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Interleukin-4 upregulates RhoA protein via an activation of STAT6 in cultured human bronchial smooth muscle cells

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ABSTRACT

Interleukin-4 (IL-4) is believed to play a role in allergic bronchial asthma, and has been suggested to cause hyperresponsiveness of airway smooth muscle. In the present study, the effects of IL-4 on the expression of RhoA protein, a monomeric GTP-binding protein that contributes to the contraction of smooth muscle, were determined in cultured human bronchial smooth muscle cells (hBSMCs). Incubation of hBSMCs with IL-4 (100 ng/mL) caused a distinct phosphorylation of signal transducer and activator of transcription 6 (STAT6), a major signal transducer activated by IL-4, indicating that IL-4 is capable of activating signal transduction in the hBSMCs directly. IL-4 also caused a significant increase in the expression level of RhoA protein: the peak of the upregulation of RhoA protein was observed at 12–24 h after the IL-4 treatment. Both the phosphorylation of STAT6 and the upregulation of RhoA protein induced by IL-4 were inhibited by the co-incubation with AS1517499, a selective inhibitor of STAT6, in a concentration-dependent fashion. These findings suggest that IL-4 is capable of inducing an upregulation of RhoA via an activation of STAT6 in cultured hBSMCs. The RhoA upregulation induced by IL-4, one of the Th2 cytokines upregulated in the airways of allergic bronchial asthmatics, might result in an augmentation of bronchial smooth muscle contractility, that is one of the causes of airway hyperresponsiveness.

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1. Introduction

The dramatic increase in the number of asthma cases over the last decades is of great concern for public health in the world [1]. Increased airway narrowing in response to nonspecific stimuli is a characteristic feature of human obstructive diseases, including bronchial asthma. This abnormality is an important sign of the disease, although the pathophysiological variations leading to the hyperresponsiveness are unclear now. It has been suggested that one of the factors that contribute to the exaggerated airway narrowing in asthmatics is an abnormality of the properties of airway smooth muscle [2,3]. Rapid relief from airway limitation in asthmatic patients by β-stimulant inhalation [4–7] may also suggest an involvement of augmented airway smooth muscle contraction in the airway obstruction. Thus, it may be important for development of asthma therapy to understand changes in the contractile signaling of airway smooth muscle cells associated with the disease.

Recently, an importance of RhoA, a monomeric GTP-binding protein, and its downstream Rho-kinases have been demonstrated in the contraction of smooth muscles, including human bronchial smooth muscle (BSM) [8]. The RhoA/Rho-kinase pathway is involved in the agonist-induced Ca^{2+} sensitization of contraction in various types of smooth muscles. When the pathway was activated by contractile agonists, the activity of myosin light chain (MLC) phosphatase reduces and the level of phosphorylated MLC then increases, resulting in an augmentation of smooth muscle contraction. Interestingly, the RhoA-mediated Ca^{2+} sensitization of BSM contraction is augmented in experimental asthma models of rats [9] and mice [10]. An upregulation of RhoA has also been demonstrated in BSMs of these animal models of allergic bronchial asthma [9–11]. It is thus possible that the RhoA/Rho-kinase signaling might be augmented in BSMs of patients with allergic bronchial asthma. The RhoA/Rho-kinase pathway has now been proposed as a novel target for the treatment of AHR in asthma [12,13].

Interleukin-4 (IL-4), one of the T-helper 2 (Th2) cytokines, is believed to play a role in asthma [14–17]. An increased expression of IL-4 has been demonstrated in bronchoalveolar lavage fluid after segmental allergen challenge to asthmatic patients [14]. IL-4 promotes eosinophilic airway inflammation by increasing eotaxin expression and inhibiting eosinophil apoptosis [15]. IL-4 induces mucus hypersecretion [16] that contributes to airway obstruction. Interestingly, IL-4 also acts on airway smooth muscle directly, and

Abbreviations: BSM, bronchial smooth muscle; AHR, airway hyperresponsiveness; MLC, myosin light chain; IL, interleukin; STAT, signal transducer and activator of transcription; hEGF, human epidermal growth factor; hFGF-b, human fibroblast growth factor-basic; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ANOVA, analysis of variance.

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has an ability to cause hyperresponsiveness of airway smooth muscle [17]. We thus hypothesized that IL-4 may be one of the causes of the RhoA upregulation in BSMs observed in animal models of antigen-induced AHR [10,11]. In the present study, the effect of IL-4 on the expression level of RhoA protein was examined in human BSM cells (hBSMCs).

2. Materials and methods

2.1. Cell culture

Normal human bronchial smooth muscle cells (hBSMCs; Cambrex Bio Science Walkersville, Inc., Walkersville, MD) were maintained in SmBM medium (Cambrex) supplemented with 5% fetal bovine serum, 0.5 ng/mL human epidermal growth factor (hEGF), 5 μg/mL insulin, 2 ng/mL human fibroblast growth factor-basic (hFGF-b), 50 μg/mL gentamicin and 50 ng/mL amphotericin B. Cells were maintained at 37 °C in a humidified atmosphere (5% CO_2), fed every 48–72 h, and passaged when cells reached 90–95% confluence. Then the hBSMCs (passages 7–9) were seeded in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 3500 cells/cm^2 and, when 80–85% confluence was observed, cells were cultured without serum for 24 h before addition of recombinant human IL-4 (PeproTech EC, Ltd., London, UK). A selective inhibitor of signal transducer and activator of transcription 6 (STAT6), AS1517499 (30 or 100 nM; kindly provided from Astellas Pharma Inc., Tokyo, Japan), or its vehicle (0.3% DMSO) was treated 30 min before the addition of IL-4 (100 ng/mL). At the indicated time after the IL-4 treatment, cells were washed with PBS, immediately collected and disrupted with 1× SDS sample buffer (250 μL/well), and used for Western blot analyses.

