TNF- α augments the expression of RhoA in the rat bronchus

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Abstract

While nonspecific airway hyperresponsiveness (AHR) is a central feature of allergic bronchial asthma, the mechanism underlying the development of AHR is not clearly understood. We have previously demonstrated *in vitro* hyperresponsiveness of bronchial smooth muscle to acetylcholine (ACh) in rats that were actively sensitized and repeatedly challenged with aerosolized antigen. It has also been demonstrated that the ACh-induced, RhoA-mediated Ca²⁺ sensitization is markedly augmented concomitantly with an increased expression and activation of RhoA protein in the bronchial smooth muscle of the antigen-treated rats. In the present study, we have investigated whether TNF- α , a proinflammatory cytokine which is involved in bronchial asthma, causes upregulation of RhoA mRNA and protein in the rat bronchus. Treatment of rat bronchial smooth muscle preparations with TNF- α (300 ng/ml for 24 hr) significantly shifted the concentration-response curve to ACh upwards, but did not alter the response to high K⁺, when compared to that of control tissues. Levels of RhoA mRNA and protein in the TNF- α -treated bronchus were significantly greater than those in the control group. In conclusion, it is suggested that the augmentation of the ACh-induced contractile response evoked by TNF- α might be mediated by an upregulation of RhoA in rat bronchial smooth muscle.

Key words: TNF- α , airway hyperresponsiveness, smooth muscle, acetylcholine, RhoA

Introduction

Bronchial asthma is a complex inflammatory disease of the lung which has had an increased incidence worldwide over the last two decades. Airway hyperresponsiveness (AHR) and inflammation leading to an increased airway resistance are characteristic features of asthma (Bousquet, 2000). The importance of AHR in the pathogenesis of asthma has been suggested by its correlation with the severity of this disease (Lotvall *et al.*, 1998). Therefore, the underlying mechanisms of AHR need to be determined to assist with the treatment of asthma.

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On the other hand, there is increasing evidence that tumor necrosis factor- α (TNF- α), one of the proinflammatory cytokines produced by a variety of cells in the airways such as epithelial cells (Devakua *et al.*, 1993; Ackerman *et al.*, 1994), macrophages (Gosset *et al.*, 1992), mast cells (Gordon *et al.*, 1990; Bradding *et al.*, 1994) and eosinophils (Costa *et al.*, 1993; Finotto *et al.*, 1994), is directly linked to airway inflammation and the hyperresponsiveness observed in asthma (Shah *et al.*, 1995). The level of TNF- α is elevated in the sputa and bronchoalveolar lavage fluid (BALF) of patients with bronchial asthma (Taki *et al.*, 1991; Broide *et al.*, 1992). In mouse (Lucacs *et al.*, 1995), guinea pig (Watson *et al.*, 1993) and rat (Escott *et al.*, 2000) models of lung inflammation, increased levels of TNF- α have been detected in the BALF of sensitized animals following challenge with an antigen. In addition, *in vivo* pretreatment of rat and human airways with aerosolized TNF- α produced an enhanced increase in airway resistance, similar to that observed in asthma, when challenged with endogenous agonists (Kips *et al.*, 1992; Thomas *et al.*, 1995). Pharmacological evidence has also pointed towards an important role of TNF- α in airway hyperresponsiveness. TNF- α receptor fusion protein, which can potently block the endogenous TNF- α -mediated reaction, is effective in reducing both the enhanced airway reactivity and the infiltration of inflammatory cells into the airways in sensitized guinea pigs and Brown-Norway rats *in vivo* (Renzetti *et al.*, 1996). Moreover, TNF- α potentiated the contractile response of the human bronchus to ACh (Sukkar *et al.*, 2001). These observations suggest that TNF- α may be one of the primary components responsible for the bronchial smooth muscle hyperresponsiveness observed in asthma.

