

A Novel STAT6 Inhibitor AS1517499 Ameliorates Antigen-Induced Bronchial Hypercontractility in Mice

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Interleukin-13 (IL-13) is one of the central mediators for development of airway hyperresponsiveness in asthma. The signal transducer and activation of transcription 6 (STAT6) is one of the major signal transducers activated by IL-13, and a possible involvement of IL-13/STAT6 pathway in the augmented bronchial smooth muscle (BSM) contraction has been suggested. In the present study, the effect of a novel STAT6 inhibitor, AS1517499, on the development of antigen-induced BSM hyperresponsiveness was investigated. In cultured human BSM cells, IL-13 (100 ng/ml) caused a phosphorylation of STAT6 and an up-regulation of RhoA, a monomeric GTPase responsible for Ca²⁺ sensitization of smooth muscle contraction: both events were inhibited by co-incubation with AS1517499 (100 nM). In BALB/c mice that were actively sensitized and repeatedly challenged with ovalbumin antigen, an increased IL-13 level in bronchoalveolar lavage fluids and a phosphorylation of STAT6 in bronchial tissues were observed after the last antigen challenge. These mice had an augmented BSM contractility to acetylcholine together with an up-regulation of RhoA in bronchial tissues. Intraperitoneal injections of AS1517499 (10 mg/kg) 1 hour before each ovalbumin exposure inhibited both the antigen-induced up-regulation of RhoA and BSM hyperresponsiveness, almost completely. A partial but significant inhibition of antigen-induced production of IL-13 was also found. These findings suggest that the inhibitory effects of STAT6 inhibitory agents, such as AS1517499, both on RhoA and IL-13 up-regulations might be useful for asthma treatment.

Keywords: airway hyperresponsiveness; bronchial smooth muscle; RhoA; STAT6; AS1517499

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 pathophysiologic variations leading to the hyperresponsiveness are unclear now. Several mechanisms have been suggested to explain the airway hyperresponsiveness (AHR), such as alterations in the neural control of airway smooth muscle (1), increased mucosal secretions (2), and mechanical factors related to remodeling of the airways (3). In addition, it has also been suggested that one of the factors that contribute to the exaggerated airway narrowing in individuals with asthma is an abnormality of the properties of airway smooth muscle (4, 5). Rapid relief from airway limitation in patients with asthma by β -stimulant inhalation 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.

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CLINICAL RELEVANCE

A signal transducer and activator of transcription 6 (STAT6) inhibitor, AS1517499, ameliorated the antigen-induced bronchial smooth muscle hyperresponsiveness by inhibiting RhoA up-regulation in bronchial smooth muscles and, at least in part, by reducing IL-13 production in the airways in mice. Both the direct and indirect effects of STAT6 inhibitory agents, such as AS1517499, on bronchial smooth muscles might be useful for the treatment of allergic bronchial asthma.

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) (6). The RhoA/Rho-kinase pathway is involved in the agonist-induced Ca²⁺ 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 Ca²⁺ sensitization of BSM contraction is augmented in experimental asthma models of rats (7) and mice (8). An up-regulation of RhoA has also been demonstrated in BSMs of these animal models of allergic bronchial asthma (7–9). 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 (10).

To date, there is increasing evidence that interleukin-13 (IL-13), one of the T-helper 2 (Th2) cytokines, is a central mediator of the induction of AHR (11–19). An increased expression of IL-13 has been demonstrated in airways of patients with symptomatic asthma (20, 21). In addition, overexpression of IL-13 in the airway epithelial cells of mouse using the Clara cell 10-kD protein gene promoter causes AHR to aerosolized methacholine (22). Intratracheal instillation of recombinant IL-13 to naive mice also evokes AHR to inhaled methacholine (23) and intravenously administered acetylcholine (ACh) (11). Interestingly, intranasal administration of recombinant IL-13 to the histamine H₁ receptor gene-deleted mouse, which fails to develop allergen-induced AHR, also induces AHR (24). In addition, the neutralization of IL-13 by systemic administration of a soluble IL-13R α 2-IgG-Fc fusion protein (11, 12) or of an antibody against IL-13 (17–19) inhibits allergen-induced AHR in sensitized mice. Mice in which targeted deletion of IL-13 was performed fail to develop allergen-induced AHR, but AHR is restored by the intranasal administration of recombinant IL-13 (13). Although several previous studies suggested that IL-13 has direct effects on BSM contractility (19, 25, 26), its mechanism of action is not fully understood.

Although the exact transcriptional regulation of RhoA expression is unknown now, the upstream genomic sequence of its gene contains several putative binding sites for signal

transducers and activations of transcriptions (STATs), suggesting that the increased expression of RhoA in BSM of the AHR animals (7–9) may be regulated by an activation of STAT6, one of the major signal transducers activated by IL-13 (16, 27–30). The reports that the development of antigen-induced AHR was inhibited by STAT6 gene knockout (31–33) and a STAT6 inhibitory peptide (34) may also support the hypothesis. In addition, our previous study revealed that IL-13 could induce both STAT6 activation and RhoA up-regulation in cultured human BSM cells, and that the latter event was inhibited by a STAT6 inhibitor, leflunomide (26). However, leflunomide is known to have nonspecific effects other than the inhibition of STAT6 (see, e.g., Refs. 35 and 36). Recently, Nagashima and colleagues (37) synthesized a potent selective STAT6 inhibitor, 4-(benzylamino)-2-[[2-(3-chloro-4-hydroxyphenyl)ethyl]amino]pyrimidine-5-carboxamide (AS1517499). So in the present study, to determine the effectiveness of an inhibition of STAT6 for the treatment of AHR, the effects of selective inhibition of STAT6 by AS1517499 on the up-regulation of RhoA and the BSM hyperresponsiveness induced by allergen challenge were investigated using a murine model of allergic bronchial asthma.

