Upregulation of *rhoA* mRNA in Bronchial Smooth Muscle of Antigen-induced Airway Hyperresponsive Rats

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Abstract

It has recently been suggested that RhoA plays an important role in the enhancement of Ca²⁺ sensitization observed in smooth muscle contraction. In the present study, the expression of *rhoA* mRNA in the bronchial smooth muscle of antigen-induced airway hyperresponsive rats was compared with that of control animals. Reverse transcription-polymerase chain reaction experiments using total RNA from these tissue specimens and the specific primers revealed *rhoA* mRNA to be expressed in bronchial smooth muscle of the rat. The *rhoA* mRNA expression in bronchial smooth muscle of the hyperresponsive rats was significantly increased in comparison to that of control animals. It is thus possible that upregulation of RhoA protein might be involved in the mechanism underlying the increased contractility of the bronchial smooth muscle which occurs with airway hyperresponsiveness.

Key words: asthma, airway hyperresponsiveness, bronchial smooth muscle, Ca²⁺ sensitization, RhoA, mRNA

Introduction

Smooth muscle contraction is mainly regulated by an increase in the concentration of cytosolic Ca²⁺. Recently, additional mechanisms have been suggested in agonist-induced smooth muscle contraction as a result of studies which used the simultaneous measurement of force development and intracellular Ca²⁺ concentration, as well as those on various smooth muscle preparations which have been chemically permeabilized. It has been demonstrated that agonist stimulation increases myofilament Ca²⁺ sensitivity in β -escin-permeabilized smooth muscle of the rat coronary artery (Satoh *et al.*, 1994), guinea pig vas deferens (Fujita *et al.*, 1995), canine trachea (Bremerich *et al.*, 1997) and rat bronchus (Chiba *et al.*, 1999b). Although the detailed mechanism is not fully understood, many investigators have suggested the

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participation of RhoA, a monomeric GTP binding protein, in the agonist-induced Ca^{2+} sensitization (*e.g.*, Fujita *et al.*, 1995; Gong *et al.*, 1997; Chiba *et al.*, 1999b).

Asthmatic patients have an increased contractility of airway smooth muscle (Roberts *et al.*, 1984), which might be a major cause of airway hyperresponsiveness (AHR). Similarly, an increased responsiveness of bronchial smooth muscle has been demonstrated in a rat model of AHR induced by repeated antigen inhalation (Chiba and Misawa, 1995a, b). In AHR rats, it has also been demonstrated that acetylcholine-induced RhoA-mediated Ca^{2+} sensitization is markedly augmented concomitantly with an increased expression of RhoA protein in the bronchial smooth muscle (Chiba *et al.*, 1999b). It is thus possible that the upregulation of RhoA protein in airway smooth muscle might be an essential event in the pathogenesis of AHR. However, the level of expression of *rhoA* mRNA in AHR has not yet been elucidated. In the present study, we have examined the level of expression of *rhoA* mRNA in the bronchial smooth muscle of antigen-induced AHR rats.

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 our previous papers (Chiba & Misawa, 1995a,b; Chiba *et al.*, 1999b). Age-matched non-sensitized normal animals were used as controls (Chiba and Misawa, 1995a,b; Chiba *et al.*, 1999b).

Isolation of total RNA

Twenty-four hours after the last antigen challenge, bronchial tissue preparations were prepared in essentially the manner described previously (Chiba *et al.*, 1999b). In brief, the airway system below the main bronchi to the lungs was removed and immediately soaked in ice-cold, oxygenated Krebs-Henseleit solution (118.0 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 25.0 mM NaHCO_3 , 1.2 mM KH_2PO_4 , 10.0 mM glucose, pH 7.4). The airways were carefully cleaned of adherent connective tissue, blood vessels and lung parenchyma using a stereomicroscope. The airway epithelium was removed as completely as possible by gently rubbing with a pair of keen-edged tweezers (Chiba *et al.*, 1999b). Then the bronchial segments (containing main and intrapulmonary bronchi) were quickly frozen with liquid nitrogen, and the tissue crushed using a CryopressTM (CP-100W; Niti-on, Co. Ltd., Japan: 15 sec $\times$ 3). Total RNA extraction was performed on each frozen tissue sample using the acid guanidium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987) and stored at -85°C until use. The total RNA experiments were performed on extracts from 5 different animals, and the mean $\pm$ s.e.m. determined.