We have already demonstrated the occurrence of both *in vivo* and *in vitro* hyperresponsiveness in rats that were actively sensitized and repeatedly challenged with aerosolized antigen (Chiba *et al.*, 1993; Chiba *et al.*, 1995). We have also previously reported that the muscarinic receptor density of bronchial tissues in the AHR rat model following repeated antigen challenge was within normal levels (Chiba *et al.*, 1995). Moreover, no significant difference in the ACh-induced increase in cytosolic Ca^{2+} concentration of the main bronchial smooth muscle was observed between control and AHR rats (Chiba *et al.*, 1999a). These findings strongly suggest that the mechanisms responsible for the augmented ACh-induced contraction of the main bronchial smooth muscle might exist within post-muscarinic receptor signaling which includes augmented Ca^{2+} sensitization. In a previous study, the level of RhoA, an important protein that mediates Ca^{2+} sensitization and ACh-induced Ca^{2+} sensitization in bronchial preparations from rats that had been repeatedly antigen challenged, was significantly increased as compared with those of control rats (Otto *et al.*, 1996). The Ca^{2+} sensitization was abolished by pretreatment with the C3 toxin, Rho inhibitor (Chiba *et al.*, 1999b). It is therefore possible that the increased RhoA enhances Ca^{2+} sensitizing signal, resulting in augmentation of the ACh-induced contractile response in the AHR rats.

There is no evidence that TNF- α directly augments agonist-induced rat bronchial smooth muscle. In the present study, the effects of TNF- α treatment on the smooth muscle responsiveness of airways were investigated in association with changes in RhoA protein and mRNA levels in TNF- α -treated rat bronchus to further investigate the mechanism involved in the development of AHR.

Materials and Methods

Animals

Male Wistar rats (6 weeks of age, specific pathogen-free, 170–190 g, Charles River Japan, Inc) were housed for appropriate time intervals in the animal center of Hoshi University after delivery, in accordance with the guidelines approved by the Animal Care Committee of Hoshi University (Tokyo, Japan) for the care and use of laboratory animals. The animals were maintained at a constant temperature and humidity ($22 \pm 1^\circ\text{C}$, $55 \pm 10\%$), with a fixed 12-hr light-dark cycle and free access to food and water.

Functional study

The animals were killed by exsanguination from the abdominal aorta under anesthesia with chloral hydrate (400 mg/kg, i.p.). An approximately 4 mm length of the left main bronchus was isolated as described previously (Chiba *et al.*, 1993). After treatment with TNF- α (300 ng/ml) or its vehicle in Krebs-Henseleit solution with the following composition (mM); NaCl 118.0, KCl 4.7, CaCl_2 2.5, MgSO_4 1.2, NaHCO_3 25.0, KH_2PO_4 1.2 and glucose 10.0 (pH 7.4) at room temperature for 1 or 24 hr, the resultant tissue ring preparations were then suspended in a 5-ml organ bath at a resting tension of 1.0 g. The isometric 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. The buffer solution was maintained at 37°C and oxygenated with 95% O_2 –5% CO_2 . 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 (ACh) were successively added after attainment of a plateau response to the previous concentration. After measurement of ACh responsiveness, the bronchial smooth muscle was also 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.

RT-PCR analysis

Three, 6, 15 or 18 hr after incubation with TNF- α (300 ng/ml), main and intrapulmonary bronchial tissues 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 was isolated from each frozen tissue sample using acid guanidium thiocyanate/phenol/chloroform extraction (Mullis *et al.*, 1989) and stored at -85°C until use.

cDNAs were prepared from the total RNA (0.5 μg) by using a Takara RNA PCR Kit (Ver. 2.1; Takara, Tokyo, Japan) in a total volume of 20 μL reaction buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM MgCl_2 , 1 mM dNTP mixture, 1 U/ml RNase inhibitor, 2.5 μM random 9-mers and 0.25 U/ml 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. The reverse transcriptase was inactivated at 99°C for 5 min. Then to the RT reaction mixture (10 μL) was added 0.5 μL of 0.1 mM forward primer, 0.5 μL of 0.1 mM reverse primer, 4 μL of 10 X amplification buffer (100 mM Tris-HCl, pH 8.3, 0.5 M KCl), 3 μL of 25 mM MgCl_2 , 31.8 μL

of H₂O, and 0.25 μ L of 5 U/ml *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'-TACTCCTTGGAGGCCATGTAGG-3' (antisense), which were made from published sequences. The thermal cycle profile used in the present study was 1) denaturing for 30 sec at 95°C, 2) annealing primers for 30 sec at 60°C and 3) extending the primers for 60 sec at 72°C. The PCR amplifications were performed for 25 cycles. A portion (10 μ L) of the PCR mixture was subjected to electrophoresis on a 2% agarose gel (E-Gel™; Invitrogen, CA, USA) and visualized using a densitometer (Atto Densitograph; Atto Co., Tokyo, Japan). The ratios of the corresponding rhoA/GAPDH were calculated as indices of rhoA mRNA levels.

