 g) were used (Nippon Zoki Pharmaceutical Co., Ltd.). All BHS had a short falling-down time (~90 sec) in a screening test with 0.08% ACh exposure at 8 weeks old, but none of the BHR showed a falling-down.

Airway responsiveness to ACh *in vivo*

Six male BHS and 6 male BHR guinea pigs were used for the *in vivo* study. Ventilatory mechanics were measured by the oscillation method applied to a bodyplethysmograph (volume = 1.3 l) according to the previous study [5]. Oscillation was set up with an electric signal generator (VP-7101A, National, Tokyo, Japan) and an amplifier (AU- α 607XR, Sansui, Tokyo, Japan) in series with a loudspeaker (FW405, Fostex, Tokyo, Japan). The frequency and intensity of sine wave oscillation were 20 Hz and 30 dB. The animal

was fitted with a face mask (volume = 6 ml) which was completely separated from the bodyplethysmograph. The respiratory airflow ($\dot{V}$) was monitored at the opening of the face mask via a differential pressure transducer (TPP-602T, Nihon Kohden, Tokyo, Japan) connected to a respiratory flow amplifier (AR-601G, Nihon Kohden, Tokyo, Japan), and the pressure (Pbox) in the bodyplethysmograph was also monitored with a pressure transducer (PD104, Toyoda, Tokyo, Japan) in series with a DC-amplifier (AA6010, Toyoda, Tokyo, Japan). The $\dot{V}$ signal during the application of the 20 Hz oscillation was electrically treated with a bandpass filter (DPA-21E, DIA Medical, Tokyo, Japan) to eliminate lower frequency components, such as a spontaneous breathing cycle; thus more pure flow ($\dot{V}_{osc}$) changes corresponding to 20 Hz-oscillation were recorded. All the signals were monitored with a thermal recorder (RT3100, NEC/San-ei, Tokyo, Japan) and stored by means of a digital tape recorder (PC208, SONY, Tokyo, Japan). The $\dot{V}$, $\dot{V}_{osc}$ and Pbox corresponding to the oscillation were served for analyzing the respiratory mechanics, i.e., respiratory resistance (Rrs), specific airway conductance (SGaw) and dynamic airway compliance (Cdyn) [8, 25, 37]. The SGaw was obtained by correcting the airway conductance (1/Rrs) by V_T during quiet breathing at the initial stage before any exposure to aerosol. The V_T was measured by an integration of the $\dot{V}$ signal without 20 Hz-oscillation.

ACh solutions at concentrations of 0.0125, 0.025, 0.05, and 0.1% (w/v) diluted with saline were prepared just before the each experiment. Twenty ml of each solution was used for aerosolization with an ultrasonic nebulizer (TUR-3200, Nihon Kohden, Tokyo, Japan) for individual inhalation. The aerosolizing rate of the drugs was 0.25 ml/min and the aerosol was delivered at a flow rate of 1 l/min with room air to the face mask as described above. Each animal inhaled ACh aerosol from the lowest to highest concentrations for 150 sec. The exposure of each aerosol concentration was done at 10–15 min intervals, ensuring almost complete recovery of the quiet breathing pattern before the next inhalation of ACh. If dyspnea (caused by a lack of respiratory airflow due to a strong bronchoconstriction) was observed during the inhalation, the aerosol exposure was immediately stopped. The changes in Pbox and $\dot{V}$ before and during inhalation at each concentration of aerosol were recorded.

Airway responsiveness to ACh and CCh in vitro

Twenty-eight male BHS and 29 male BHR guinea pigs were used for the *in vitro* study. The animals were killed by stunning and exsanguination. The trachea was rapidly excised and placed in physiological solution (mM): NaCl 136.9, KCl 5.4, CaCl₂ 1.5, MgCl₂ 1.0, NaHCO₃ 23.8, glucose 5.5, EDTA 0.01. Fat and connective tissue were cleaned from the trachea which was opened by cutting longitudinally opposite the tracheal muscle and cut into small strips (approx. 3 mm wide and 7 mm long). The luminal surface was scraped with a cotton wool swab to remove the epithelium. Each strip was mounted in a 20 ml organ bath in physiological solution. Indomethacin (3×10^{-6} M) was routinely included to prevent synthesis of cyclooxygenase products, which could contribute to the contractile responses. High-K⁺ (72.7 mM) solution was made by replacing NaCl with equimolar KCl. This solution-induced sustained change in muscle tension was used as a reference (100%) in Figs. 3, 4 and 6. In Ca²⁺-free experiments, the same physiological solution was used except that 1.5 mM CaCl₂ was omitted and 0.5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) was added. These solutions were saturated with a 95% O₂ and 5% CO₂ mixture at 37°C and pH 7.4.