Reverse transcription-polymerase chain reaction (RT-PCR)

The level of *rhoA* mRNA was examined by RT-PCR, a simple method that is widely used for semi-quantitative measurement of mRNA levels (*e.g.*, Niiro *et al.*, 1997; Ma *et al.*, 2002; Murray

et al., 2002; Chen *et al.*, 2003; Medina *et al.*, 2003; Zhu *et al.*, 2003). Briefly, cDNAs were prepared from the total RNA (0.5 μ g) by using a Takara RNA PCR Kit (Ver. 2.1; Takara, Tokyo, Japan) in a total volume of 20 μ l reaction buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM MgCl₂, 1 mM dNTP mixture, 1 U μ l⁻¹ RNase inhibitor, 2.5 μ M random 9 mers and 0.25 U μ l⁻¹ avian myeloblastosis virus reverse transcriptase. The reaction mixture was incubated for 10 min at 30°C followed by 60 min at 42°C to initiate the synthesis of the cDNAs. Reverse transcriptase was inactivated at 99°C for 5 min. Then the followings were added to the RT reaction mixture (10 μ l): 0.5 μ l of 0.1 mM forward primer, 0.5 μ l of 0.1 mM reverse primer, 4 μ l of 10 $\times$ amplification buffer (100 mM Tris-HCl, pH 8.3, 0.5 M KCl), 3 μ l of 25 mM MgCl₂, 31.8 μ l of H₂O, and 0.25 μ l of 5 U μ l⁻¹ *Taq* polymerase. The PCR primers for rat *rhoA* were 5'-GTGATTGTTGGTGATGGAGC-3' (sense) and 5'-CTCGTGGCCATCTCAAAAAC-3' (antisense) (Yoshimura *et al.*, 1997). The PCR primers for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5'-CCATCACTGCCACTCAGAAGAC-3' (sense) and 5'-TACTCCTTGAGGCCATGTAGG-3' (antisense), which were designed from published sequences. The thermal cycle profile used in the present study was: 1) denaturing for 30 sec at 95°C, 2) annealing the primers for 30 sec at 60°C and then 3) extending the primers for 60 sec at 72°C. PCR amplifications were performed for 15, 20, 25, 30 and 35 cycles. A portion (10 μ l) of the PCR mixture was subjected to electrophoresis on 2% agarose gel (E-Gel™; Invitrogen, CA, USA) and visualized by ethidium bromide staining. The intensity of bands was quantified by a densitometer (Atto Densitograph; Atto Co., Tokyo, Japan). The ratios of the corresponding *rhoA*/GAPDH were calculated as indices of *rhoA* mRNA levels.

Statistical analyses

All the data are expressed as the mean $\pm$ standard error of the mean (s.e.m.). Statistical significance of differences was determined by Dunnett's multiple analysis.

Results

Figure 1 shows the expression of *rhoA* and GAPDH mRNAs in the rat bronchial smooth muscle, as determined by RT-PCR using the total RNA estimated in preparations from non-sensitized normal rats. The PCR amplifications were performed for 15, 20, 25, 30 and 35 cycles. Bands for *rhoA* (503 bp) and GAPDH (468 bp) were clearly detected in the rat bronchial smooth muscle at a size close to that predicted (Fig. 1a). Both the band intensities for *rhoA* and GAPDH were PCR amplification-cycle-dependently increased and had nearly reached a plateau by 30–35 and 30 cycles, respectively (Fig. 1b). On the other hand, the PCR products obtained without RT reaction had no distinct bands (lanes 6 and 11 of Fig. 1a), indicating that there was no genomic DNA contamination in the RNA samples used in the present study.

In order to estimate the extent of the expression of *rhoA* mRNA, the ratios of the band intensity of *rhoA* mRNA to that of GAPDH were calculated. Our previous study revealed that the antigen sensitization procedure used in the present study had no significant effect by itself on either the ACh responsiveness of rat bronchial smooth muscle or on their muscarinic receptor properties (Chiba and Misawa, 1995a). So in the present study, age-matched non-

Fig. 1. **a)** Detection of *rhoA* (lanes 2–6) and GAPDH (as an inner control; lanes 7–11) mRNAs by RT-PCR in bronchial smooth muscle preparations of control (nonsensitized, normal) rats. PCR amplifications for *rhoA* and GAPDH were performed for 20 (lanes 2 and 7), 25 (lanes 3 and 8), 30 (lanes 4 and 9) and 35 cycles (lanes 5, 6, 10 and 11), respectively. The predicted sizes of each PCR product are shown beside the photograph. Lanes 6 and 11 represent a negative control, where the RT reaction was performed in the absence of the reverse transcriptase. Lanes 1 and 12 represent the DNA size marker (100 bp DNA ladder). **b)** The PCR cycle-dependency on the amplifications of the synthesized cDNAs for *rhoA* (triangles) and GAPDH (circles). The total RNA experiments were performed on extracts from 5 different animals, and the mean $\pm$ s.e.m. shown.