Western blot analysis

In the rhoA expression study, the samples were subjected to 15% SDS-polyacrylamide gel electrophoresis. Proteins were then electrophoretically transferred to PVDF membranes. After blocking, the membranes were then incubated with the primary antibodies. The primary antibodies used were rabbit anti-RhoA (1:2000 dilution; Santa Cruz Biotechnology, Inc) or mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (1:3000 dilution; Chemicon). Then the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig) G (1:5000 dilution; Amersham) and goat anti-mouse IgG (1:5000 dilution; Amersham) and detected by an ECL System. The ratio of the corresponding RhoA/GAPDH in each lane was calculated as an index of the RhoA level. We used GAPDH as an internal control because this protein is also constitutively expressed in most tissues and is the most widely accepted internal control in the molecular biology literature.

Statistical analyses

All the data were expressed as the mean and S.E.M. Statistical significance of differences was determined by two way analysis of variance (ANOVA).

Results

Functional study

Figure 1 shows the effects of a 24-hr incubation with TNF- α on bronchial smooth muscle responsiveness. As compared with vehicle-incubated control preparations, the ACh responsiveness in TNF- α (300 ng/ml)-treated groups was significantly augmented (Fig. 1A): the Emax of TNF- α -treated groups (1.63 ± 0.07 g tension, $p < 0.01$) was significantly greater than that of the vehicle-incubated group (1.13 ± 0.10 g). On the other hand, no significant difference in the response to isotonic high K⁺ (10, 30 and 60 mM) was observed between the groups (Fig. 1B).

RT-PCR analysis

Figure 2 shows the expression of rhoA and GAPDH mRNAs in rat bronchial preparations, as

Fig. 1. Acetylcholine (ACh; A) and high K⁺ (B) concentration-response curves in rat bronchial ring preparations following treatment with tumor necrosis factor- α (TNF- α) (300 ng/ml) or its vehicle control (Control) for 24 hr. Each point represents the mean and S.E. from 5 experiments. In the TNF- α -treated group, ACh responsiveness of the main bronchial smooth muscles was significantly ($p<0.05$, by ANOVA; 300 ng/ml and $p<0.01$) augmented as compared to that of the vehicle control group. *: $p<0.05$ and **: $p<0.01$ (TNF- α , 300 ng/ml) vs. vehicle control.

determined by RT-PCR using total RNA. The PCR amplification was performed for 25 cycles and showed submaximal but distinct bands. The expected bands for rhoA (503 bp) and GAPDH (468 bp) were clearly detected in the rat bronchial tissue (data not shown). The band intensities for rhoA, but not GAPDH, were TNF- α -treated time-dependently increased. In order to estimate the extent of expression of rhoA mRNA, the ratios of the band intensity of rhoA mRNA to that of GAPDH were calculated. The level of expression of rhoA mRNA was time-dependently increased in TNF- α -treated bronchial tissue. The relative rhoA mRNA obtained from the TNF- α -treated (for 18 hr) bronchial tissue was significantly increased as compared to that from non-treated bronchial tissue, whereas the band intensity for GAPDH remained at the same level (data not shown).

Western blot analysis

In the present study, anti-RhoA antibody was used to detect RhoA protein in TNF- α - and vehicle-treated bronchial preparations. Representative immunoblots for RhoA and GAPDH from TNF- α -treated and its vehicle control preparations are shown in Fig. 3A. Immunoblotting with the antibody against RhoA showed a single 21 kD band, indicating the existence of RhoA proteins in the rat bronchi. The ratios of corresponding RhoA/GAPDH were calculated and represented in Fig. 3B. The relative expression of RhoA protein in the TNF- α -treated bronchial preparation was significantly increased as compared with that in the vehicle control preparation ($p<0.05$).

Fig. 2. The level of expression of rhoA mRNA in the TNF- α or non (0)-treated rat bronchial tissue. The experiments were done using the total RNA from each of 4 different animals. The expression of rhoA mRNA was increased in a time-dependent manner in the TNF- α -treated rat bronchial tissue. The expression of rhoA mRNA was significantly increased in the 18 hr TNF- α -treated group (*: $p < 0.05$ vs. 0 hr).