MATERIALS AND METHODS

Cell Culture and Sample Collection

Normal human BSM 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% CO₂), fed every 48 to 72 hours, and passaged when cells reached 90 to 95% confluence. Then the hBSMCs (passages 7–9) were seeded in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) and 8-well chamber slides (Nalge Nunc International, Naperville, IL) at a density of 3,500 cells/cm² and, when 80 to 85% confluence was observed, cells were cultured without serum for 24 hours before addition of recombinant human IL-13 (PeproTech EC, Ltd., London, UK). AS1517499 (100 nM; kindly provided from Astellas Pharma Inc., Tokyo, Japan) or its vehicle (0.3% DMSO) was treated 30 minutes before the addition of IL-13 (100 ng/ml). In some experiments, AS1517499 was treated 0 (co-incubation), 3, or 12 hours after the addition of IL-13. In another series of experiments, a selective Rho-kinase inhibitor Y-27632 (1 µM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) or its vehicle (0.3% DMSO) was also applied 15 minutes before the IL-13 application. At the indicated time after the IL-13 treatment, cells were washed with PBS, immediately collected, and disrupted with 1× SDS sample buffer (250 µl/well), and used for Western blot analyses.

RNA Interference

The synthetic siRNA duplex targeting the human STAT6 gene was purchased from Applied Biosystems/Ambion (Foster City, CA; siRNA ID: s13541). The Cy3-labeled negative control siRNA was also purchased from Applied Biosystems/Ambion (catalog number AM4621). The hBSMCs (30–40% confluent) were transfected with 100 pmol of siRNA duplex in 6-well plates using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Forty-eight hours after the transfection, IL-13 was applied to the cells under the starved condition as described above. Twenty-four hours after the IL-13 application (i.e., 72 h after the transfection), protein expressions were assessed by Western blottings.

Immunocytochemistry

The hBSMCs cultured on the 8-well chamber slides were subjected to immunocytochemistry. In brief, cells were fixed with 10% formaldehyde in PBS (10 min) and permeabilized by incubation with 0.5% Triton X-100 in PBS for 10 minutes. Endogenous peroxidase activity was blocked by incubation with 0.3% H₂O₂ in 100% methanol for

10 minutes. After blocking with 5% skim milk in PBS for 1 hour, the cells were incubated with anti-STAT6 or anti-phospho-STAT6 antibody (1:100 dilution in 1% skim milk-PBS, respectively) for 1 hour. After washing with PBS, the cells were incubated with anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) for 1 hour. Detection was performed by using an ABC reagent (Vector Laboratories) with 3,3'-diaminobenzidine (DAB) (Sigma FAST; Sigma, St. Louis, MO) according to the manufacturers' instructions. Counterstaining was also performed by hematoxylin (Vector Laboratories) before examination by light microscopy.

Animals and Treatments

Male BALB/c mice were purchased from the Charles River Japan, Inc. (Kanagawa, Japan) and housed in a pathogen-free facility. All animal experiments were approved by the Animal Care Committee of the Hoshi University (Tokyo, Japan).

Preparation of a murine model of allergic bronchial asthma, which has an *in vivo* AHR (38), was performed as described previously (8). In brief, BALB/c mice (8 wk of age) were actively sensitized by intraperitoneal injections of 8 µg ovalbumin (OVA; Seikagaku Co., Tokyo, Japan) with 2 mg Imject Alum (Pierce Biotechnology, Inc., Rockford, IL) on Day 0 and Day 5. The sensitized mice were challenged with aerosolized OVA-saline solution (5 mg/ml) for 30 minutes on Days 12, 16, and 20. A control group of mice received the same immunization procedure but inhaled saline aerosol instead of OVA challenge. The aerosol was generated with an ultrasonic nebulizer (Nihon Kohden, Tokyo, Japan) and introduced to a Plexiglas chamber box (130 × 200 mm, 100 mm height) in which the mice were placed. Animals also received intraperitoneal injection with AS1517499 (1 or 10 mg/kg/d; dissolved in 20% DMSO in saline) or its vehicle 1 hour before each antigen inhalation (Days 12, 16, and 20). Twenty-four hours after the last OVA challenge, mice were killed by exsanguination from abdominal aorta under urethane (1.6 g/kg, intraperitoneally; Sigma) anesthesia.

Analyses of Bronchoalveolar Lavage Fluids

After the exsanguinations, the chest of each animal was opened and a 20-gauge blunt needle was tied into the proximal trachea. Bronchoalveolar lavage (BAL) fluid was obtained by intratracheal instillation of 1 ml/animal of phosphate-buffered saline (PBS; pH 7.5, room temperature) into the lung while it was kept located within the thoracic cavity. The lavage was refused into the lung twice before final collection. BAL cells were isolated by centrifugation at 500 × *g*. The resultant pellet was resuspended in 500 µl of 10% formaldehyde and incubated for 10 minutes. Then the cells were washed by PBS and resuspended in 500 µl of PBS. An aliquot of BAL cell suspension was used for cell counts with a hemocytometer. The resultant supernatants of the lavage fluids were subjected to cytokine analysis. The levels of IL-13 were measured by an IL-13 ELISA system (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Determination of BSM Responsiveness

Mice were killed by exsanguination from abdominal aorta under urethane (1.6 g/kg, intraperitoneally) anesthesia and the airway tissues under the larynx to lungs were immediately removed. About 3 mm length of the left main bronchus (~0.5 mm diameter) was isolated and the epithelium was removed by gently rubbing with sharp tweezers (7, 8). The resultant tissue ring preparation was then suspended in a 5-ml organ bath by two stainless-steel wires (0.2 mm diameter) passed through the lumen. For all tissues, one end was fixed to the bottom of the organ bath while the other was connected to a force-displacement transducer (TB-612T; Nihon Kohden) for the measurement of isometric force. A resting tension of 0.5 g was applied. The buffer solution contained modified Krebs-Henseleit solution with the following composition (in mM): NaCl 118.0, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 25.0, KH₂PO₄ 1.2, and glucose 10.0. The buffer solution was maintained at 37°C and oxygenated with 95% O₂–5% CO₂. After the equilibration period, the concentration–response curve to ACh (10^{–7}–10^{–3} M in final concentration) was constructed cumulatively. A higher concentration of ACh was successively added after attainment of a plateau response to the previous concentration. In another series of experiment, isotonic K⁺ solution (10–90 mM in final concentration) was cumulatively administered in the presence of atropine and in-

domethacin (both 10^{-6} M) to determine the BSM responsiveness to high K^{+} -depolarization.