2.2. Western blot analyses

Protein samples were subjected to 15% (for RhoA) or 7.5% SDS-PAGE (for the others) and the proteins were then electrophoretically transferred to a PVDF membrane. After blocking with 3% skim milk (for RhoA) or 1% BlockAce™ (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan; for the others), the PVDF membrane was incubated with the primary antibody. The primary antibodies used in the present study were polyclonal rabbit anti-RhoA (1:2500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-STAT6 (1:1000 dilution; Santa Cruz Biotechnology, Inc.), and anti-phospho-STAT6 (1:1000 dilution; Santa Cruz Biotechnology, Inc.) antibodies. Then the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:2500 dilution; Amersham Biosciences, Co., Piscataway, NJ), detected by an enhanced chemiluminescent system (Amersham Biosciences, Co.) and analyzed by a densitometry system. Detection of housekeeping gene was also performed on the same membrane by using monoclonal mouse anti-GAPDH (1:10,000 dilution; Chemicon International, Inc., Temecula, CA) and horseradish peroxidase-conjugated sheep anti-mouse IgG (1:2500 dilution; Amersham Biosciences, Co.) to confirm the same amount of proteins loaded.

2.3. Statistical analyses

All the data were expressed as the mean with S.E. statistical significance of difference was determined by unpaired Student's *t*-test or two-way analysis of variance (ANOVA) with *post hoc* Bonferroni/Dunn (StatView for Macintosh ver. 5.0, SAS Institute, Inc., NC). A value of $p < 0.05$ was considered significant.

3. Results

3.1. Effects of IL-4 on RhoA protein expression in cultured hBSMCs

To investigate the direct effects of IL-4 on bronchial smooth muscle cells, cultured hBSMCs were treated with recombinant human IL-4 under the serum-free condition as described in Section 2. Our previous study [18] demonstrated that the hBSMCs used expressed IL-4 receptor α (IL4Rα) and IL-13 receptor α chains (IL13Rα1) and STAT6, a major signal transducer activated by IL-4 [19–21]. IL-4 is thus capable of activating signal transduction in hBSMCs directly. To confirm this, tyrosine phosphorylation of STAT6 in the IL-4-stimulated hBSMCs was determined by Western blotting with specific antibody against 641-phosphotyrosine-

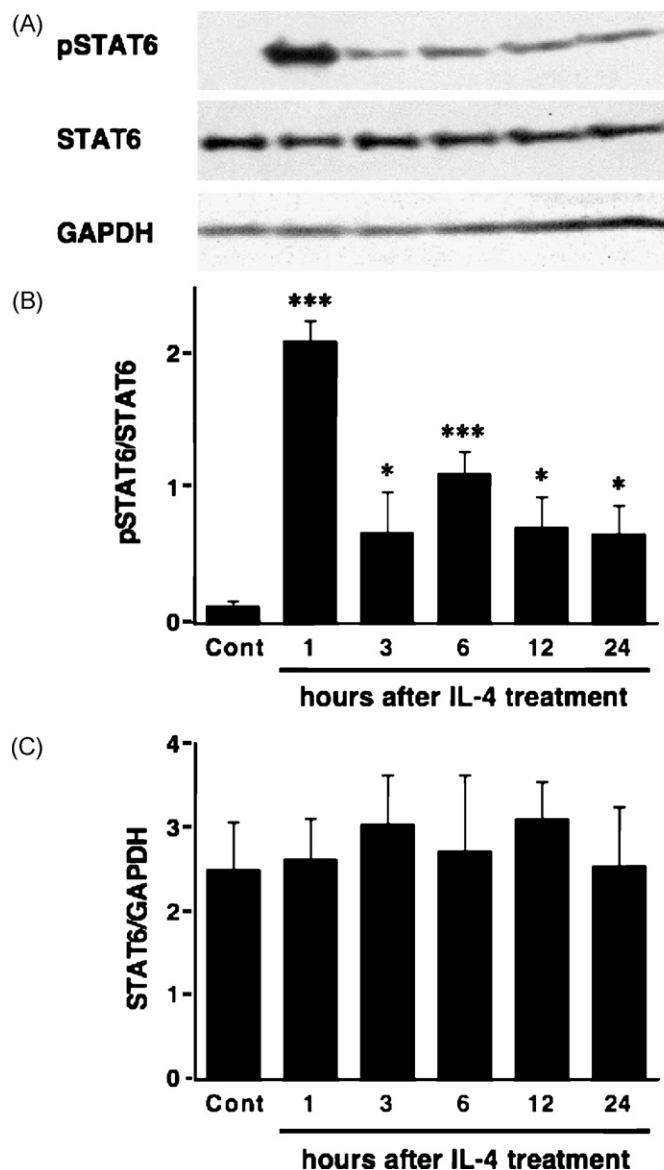

Fig. 1. Effects of interleukin-4 (IL-4) on the expression and phosphorylation of signal transducer and activator of transcription 6 (STAT6) in cultured human bronchial smooth muscle cells (hBSMCs). The hBSMCs were incubated with IL-4 (100 ng/mL) for the indicated time, and total protein samples were analyzed by immunoblottings. (A) Representative Western blots of phosphorylated STAT6 (*upper*), total STAT6 (*middle*) and GAPDH (*lower*). The bands were analyzed by a densitometer and pSTAT6/STAT6 and STAT6/GAPDH were calculated in each protein sample, and the data are summarized in (B) and (C) respectively. Each column represents the mean ± SEM from three independent experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus control (Cont) by Bonferroni/Dunn's test.

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