Muscle strips were placed under an initial resting tension of 1 g and allowed to equilibrate for 1 hr before the experiment was begun. At first the maximum contractile response to high-K⁺ solution was determined. Cumulative concentration response curves were obtained with ACh and CCh by increasing the concentration of each agent from 10⁻⁹ to 10⁻³ M. Each succeeding concentration was given after the response to the previous concentration had reached a plateau. Muscle contractions were measured continuously with an isometric force transducer (TB-612T, Nihon Kohden, Tokyo, Japan).

Muscle strips were also incubated with muscarinic receptor antagonists for 30 min to characterize the muscarinic receptor subtypes: 10⁻⁵ M methoctramine (M₂ receptor) or 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP; M₃ receptor). Then responses to 10⁻⁴ M ACh or CCh were obtained.

Drugs

The following drugs were used: acetylcholine chloride, indomethacin, nifedipine (Wako Pure Chemical Industries Ltd., Tokyo, Japan); carbachol chloride (Sigma Chemical Co., St. Louis, MO, USA); methoctramine and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (Research Biochemicals International, Natick, MA, USA).

Statistical analysis

Results are expressed as the means $\pm$ SEM. Statistical analysis was performed by an analysis of variance (ANOVA). A value of $P < 0.05$ was considered significant.

Results*Airway responsiveness to ACh in vivo*

The basal values for Rrs, V_T, SGaw and Cdyn are summarized in Table 1. Because no noticeable differences between the 'end-inspiratory' and 'end-expiratory' phases were observed in these parameters throughout the ACh inhalation, all changes in ventilatory mechanics were expressed as the 'end-inspiratory' phase. There was no significant difference between BHS and BHR in these parameters. As shown in Fig. 1, Rrs to 0.05% ACh inhalation in BHS was progressively increased in a time-dependent manner, but that in BHR was slightly decreased. V_T, SGaw and Cdyn response to 0.05% ACh inhalation in BHR was progressively increased in

Table 1. Basal values for ventilatory mechanics in bronchial hypersensitive and hyposensitive strains of guinea pigs

	Rrs (cmH ₂ O/ml/sec)	V _T (ml)	SGaw (ml/sec/cmH ₂ O/ml)	Cdyn (ml/cmH ₂ O)
Bronchial hypersensitive strain (n=6)	89.3 $\pm$ 12.9	5.18 $\pm$ 0.83	2.72 $\pm$ 0.48	314.2 $\pm$ 48.8
Bronchial hyposensitive strain (n=6)	107.8 $\pm$ 15.7	3.46 $\pm$ 0.49	3.01 $\pm$ 0.33	215.5 $\pm$ 31.6

Values are expressed as the mean $\pm$ SEM. Rrs: respiratory resistance; V_T: tidal volume; SGaw: specific airway conductance; Cdyn: dynamic compliance.

Fig. 1. Changes in respiratory resistance (Rrs), tidal volume (V_T), specific airway conductance (SGaw), and dynamic compliance (Cdyn) during inhalation of acetylcholine (0.05%) in BHS (○) and BHR (●). Data are expressed as percentages of the pre-inhalation values. Each point represents the mean $\pm$ SEM for 6 animals. * $P < 0.05$ vs pre-inhalation; # $P < 0.05$ vs BHR.

a time-dependent manner, but that in BHS was slightly decreased. These changes were also confirmed at all doses (data not shown). Furthermore, dose-dependent responses on Rrs, V_T , SGaw and Cdyn at 90 sec during inhalation to ACh were observed in BHS (Fig. 2), but these changes were not seen in BHR.

Airway responsiveness to ACh and CCh *in vitro*

Figure 3 shows concentration response curves of tracheal muscle strips to ACh in BHS and BHR. ACh (10^{-9} to 10^{-3} M) induced an increase of contraction in a concentration-dependent manner. The contraction response to ACh was significantly ($P < 0.05$) larger in BHS than in BHR. EC_{50} values for BHS and BHR were 2.3×10^{-6} and 4.3×10^{-6} M, respectively.

As shown in Fig. 4, CCh-induced concentration response curves were similar in BHS and BHR. There was no significant difference at any concentrations of CCh.

When the mean contractile responses to CCh (10^{-4} M) were taken as a reference (100%), there was a significant difference between ACh and CCh in BHR (Fig. 5), but no such difference was observed in BHS.

In the absence of extracellular Ca^{2+} , ACh- and CCh-induced contractions were noticeably reduced in both BHS and BHR (Fig. 6), but the CCh-induced contractions in BHR were larger than the ACh-induced ones. In BHS, ACh- and CCh-induced contractions were almost the same.