Protein Samples of Bronchial Tissues

The airway tissues below the main bronchi to lungs were removed and immediately soaked in ice-cold, oxygenated Krebs-Henseleit solution. The airways were carefully cleaned of adhering connective tissues and blood vessels under a stereomicroscopy, and the resultant tissues containing lungs and the main bronchi were used as the "whole lung" samples. In some experiments, only the main bronchi were isolated and the epithelium was removed as much as possible by gently rubbing with sharp tweezers (8), and the resultant tissues were used as the "bronchial tissue" samples. Then each whole lung and bronchial tissue were quickly frozen with liquid nitrogen, and the tissue was crushed to powders by using a mortar (39). The tissue powder was homogenized in ice-cold tris(hydroxymethyl)aminomethane (Tris, 10 mM; pH 7.5) buffer containing 5 mM $MgCl_2$, 2 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin, 1% Triton X-100, and 1% sodium cholate. The tissue homogenate was then centrifuged ($3,000 \times g$, $4^{\circ}C$ for 15 min) and the resultant supernatant was stored at $-85^{\circ}C$ until use.

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 (1:2,500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-STAT6 (1:1,000 dilution; Santa Cruz Biotechnology), and anti-phospho-STAT6 (1:1,000 dilution; Santa Cruz Biotechnology) antibodies. Then the membrane was incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (1:2,500 dilution; Amersham Biosciences, Co., Piscataway, NJ), detected by an enhanced chemiluminescent system (Amersham Biosciences) 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 HRP-conjugated sheep anti-mouse IgG (1:2,500 dilution; Amersham Biosciences) to confirm the same amount of proteins loaded.

Quantitation of IgE Levels in Serum

Serum was obtained from the blood sample by centrifugation at $3,000 \times g$ for 10 minutes at $4^{\circ}C$. Total IgE in serum was measured by an ELISA system (Bethyl Laboratories, Inc., Montgomery, TX) according to the manufacturer's instructions. OVA-specific IgE in the serum was measured by the ELISA system with modification. Briefly, 96-well microtitre plates (Nunc, Roskilde, Denmark) were coated with 100 μ l/well of 100 μ g/ml OVA (Sigma) in 0.05 M bicarbonate buffer (pH 9.6) and incubated for 1 hour at $4^{\circ}C$. After washing with 0.5% Tween 20/Tris-buffered saline (TBS, pH 8.0) and blocking with 1% bovine serum albumin (BSA)/TBS, samples (five time diluted, 100 μ l/well) were added to the wells and incubated for 1 hour at room temperature. Subsequently, plates were washed and 1:20,000 diluted HRP-conjugated goat anti-mouse IgE (100 μ l/well; Bethyl Laboratories) was added and incubated for 1 hour. After a further wash, all plates were developed with tetramethylbenzidine substrate solution (100 μ l/well; Bethyl Laboratories), stopped with 1 N H_2SO_4 and read at 450 nm using a Bio-Rad microplate reader (Bio-Rad, Munich, Germany). For lack of standard OVA-specific murine IgE, the OVA-specific IgE level in each group was expressed as the O.D.450 ratio to the nonsensitized normal animals.

Statistical Analyses

All the data were expressed as the mean with SE. Statistical significance of difference was determined by unpaired Student's *t* test or two-way 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.

RESULTS

STAT6 Activation and RhoA Up-Regulation by IL-13 in hBSMCs

To investigate the direct effects of IL-13 on BSM cells, cultured hBSMCs were treated with recombinant human IL-13 under the serum-free condition as described in MATERIALS AND METHODS. Our previous study revealed that both IL-13 receptor $\alpha 1$ (IL13R $\alpha 1$) and IL-4 receptor α (IL4R α) were expressed in the cultured hBSMC (26). STAT6, a major signal transducer activated by IL-13 (27–30), was also detected (26), indicating that, as reported in human tracheal smooth muscle cells (25), IL-13 is capable of activating signal transduction in BSM cells directly. To confirm the activation of STAT6 by IL-13, tyrosine phosphorylation of STAT6 in the IL-13-stimulated BSM cells was determined by immunoblotting with specific antibody against 641-phosphotyrosine-STAT6. As shown in Figure 1A, the protein expression of STAT6 was detected in the cultured hBSMCs. Although the expression level of total STAT6 protein was not affected by the IL-13 treatment, a distinct phosphorylation of STAT6 was observed when the cells were treated with IL-13 for 1 hour. The phosphorylation of STAT6 induced by IL-13 seems to be a transient reaction because the band for phosphorylated STAT6 disappeared in the cells treated with IL-13 for 3 hours. A lesser but distinct phosphorylation of STAT6 was also observed again at 6 hours after treatment with IL-13 (Figure 1A). Immunocytochemical examination also revealed a distinct activation of STAT6 by the IL-13 stimulation: immunostaining for phosphorylated STAT6, mainly located in cell nuclei, was observed only in cells incubated with IL-13 (Figure 2A). In addition to the activation of STAT6, the RhoA protein expression in hBSMCs was significantly increased 24 hours after the application of IL-13 (Figure 1B). Similarly, the phosphorylation of STAT6 and up-regulation of RhoA were observed when IL-4 (100 ng/ml) was used instead of IL-13 (data not shown). The up-regulation of RhoA protein induced by IL-13 also seems to be transient: no significant increase in the level of RhoA protein expression was observed when cells were incubated with IL-13 for 48 or 72 hours (Figure 1B). As shown in Figure 1C, the IL-13-induced up-regulation of RhoA was inhibited when STAT6 was depleted by its siRNA, indicating an involvement of STAT6 in the phenomenon. On the other hand, the IL-13-induced up-regulation of RhoA protein tended to be augmented by the co-incubation with Y-27632 (an inhibitor of Rho-kinase), but no significant difference was observed when compared with the cells treated with IL-13 in the presence of DMSO (0.3%; vehicle for Y-27632) (Figure 1D).