sensitized normal rats were used as controls. The PCR amplifications were performed for 25 cycles, respectively, which showed submaximal but distinct bands (Fig. 1). As shown in Fig. 2, the level of mRNA expression of *rhoA* was significantly increased in the bronchial smooth muscle of repeatedly antigen-challenged rats. The band intensity for *rhoA* of the antigen-treated rats (395.2 ± 34.9 , $n=5$) was significantly increased when compared to that of the control animals (247.4 ± 25.4 , $n=5$, $P<0.01$), whereas the band intensity for GAPDH was at an equivalent level (734.6 ± 93.6 and 818.2 ± 115.7 , $n=5$, respectively).

Discussion

The present study has clearly demonstrated the upregulation of the expression of *rhoA*

Fig. 2. The level of expression of *rhoA* mRNA in bronchial smooth muscle from nonsensitized normal (Control) and repeatedly antigen-challenged rats (Antigen-treated). **a)** Typical photographs of RT-PCR product bands for *rhoA* (*upper*) and GAPDH (*lower*) mRNAs. The PCR amplifications were performed for 25 cycles, respectively. The bands were scanned and the level of *rhoA* mRNA expression was expressed as density ratio of the *rhoA* to the GAPDH bands and the data were summarized in **b)**. The total RNA experiments were performed on extracts from 5 different animals, and the mean $\pm$ s.e.m. shown. The expression of *rhoA* mRNA is significantly increased in the bronchial smooth muscle of antigen-treated rats as compared to that of control animals ($P < 0.01$).

mRNA in bronchial smooth muscle of antigen-induced AHR rats. We previously reported an involvement of RhoA-mediated Ca^{2+} sensitization in agonist-induced contraction of bronchial smooth muscle in AHR rats and an augmentation of the Ca^{2+} sensitizing effect, together with an upregulation of RhoA protein (Chiba *et al.*, 1999b). We have shown that the increased expression of *rhoA* mRNA in the bronchial smooth muscle causes an upregulation of RhoA protein, resulting in an augmented contraction in the AHR by the enhancement of RhoA-mediated Ca^{2+} sensitization.

In smooth muscle, Hirata *et al.* (1992) first reported that a small GTPase, RhoA, is involved in the mechanism for the increased sensitization of myofilament Ca^{2+} . It was then shown that RhoA is responsible for the inhibition of myosin light chain phosphatase through the activation of Rho-associated kinases (Kimura *et al.*, 1996). The RhoA-mediated Ca^{2+} sensitization in agonist-induced contraction has also been reported in airway smooth muscle (Chiba *et al.*, 1999b; Iizuka *et al.*, 1999; Yoshii *et al.*, 1999; Yamagata *et al.*, 2000). On the other hand, an increase in the responsiveness of airway smooth muscle to contractile agonists has been

demonstrated in animal models of AHR (Gavett *et al.*, 1993; Lee *et al.*, 1994; Chiba and Misawa, 1995a, b) and asthmatic patients (Roberts *et al.*, 1984), although no change in the levels of plasma membrane receptors was observed (Gavett *et al.*, 1993; Lee *et al.*, 1994; Chiba and Misawa, 1995a). Moreover, the agonist-stimulated increase in the cytosolic Ca^{2+} level was within the normal range even in the hyperresponsive bronchial smooth muscle (Chiba *et al.*, 1999a). Thus, it is likely that the enhanced contractility to agonists reflects, at least in part, the augmentation of RhoA-mediated Ca^{2+} sensitization. In fact, we have clearly demonstrated the upregulation of both RhoA protein (Chiba *et al.*, 1999b) and *rhoA* mRNA (in the current study) in the bronchial smooth muscle of AHR rats. Although the mechanism of transcription of *rhoA* mRNA in the airway smooth muscle is presently unclear, it might be important to clarify the transcriptional mechanism to enable an understanding of the underlying mechanism involved in the augmented bronchial smooth muscle contraction in the AHR.

An increased expression of *rhoA* mRNA associated with augmentation of smooth muscle contractility has also been reported in both the rat myometrium during pregnancy (Niïro *et al.*, 1997) and the dog femoral artery in heart failure (Hisaoka *et al.*, 2001). Thus, the upregulation of RhoA might be widely involved in enhanced contraction of abnormal smooth muscle including that of hyperresponsive bronchial smooth muscle.

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