Discussion

In the present study, treatment of rat bronchial smooth muscle preparations with TNF- α (300 ng/ml for 24 hr) significantly shifted the concentration-response curve to ACh upwards, but not that due to high K^+ , as compared to that of control preparations. The immunoblot study revealed that the expression of RhoA protein was significantly increased by TNF- α treatment in the rat bronchus. A semi-quantitative analysis of rhoA mRNA by RT-PCR also suggested an up-regulation of RhoA in TNF- α -treated bronchi.

Observations that TNF- α is released via the IgE-dependent activation of mast cells (Gordon *et al.*, 1990) or in the sensitized human lung (Ohkawara *et al.*, 1992) suggest that TNF- α contributes to the allergen-induced inflammatory response. Amrani *et al.* (1995) showed that exposure of human airway smooth muscle cells to TNF- α for 24 hr potentiates the increase in cytosolic free calcium induced by contractile agonists such as carbachol and bradykinin. However, in the present study, TNF- α potentiated the ACh-, but not high K^+ -, induced contraction of rat bronchial smooth muscle. Accordingly, TNF- α may increase bronchial smooth muscle contractility by augmenting receptor-mediated signaling such as that via muscarinic receptors.

We have previously demonstrated that the increased expression of RhoA protein by allergic stimulation seems to enhance the Ca^{2+} sensitizing signal, resulting in an augmentation of the

Fig. 3. (A) Typical immunoblots of RhoA (upper) and GAPDH (lower) in the TNF- α -treated (300 ng/ml, for 24 hr) or vehicle (Control)-treated bronchial preparations. (B) Relative densities of RhoA to GAPDH (RhoA/GAPDH) in the TNF- α - or its vehicle-treated bronchial preparations. Values are the mean and S.E. from 6 different animals. The expression of RhoA was significantly increased in the TNF- α -treated bronchial preparations as compared to that in the vehicle-treated ones (*: $p < 0.05$ vs. control).

ACh-induced contractile response in antigen-challenged AHR rats (Chiba *et al.*, 1999b). Presently, we have found that TNF- α treatment augments the ACh-induced bronchial smooth muscle contraction; ACh has been reported as one of the activators of RhoA in bronchial smooth muscle (Chiba *et al.*, 2001). We have now demonstrated that the TNF- α augmented expression of RhoA protein occurs in rat bronchial preparations. Thus, the TNF- α that was produced by allergic stimulation appears to increase the agonist-induced Ca^{2+} sensitizing signal via an enhanced expression of RhoA protein.

It has previously been reported that a short-term exposure (for 30 min) to TNF- α directly affects airway smooth muscle cells by augmenting muscarinic agonist-mediated contraction of both isolated guinea-pig (Pennings *et al.*, 1998) and bovine (Nakatani *et al.*, 2000) tracheal smooth muscle. However, although we studied a short-term exposure effect of TNF- α (300 ng/ml for 1 hr), no significant difference was observed in the contraction response to ACh between treatments with or without TNF- α (data not shown).

The mechanisms underlying the TNF- α -induced increase in bronchial smooth muscle contractility have not been fully elucidated. TNF- α initiates its pleiotropic action by binding to two receptors designated as p55 (TNFR1) and p75 (TNFR2) according to their apparent

molecular mass. These receptors are coexpressed on the surface of mast cells (Tartaglia *et al.*, 1992). Although both TNFR1 and TNFR2 were found to be coexpressed on airway smooth muscle cells (Amrani *et al.*, 1996; Amrani *et al.*, 2000a), the majority of TNF- α effects on airway smooth muscle are mediated by TNFR1 (Amrani *et al.*, 2000b). TNFR1 is shown to regulate TNF- α -induced expression of adhesion molecules (Amrani *et al.*, 2000b). However, the receptor subtype for the TNF- α -induced increase in bronchial smooth muscle contractility is unclear. It has been reported that TNF- α stimulation of TNFR1 elicited NF- κ B (nuclear factor- κ B transcription factor) activation in airway smooth muscle cell (McFarlane *et al.*, 2001). TNF- α may thus stimulate RhoA expression through NF- κ B activation.

In summary, we suggest that TNF- α might be one of the important mediators that are involved in the pathogenesis of the augmented bronchial smooth muscle contractility in AHR and that the augmentation of the ACh-induced contractile response evoked by TNF- α might be mediated by upregulation of RhoA in bronchial smooth muscle.

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