Abstract. Objective: The present study compared the effects of repeated antigen exposure on the development of hyperresponsiveness and the expression of RhoA in the main bronchial and lower tracheal smooth muscles of sensitized rats.

Methods: Actively sensitized rats were repeatedly challenged by antigen inhalation. Twenty-four hours after the final antigen challenge the isometrical contractions of the bronchial and tracheal smooth muscles were measured. Immunoblottings were also performed using bronchial and tracheal homogenates and the density ratios of RhoA/β-actin were calculated to quantify the levels of RhoA.

Results: Acetylcholine-induced contraction of bronchial, but not tracheal, smooth muscle of antigen-treated rats was significantly augmented as compared with that of control rats, indicating that airway hyperresponsiveness appeared by antigen challenge in bronchial smooth muscle. RhoA expression in bronchial, but not tracheal, smooth muscle was significantly increased in the antigen-treated animals.

Conclusion: The increased expression of RhoA is suggested to have an important role in developing hyperresponsiveness of bronchial smooth muscle.

Key words: Airway hyperresponsiveness – Trachea – Bronchus – Acetylcholine – RhoA

Introduction

Airway hyperresponsiveness (AHR) is a common feature of allergic bronchial asthma, but the underlying mechanism(s) of AHR have yet to be elucidated. The importance of AHR in the pathogenesis of asthma has been suggested by its relevance to the severity of this disease [1, 2]. The authors previously reported that repeated antigen inhalation to actively sensitized rats causes bronchial inflammation and marked AHR to inhaled contractile agonists [3]. In this animal model of AHR, the isolated smooth muscle of the main bronchus also exhibited hyperresponsiveness [3, 4, 5]. Thus, it is possible that the mechanisms responsible for the AHR exist, at least in part, in the smooth muscle itself.

It has been demonstrated that agonist stimulation increases myofilament Ca\(^{2+}\) sensitivity in variety types of smooth muscles which were permeabilized by β-escin or α-toxin [6, 7, 8]. A participation of Rho protein in the agonist-induced Ca\(^{2+}\) sensitization has been suggested by many investigators [e.g. 7, 9, 10]. Recently, we also demonstrated an existence of Ach-induced, RhoA-mediated Ca\(^{2+}\) sensitization in smooth muscle contraction of β-escin-permeabilized intrapulmonary bronchial strips and, furthermore, a strong augmentation of this Ca\(^{2+}\) sensitizing effect in the AHR state [11]. It is thus possible that the augmented Ach-induced contraction of intact bronchial smooth muscle might be due to an enhanced Ca\(^{2+}\) sensitizing signal, especially RhoA-mediated pathways, in antigen-induced AHR rats. Our previous study has also reported that the smooth muscle hyperresponsiveness observed in the main bronchus of the AHR animals was not found in the lower trachea even at the AHR [3].

In the present study, the expression levels of RhoA in the main bronchial and lower tracheal smooth muscles of antigen-induced AHR rats were investigated to understand the reason of the site difference in the induction of hyperresponsiveness and to get an information on the pathogenesis of airway hyperresponsiveness.

Materials and methods

Sensitization and antigenic challenge

Male Wistar rats (6 weeks of age, specific pathogen-free, 170–190 g, Charles River Japan, Inc) were sensitized and repeatedly challenged with 2,4-dinitrophenylated Ascaris suum antigen (DNP-Asc) by the method described in the previous papers [4, 5]. Our previous study
revealed that the sensitization procedure to antigen used in the present study had no significant effect by itself on the ACh responsiveness of the bronchial muscle and muscarinic receptors property in rats [4]. So in the present study, the age-matched nonsensitized normal rats were used as control.

**Functional study**

The airway tissues under the larynx to lungs were removed under chloral hydrate (400 mg/kg, intraperitoneal) anesthesia as approved by the local Animal Care Committees at Hoshi University (Tokyo, Japan). About 4 mm length of the left main bronchus and lower trachea was isolated by the method described previously [3] and the resultant tissue ring preparations were then suspended in a 5-ml organ bath at a resting tension of 1.0 g. The isometrical contraction of the circular smooth muscle was measured with a force-displacement transducer (TB-612T, Nihon Kohden, Japan). The organ bath contained modified Krebs-Henseleit solution with the following composition (mM): NaCl 118.0, KCl 4.7, MgSO4 1.2, NaHCO3 25.0, KH2PO4 1.2 and glucose 10.0 (pH 7.4). The buffer solution was maintained at 37 °C and oxygenated with 95% O2-5% CO2. During an equilibration period in the organ bath, the tissues were washed four times at 15 min intervals and equilibrated slowly to a baseline tension of 1.0 g. Fifteen min after the last washing, higher concentrations of acetylcholine were successively added after attainment of a plateau response to the previous concentration. In another series of experiments, bronchial smooth muscle was depolarized with isotonic high K+ solution prepared by iso-osmotic replacement of NaCl by KCl in the presence of 10−6 M atropine and 10−6 M indomethacin.

**Western blot analyses**

Immunoblotting was performed by the method described previously [11] with a minor modification. To quantify the expression of RhoA and b-actin protein, western blot was performed in the homogenates of the main bronchi and lower trachea. The samples were subjected to 15% SDS-polyacrilamide gel electrophoresis (SDS-PAGE). Proteins were then electrophoretically transferred to nitrocellulose membranes (Hybond-ECL, Amersham). After blocking with 3% gelatin, the nitrocellulose membranes were then incubated with the primary antibodies. The primary antibodies used rabbit anti-RhoA C-terminal (1:2000 dilution; Santa Cruz Biotechnology, Inc) or mouse anti- b-actin N-terminal (1:5000 dilution; Sigma). Then the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG) (1:5000 dilution; Amersham) and goat anti-mouse IgG (1:5000 dilution; Amersham), detected by an enhanced chemiluminescent System (Amersham). The resultant images were scanned (Canoscan FB 636U; Canon) into a computer (iMac 233; Apple Computer, Inc.) and analyzed by densitometry system (Atto Densitograph Software for Macintosh version 4.0; Atto Co., Japan). The ratio of corresponding RhoA/b-actin in each lane was calculated as an index of RhoA level.

**Statistical analyses**

All the data were expressed as the mean with S.E. Statistical significance of difference was determined by Dunnett’s multiple analysis or two way analysis of variance (ANOVA).

**Results**

**Functional study**

Fig. 1 shows the acetylcholine responsiveness of the bronchial and tracheal smooth muscles isolated from control and repeatedly antigen challenged rats. Acetylcholine elicited a concentration-dependent contractile response. In bronchial smooth muscle, the concentration-response curve to acetylcholine was significantly shifted upward after repeated antigenic challenge (p < 0.05 by ANOVA), whereas no significant difference was observed in the case of tracheal smooth muscle. Application of isotonic high K+ solution (10, 30 and 60 mM) also elicited a concentration-dependent contractile response in all tissues used (Fig. 1). No significant difference in K+ responsiveness was observed between groups in either bronchial or tracheal smooth muscles.

**Western blot**

In the present study, anti-RhoA antibody was used to detect RhoA protein of rat bronchial and tracheal preparations. Representative immunoblots for RhoA and β-actin of bronchial and tracheal preparations from control and repeat-