Effects of nifedipine treatment on contractile response

Fig. 2. Concentration responses on respiratory resistance (Rrs), tidal volume (V_T), specific airway conductance (SGaw), and dynamic compliance (Cdyn) at 90 sec during inhalation of acetylcholine in BHS (○) and BHR (●). Data are expressed as percentages of the pre-inhalation values. Each point represents the mean $\pm$ SEM for 6 animals. * $P < 0.05$ vs pre-inhalation; # $P < 0.05$ vs BHR.

are shown in Fig. 7. KCl (60 mM)-induced contractions in BHS and BHR were completely inhibited by nifedipine (10^{-6} M) pretreatment, but nifedipine had no effect on ACh- or CCh-induced contractions in BHS or BHR. There was no difference between BHS and BHR in these responses.

As shown in Fig. 8, both ACh- and CCh-induced (10^{-4} M) contractions were still present after M_2 antagonist (10^{-5} M) treatment, but because M_3 antagonist (10^{-5} M) completely blocked ACh- and CCh-induced (10^{-4} M) contractions, no contractions were observed in either BHS or BHR.

Discussion

This study has demonstrated the characteristics of respiratory function and contractile response of airway smooth muscle to ACh in BHS and BHR. The airway responsiveness to inhaled ACh was remarkably different from that in BHS and BHR. The decreases in V_T , SGaw and Cdyn as well as the increase in Rrs indicate the strong constriction of airway in BHS. The contractile responses to ACh in BHS were significantly greater than those in BHR, but CCh-induced responses were similar in BHS and BHR. Moreover, ACh-induced airway smooth muscle contractions in BHS and BHR were mediated via M_3 receptors. These results suggest that

Fig. 3. Concentration response curves for acetylcholine in trachea from BHS (○) and BHR (●). 100% represents the 72.7 mM KCl-induced sustained contraction. Each point represents the mean $\pm$ SEM from 10 separate experiments. * $P < 0.05$ vs BHR.

Fig. 4. Concentration response curves for carbachol in trachea from BHS (○) and BHR (●). 100% represents the 72.7 mM KCl-induced sustained contraction. Each point represents the mean $\pm$ SEM from 6–7 separate experiments.

Fig. 5. Contractile responses to acetylcholine (10^{-4} M: ■) and carbachol (10^{-4} M: □) in trachea from BHS and BHR. 100% represents the mean contractile responses to carbachol (10^{-4} M). Each bar represents the mean $\pm$ SEM from 6–10 separate experiments. * $P < 0.05$ vs carbachol.

Fig. 6. Effects of acetylcholine (10^{-4} M: ■) and carbachol (10^{-4} M: □) on contractile responses in trachea from BHS and BHR in absence of extracellular Ca^{2+} . 100% represents the 72.7 mM KCl-induced sustained contraction. Each bar represents the mean $\pm$ SEM from 8–10 separate experiments.

the AHR to ACh in BHS is partly dependent on the change in AChE activity.

AHR is a risk factor for the development of asthma, and may precede the expression of the asthmatic phe-

notype [10, 11]. Human and animal studies have demonstrated a genetic predisposition to AHR [16, 17, 24, 26, 33], such as an increased incidence of AHR in monozygotic twins as compared with dizygotic twins

Fig. 7. Effects of nifedipine on KCl (60 mM)-, acetylcholine (ACh: 10^{-4} M)-, and carbachol (CCh: 10^{-4} M)-induced contractile responses in trachea from BHS (□) and BHR (■). 100% represents the contractile responses without nifedipine treatment. Each bar represents the mean $\pm$ SEM from 6–7 separate experiments.

Fig. 8. Effects of muscarinic M_2 receptors antagonists (10^{-5} M) on acetylcholine (ACh: 10^{-4} M)- and carbachol (CCh: 10^{-4} M)-induced contractile responses in trachea from BHS (□) and BHR (■). 100% represents the contractile responses without muscarinic M_2 receptors antagonists treatment. Each bar represents the mean $\pm$ SEM from 6–7 separate experiments.

[15, 34]. If inbred strains are tested under controlled environmental conditions, any differences observed among strains can be attributed to genetic determinants. Numerous animal models have been developed to study asthma [9, 16, 31], but the physiological changes that account for increases in responsiveness are poorly understood. We have developed two inbred lines of guinea pigs that differed in the onset time of falling-down to ACh [20]. Furthermore, we have recently clarified that AChE-positive nerves around bronchi are morphometrically greater in BHR than BHS [30]. Other investigators have also reported studies on reduced AChE activity in airways [14, 22, 23]. In view of these lines of evidence, it is conceivable that the reduction in AChE activity on airway smooth muscle may play an important role in the expression of AHR.