Inhibitory Effects of AS1517499 on the IL-13-Induced Activation of STAT6 and Up-Regulation of RhoA in hBSMCs

To determine the ability of AS1517499 to inhibit the activation of STAT6 in hBSMCs, the effect of AS1517499 on STAT6 phosphorylation observed at 1 h after the treatment with IL-13 (100 ng/ml) was examined. As shown in Figure 2A, immunocytochemical examination revealed that AS1517499 (100 nM) blocked the STAT6 phosphorylation induced by IL-13 almost completely. Moreover, Western blot analyses also revealed that the band for phosphorylated STAT6 in the IL-13-stimulated hBSMCs disappeared when cells were pretreated with AS1517499 (data not shown). These observations indicate that AS1517499, at least at the concentration used, can inhibit the activation of STAT6 by blocking its phosphorylation. Under these conditions, the up-regulation of RhoA protein observed at 24 hours after the treatment with IL-13 was attenuated when cells were pretreated with AS1517499 (100 nM) 30 minutes before the administration of IL-13 (Figure 2B). AS1517499 also inhibited

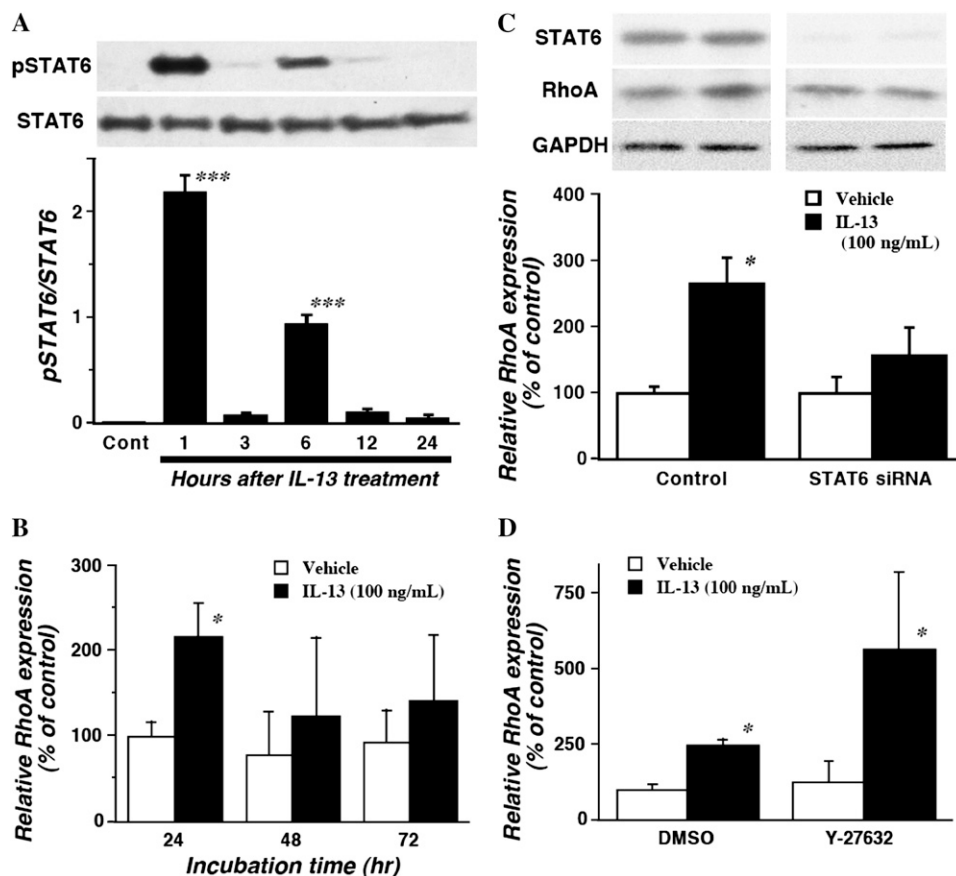


Figure 1. Interleukin-13 (IL-13)-induced activation of signal transducer and activator of transcription 6 (STAT6) and up-regulation of RhoA in cultured human bronchial smooth muscle cells (hBSMCs). (A) Time-course change in the phosphorylation of STAT6 after treatment with IL-13 (100 ng/ml) determined by immunoblotting. Significant increases in the levels of phosphorylated STAT6 (pSTAT6) without change in total STAT6 protein were observed when cells were incubated with IL-13 for 1 and 6 hours. (B) Duration of IL-13-induced up-regulation of RhoA. hBSMCs were cultured with IL-13 (100 ng/ml) or its vehicle for 24 to 72 hours. (C) Effect of STAT6 depletion on the IL-13 (100 ng/ml, for 24 h)-induced up-regulation of RhoA protein. The STAT6 siRNA-mediated depletion of STAT6 protein is also shown in the upper photos. (D) Effect of a selective Rho-kinase inhibitor, Y-27632, on the IL-13-induced up-regulation of RhoA. hBSMCs were cultured with IL-13 (100 ng/ml) or its vehicle for 24 hours in the absence or presence of Y-27632 (1 μ M). In B and D, the levels of RhoA were expressed as % of control (i.e., Vehicle-24 h and Vehicle-DMSO groups, respectively). Each column represents the mean $\pm$ SEM from three to six independent experiments. * P < 0.05 and *** P < 0.001 versus respective control groups by Bonferroni/Dunn's test.

the STAT6 phosphorylation and RhoA up-regulation induced by IL-4 (data not shown). On the other hand, the inhibitory effect was not observed when cells were treated with AS1517499 (100 nM) 3 or 12 hours after the administration of IL-13 (Figure 2C).

STAT6 Activation and RhoA Up-Regulation by Antigen Challenge in Mouse Bronchial Tissues

In the murine allergic bronchial asthma model currently used, increased expression of IL-13 (Figure 3A) and IL-4 (data not shown) in BAL fluids was observed: the peak expression was found at 6 to 12 hours after the last OVA challenge. Our previous study also revealed the expressions of IL-13/IL-4 signaling molecules, such as IL-4R α , IL-13R α 1, and STAT6, in the bronchial tissues of naive mice (26). The OVA challenge caused a phosphorylation of STAT6 in lung tissues: the time-course examination revealed a peak of the STAT6 phosphorylation at 3 hours after the OVA challenge (Figure 3A). At this time point, a marked and significant increase in the phosphorylated STAT6 was also detected in tissues of the main bronchi (Figure 3B). These findings indicate that IL-13 and/or IL-4, generated by antigen exposure in the airways, directly act on the bronchial tissue (probably including bronchial smooth muscle) and activate its STAT6. In addition, as well as the results observed in the IL-13-treated hBSMCs (Figures 1 and 2), the RhoA protein expression in bronchial tissues of the antigen-challenged mice (AC) was significantly increased as compared with that of the sensitized control animals (SC, P < 0.01; Figure 4).

Effects of AS1517499 on the Antigen-Induced Up-Regulation of RhoA and BSM Hyperresponsiveness in Mice

To determine the effect of AS1517499 in the development of antigen-induced BSM hyperresponsiveness, animals were treated

with AS1517499 (1 or 10 mg/kg, intraperitoneally) 1 hour before each antigen inhalation. The STAT6 phosphorylation in bronchial tissues observed at 3 hours after the last OVA challenge (Figure 3B) was completely blocked when the dosage of 10 mg/kg was used (data not shown). As reported previously (8), the RhoA protein expression in bronchial tissues was significantly increased 24 hours after the last OVA challenge (Figure 4). Pretreatment with AS1517499 inhibited the up-regulation of RhoA protein induced by antigen exposure, dose-dependently: a significant and complete inhibition was observed when 10 mg/kg of AS1517499 was administered 1 hour before each antigen exposure (Figure 4). On the other hand, the 10 mg/kg of AS1517499 had no effect on the RhoA protein expression in the sensitized control animals (Figure 4).

As shown in Figure 5, the contractile responsiveness to acetylcholine (ACh) of the BSMs isolated from the repeatedly antigen-challenged mice was significantly augmented 24 hours after the last OVA challenge as compared with that from the sensitized control animals (P < 0.05). Although the AS1517499 (10 mg/kg) pretreatments had no effect on the BSM responsiveness to ACh in the sensitized control animals, the BSM hyperresponsiveness to ACh induced by antigen exposure was significantly inhibited by the pretreatments (P < 0.001; Figure 5, upper panel). The BSM responsiveness to high K⁺ depolarization was not changed by any treatments (Figure 5, lower panel).

Effects of AS1517499 on Serum IgE, IL-13 in BAL Fluids, and Inflammatory Cell Infiltration

Both the levels of total and OVA-specific IgE in sera of the OVA-challenged mice were significantly greater as compared with those of the sensitized control animals. In either group of animals, pretreatments with AS1517499 (1 or 10 mg/kg, intra-

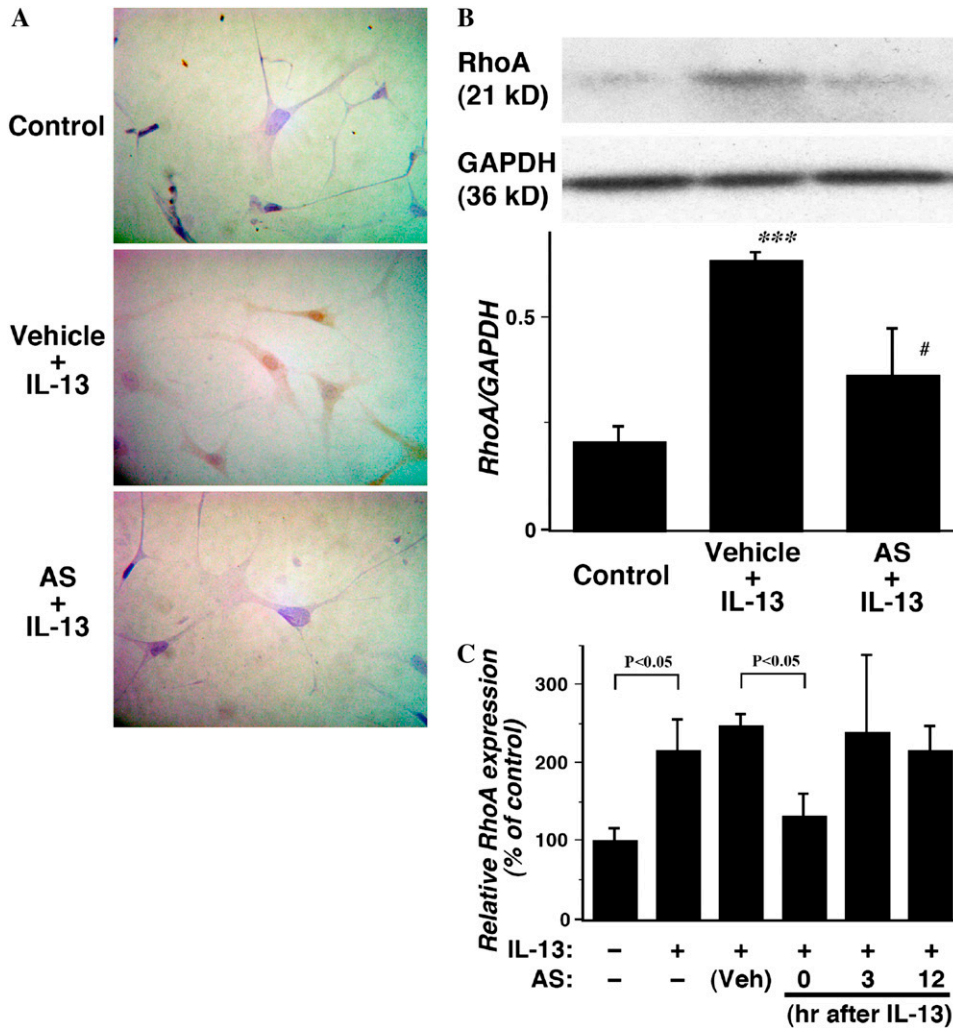


Figure 2. Inhibitory effects of AS1517499 (AS) on the IL-13-induced activation of STAT6 and up-regulation of RhoA in cultured hBSMCs. (A) Inhibition of IL-13-induced phosphorylation of STAT6 by AS. The hBSMCs without any treatment (Control; upper panel) and stimulated with IL-13 (100 ng/ml for 1 h) in the absence (Vehicle+IL-13; middle panel) or presence (AS+IL-13; lower panel) of AS (100 nM) were immunostained with an antibody against phosphorylated STAT6 and visualized by DAB (brown color). The photos are representative of three independent experiments. (B) Inhibition of IL-13 (100 ng/ml for 24 h)-induced upregulation of RhoA protein by AS (100 nM). The RhoA expression levels in hBSMCs were determined by immunoblotting. (C) Effect of post-treatment with AS on IL-13-induced up-regulation of RhoA. hBSMCs were treated with AS (100 nM) 3 or 12 hours after the administration of IL-13 (100 ng/ml). The levels of RhoA were expressed as % of control (IL-13(-)-AS(-) group). Each column represents the mean $\pm$ SEM from three independent experiments. *** P < 0.001 versus control and # P < 0.05 versus Vehicle+IL-13 by Bonferroni/Dunn's test.

peritoneally) had no significant effect on the production of IgE (Figures 6A and 6B).