BHS had greater airway responsiveness to ACh inhalation. Our results clearly showed that the falling-down of BHS to ACh inhalation was caused by the decreased V_T , SGaw and Cdyn as well as the increased Rrs, because all of these changes indicated the strong constriction of airways and the reduction of ventilation, and the airway responsiveness to ACh inhalation in BHR was rather in contrast to that in BHS. BHR was highly resistive to ACh inhalation, because

BHR could maintain the ventilatory function in response to ACh inhalation due to the increasing V_T , SGaw and Cdyn.

In BHS, contractile responses to ACh and CCh were very similar under conditions in the presence and absence of extracellular Ca^{2+} . In BHR, however, CCh-induced contraction was greater than the ACh-induced one under those conditions. Moreover, 0.0125% CCh aerosol induced the falling-down with almost the same onset time in both BHS and BHR (unpublished observation). Of course the possibility of an altered number and/or binding affinity of muscarinic receptors cannot exclude the differences in contractile responses, because those in lung tissue were significantly different in BHS and BHR [21], but our results showed that the reduction in AChE activity on airway smooth muscle was relevant to AHR to ACh in BHS.

AChE and butyrylcholinesterase are present in the smooth muscle and ganglia of guinea pig and rat trachea [23, 28]. Although both of these can hydrolyze ACh, AChE has a much greater potential than butyrylcholinesterase [32]. Itabashi *et al.* [14] have reported that the choline-deficient diets-induced changes in AChE activity have altered the contractile response of lung parenchymal strips to ACh in rats, and they

suggest that the amount of intact ACh inducing the contraction may depend on the cleavage of ACh by AChE. It is therefore likely that the hyperresponsiveness to ACh in BHS is dependent on the decreased degradation of ACh.

Muscarinic receptors have been functionally classified into three major subtypes which are present in the airways: M_1 , M_2 and M_3 receptors [2]. M_1 receptors facilitate neurotransmission through parasympathetic ganglia and enhance cholinergic reflexes. M_2 receptors act as autoreceptors on post-ganglionic cholinergic nerves and inhibit ACh release. M_3 receptors mediate contractile responses in airway smooth muscle. Because M_3 receptor antagonist completely abolished ACh- and CCh-induced contractions in both BHS and BHR, airway smooth muscle contractions in BHS and BHR were mediated via M_3 receptors. These results are coincident with an earlier report which has shown that M_3 receptors are localized predominantly to the smooth muscle of airways in guinea pig [18]. Recently it has been established that M_2 receptors may play a functional role in counteracting the bronchodilator response to β agonists due to activation of adenylyl cyclase [7, 36], and M_2 receptors on sympathetic nerve terminals inhibit the release of norepinephrine from these nerves [27], so that further studies will be needed to clarify the role of M_2 receptors in airway responsiveness in both BHS and BHR.

The major routes of Ca^{2+} entry into airway smooth muscle cells are voltage-operated Ca^{2+} channels (VOCs) and receptor-operated Ca^{2+} channels (ROCs), and Ca^{2+} release from intracellular stores contributes to the airway smooth muscle contractions. Therefore we have partially clarified the role of Ca^{2+} influx and intracellular Ca^{2+} release to ACh- and CCh-induced contractions in BHS and BHR. Responses to KCl were completely abolished by nifedipine, whereas ACh- and CCh-induced contractions were resistant to nifedipine. Because KCl and nifedipine act on L-type VOCs in smooth muscle, it seems that ACh- and CCh-induced contractions in BHS and BHR occur through ROCs. Furthermore, the removal of extracellular Ca^{2+} noticeably reduced the contractile responses induced by ACh and CCh. These data are generally consistent with earlier studies on normal guinea pigs [1, 4, 6, 19].

The regulatory mechanisms of the airway smooth muscle contraction in BHS and BHR were not clarified

by this study. Several possible signal transduction cascades involving protein kinase C in the mechanism of smooth muscle contraction have been outlined [12]. Of course further studies will be needed to clarify the mechanisms of the airway smooth muscle contraction in these animals, but because BHS showed an enhanced contractile airway responsiveness to bradykinin [35], histamine [5] and leukotriene D_4 [21], BHS might have airway hyperresponsiveness to a wide range of airway stimulants. Therefore we believe that further studies on BHS and BHR guinea pigs may contribute to the clarification of the pathogenic mechanism underlying the airway smooth muscle hyperresponsiveness in asthma.

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