Although no detectable level of IL-13 was contained in BAL fluids of the sensitized control mice, a significant increase in IL-13 was observed in those of the antigen-challenged animals (24 h after the last antigen challenge; Figure 6C). Concurrently with the up-regulation of IL-13, a significant increase in the

cells, mainly eosinophils (39, 40), in BAL fluids was also found 24 hours after the last OVA challenge (Figure 6D). Both the up-regulation of IL-13 and the increment of cell number were inhibited by pretreatment with AS1517499, dose-dependently: a significant inhibition was observed when 10 mg/kg of AS1517499 was treated 1 hour before each antigen challenge (Figures 6C and 6D).

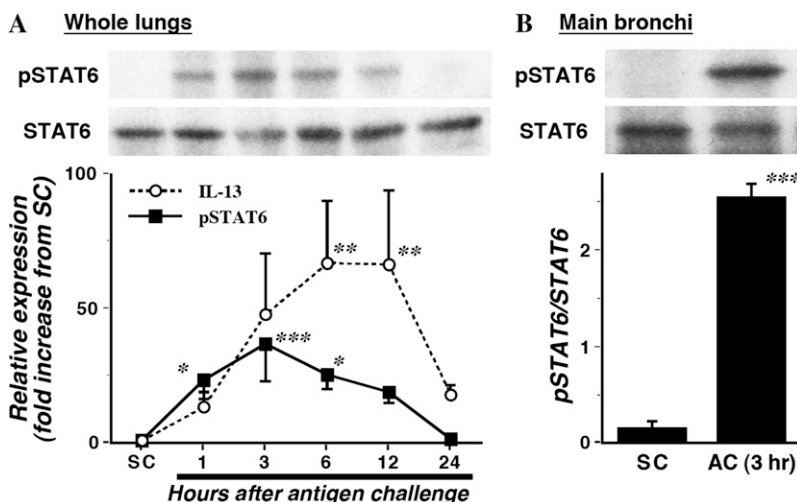


Figure 3. Antigen-induced activation of signal transducer and activator of transcription 6 (STAT6) in lungs and bronchial tissues of sensitized mice. (A) Time-course changes in levels of IL-13 in BAL fluids (open circles) and phosphorylation of STAT6 in lungs (closed squares) after challenge with ovalbumin (OVA) antigen. Male BALB/c mice were actively sensitized and repeatedly challenged with OVA as described in MATERIALS AND METHODS. The levels of IL-13 and phosphorylated STAT6/total STAT6 (pSTAT6/STAT6) were determined by ELISA and immunoblots, respectively, and expressed as the fold increase from sensitized control group (SC). (B) Antigen-induced phosphorylation of STAT6 in bronchial tissues of the sensitized mice. A significant increase in the levels of pSTAT6 was also observed at 3 hours after the OVA challenge in the bronchial tissues. Each value represents the mean $\pm$ SEM from six independent experiments. SC, sensitized control; AC, antigen-challenged groups. * P < 0.05, ** P < 0.01, and *** P < 0.001 versus SC by Bonferroni/Dunn's test.

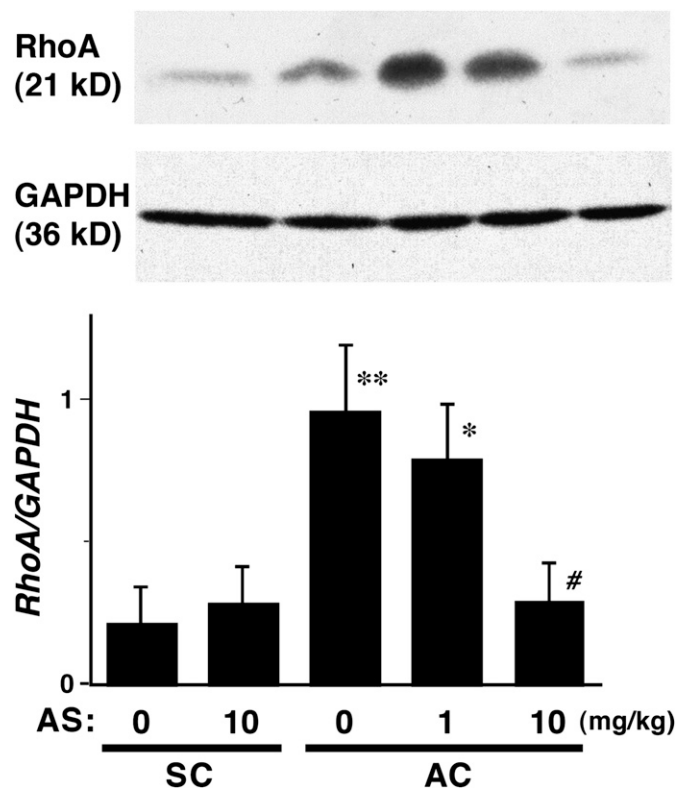


Figure 4. Inhibitory effects of AS on the antigen-induced up-regulation of RhoA in bronchial tissues of sensitized mice. Male BALB/c mice were actively sensitized and repeatedly challenged with OVA antigen as described in MATERIALS AND METHODS. Animals also received intraperitoneal injection of AS (1 or 10 mg/kg) or its vehicle (AS [0]; 20% DMSO in saline) 1 hour before each OVA challenge. Twenty-four hours after the last OVA challenge, the RhoA protein expression levels in bronchial tissues were determined by immunoblottings. Representative blots for RhoA and GAPDH are shown in the upper panels. The bands were analyzed by a densitometer and the data are summarized in the lower panel. Each column represents the mean $\pm$ SEM from four to six independent experiments. SC, sensitized control; AC, antigen-challenged groups. * $P < 0.05$ and ** $P < 0.01$ versus SC-AS (0) group and # $P < 0.05$ versus AC-AS (0) group by Bonferroni/Dunn's test.

DISCUSSION

Airway smooth muscle is an important effector tissue regulating bronchomotor tone. It has been suggested that modulation of airway smooth muscle by inflammatory mediators such as cytokines may play an important role in the development of AHR (41). In the BALB/c strain of mice that were actively sensitized and repeatedly challenged using the same procedures as the current study, an *in vivo* AHR accompanied by the increased IgE production and pulmonary eosinophilia has been demonstrated (38). In this animal model of allergic bronchial asthma, an increased contractility of isolated BSM to contractile agonists has also been found (8, 42). The augmented BSM contraction induced by antigen challenge has reportedly been associated with an up-regulation of RhoA (8), a small GTPase that is involved in the agonist-induced Ca^{2+} sensitization of smooth muscle contraction (43, 44). An importance of RhoA and its downstream Rho-kinases was also demonstrated in contraction of human BSM (6), and the RhoA/Rho-kinase pathway has now been proposed as a new target for the treatment of AHR in asthma (10). In the present study, the IL-13-induced up-regulation of RhoA in hBSMCs was inhibited both by

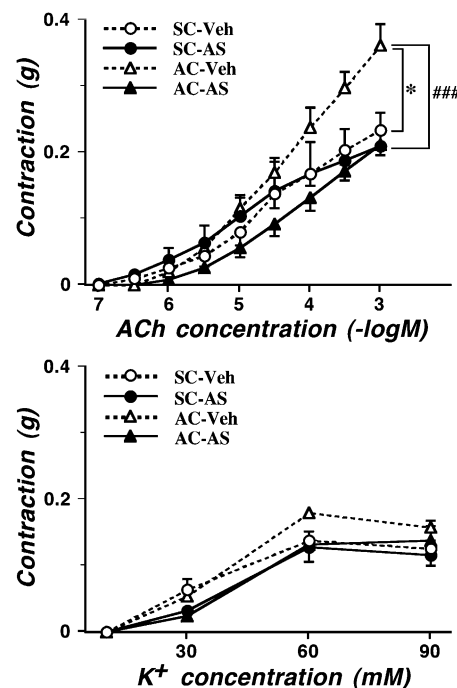


Figure 5. Inhibitory effects of AS on the antigen-induced bronchial smooth muscle (BSM) hyperresponsiveness in mice. Male BALB/c mice were actively sensitized and repeatedly challenged with OVA antigen as described in MATERIALS AND METHODS. Animals also received intraperitoneal injection of AS (10 mg/kg) or its vehicle (Veh; 20% DMSO in saline) 1 hour before each OVA challenge. Twenty-four hours after the last OVA challenge, the BSM responsiveness to acetylcholine (ACh; upper panel) and isotonic high K^+ (lower panel) were measured. Each point represents the mean $\pm$ SEM from six independent experiments. SC, sensitized control; AC, antigen-challenged groups. * $P < 0.05$ versus SC-Veh group and ### $P < 0.001$ versus AC-AS group by Bonferroni/Dunn's test.

STAT6 depletion using RNA interference and by co-incubation with a STAT6 inhibitor, AS1517499 (37). Moreover, in the mice with allergic bronchial asthma, the *in vivo* treatment with AS1517499 inhibited both the up-regulation of RhoA and the BSM hyperresponsiveness induced by antigen exposure. These findings suggest that STAT6 inhibitory agents might have an ability to inhibit BSM hyperresponsiveness, one of the factors of the AHR.

Currently, treatment of hBSMCs with AS1517499 inhibited the STAT6 phosphorylation induced by IL-13 (Figure 2A). AS1517499 is a novel selective STAT6 inhibitor synthesized based on the structure of a reported STAT6 inhibitor, TMC-264, discovered from the fungus *Phoma* (45, 46). Nagashima and colleagues (37) synthesized a series of 2-[[2-(4-hydroxyphenyl)ethyl]amino}pyrimidine-5-carboxamide derivatives and evaluated their STAT6 inhibitory activities using a STAT6 reporter assay in cells stably transfected with an IL-4-responsive luciferase reporter plasmid. Among these compounds, AS1517499 showed a potent STAT6 inhibition with a 50% inhibitory concentration (IC_{50}) of 21 nM (37). AS1517499 also inhibited the IL-4-induced Th2 cell differentiation of mouse spleen T cells with an IC_{50} value of 2.3 nM without influencing the IL-12-induced Th1 cell differentiation (37). Although the exact mechanism of inhibition of STAT6 by AS1517499 is unclear, the parent compound TMC-264 is known to inhibit both tyrosine phosphorylation of STAT6, with an IC_{50} value of 1.6 μM (1,600 nM), and the complex formation of phosphorylated STAT6 with its

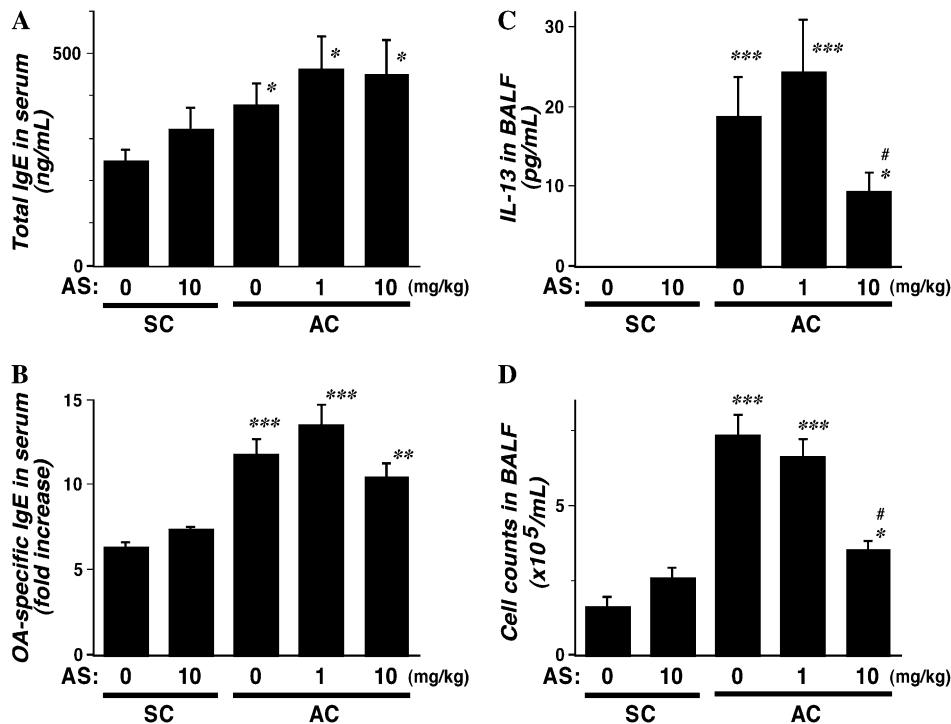


Figure 6. Effects of AS on the antigen-induced production of IgE and IL-13 and infiltration of inflammatory cells into the airways in mice. Male BALB/c mice were actively sensitized and repeatedly challenged with OVA antigen as described in MATERIALS AND METHODS. Animals also received intraperitoneal injection of AS (1 or 10 mg/kg) or its vehicle (AS (0); 20% DMSO in saline) 1 hour before each OVA challenge. Twenty-four hours after the last OVA challenge, the levels of (A) total and (B) OVA-specific IgE in sera and (C) IL-13 levels and (D) total cell counts in bronchoalveolar lavage fluids (BALFs) were determined. Each column represents the mean $\pm$ SEM from four to five independent experiments. SC, sensitized control; AC, antigen-challenged groups. * P < 0.05, ** P < 0.01 and *** P < 0.001 versus SC-AS (0) group and # P < 0.05 versus AC-AS (0) group by Bonferroni/Dunn's test.

recognition DNA sequence (46). The present study suggests that mode of action of AS1517499 is at least the inhibition of phosphorylation of STAT6. However, nonspecific effect(s) other than the inhibition of STAT6 phosphorylation might also be considerable, since other small molecule STAT6 inhibitors, such as leflunomide and niflumic acid, have been known to act on various protein kinases and/or ion channels (*see, e.g., Refs. 35 and 36*).

As well as our previous findings that both the STAT6 phosphorylation and RhoA up-regulation induced by IL-13 were inhibited by leflunomide (250 μ M) (26), treatment of hBSCs with a STAT6 siRNA (Figure 1C) or a STAT6 inhibitor AS1517499 inhibited the up-regulation of RhoA induced by IL-13 (Figure 2B). These findings indicate that STAT6 activation in BSM cells is involved in the IL-13-induced up-regulation of RhoA. The importance of STAT6 activation in the IL-13-induced up-regulation of RhoA might also be supported by the observations that AS1517499 did not inhibit the phenomenon when administered after the IL-13 treatment (Figure 2C). The *in vivo* study revealed that the RhoA up-regulation in bronchial tissues of the antigen-challenged mice was inhibited by AS1517499, dose-dependently (Figure 4). Moreover, the antigen-induced BSM hyperresponsiveness, which is associated with an augmented RhoA/Rho-kinase signaling (8), was abolished by the AS1517499 treatments (Figure 5). We have previously reported that an increase in the active form of RhoA (*i.e., membrane-associated and/or GTP-bound forms of RhoA proteins*) induced by contractile agonists, such as ACh and endothelin-1, was augmented in BSMs of antigen-induced AHR animals (8, 47–49), suggesting that agonist stimulation activates the up-regulated RhoA proteins, resulting in a greater contraction of BSMs. Taken together, IL-13, generated by antigen exposure in the airways (Figure 6C), directly acts on BSM cells and activates their STAT6, and then up-regulates RhoA protein in the cells, resulting in an augmentation of the agonist-induced, RhoA/Rho-kinase-mediated contraction of BSM. As described above, a critical role of STAT6 signal transduction in the development of allergen-induced AHR has also been suggested

in STAT6 knockout mice (31–33), although the change in BSM itself in these animals was not studied. Alternatively, the inhibitory effects of AS1517499 on the IL-13 production (Figure 6C) and inflammatory cells infiltration (Figure 6D) might also be involved in the inhibition of BSM hyperresponsiveness, indirectly.

In the present study, AS1517499 was treated 1 hour before each antigen challenge (but not before the first immunization) to determine its effect on BSM hyperresponsiveness induced by the antigen exposure. As shown in Figure 6C, the IL-13 level in BALF was significantly reduced in the AS1517499 (10 mg/kg)-treated group. It is thus likely that STAT6 inhibitory agents might also have effects on many types of cells other than BSMs *in vivo*. However, the inhibition of IL-13 level in BALF was only partial (Figure 6C), whereas the RhoA up-regulation (Figure 4) and BSM hyperresponsiveness (Figure 5) were almost completely inhibited at the dosage of 10 mg/kg. In addition, AS1517499 had no significant effect on IgE production, either in the sensitized control or in the repeatedly antigen-challenged groups (Figures 6A and 6B). Since IgE production by B cells is believed to be induced after Th2 differentiation (*see, e.g., Ref. 50*), the inhibitory effects of AS1517499 on antigen-induced BSM hyperresponsiveness and RhoA up-regulation might not be due to the inhibition of Th2 differentiation, at least under the experimental condition used. The findings that BSM hyperresponsiveness and RhoA up-regulation induced by intranasal administration of IL-13 in nonsensitized normal animals were inhibited by AS1517499 (our personal observations) may also support an involvement of IL-13–STAT6 signaling pathway in the development of antigen-induced BSM hyperresponsiveness. On the other hand, we have some discrepancy between time-course changes in the STAT6 phosphorylation (Figure 1A) and the RhoA up-regulation (26) induced by IL-13 in hBSCs. It may also be possible that unknown mediator(s) produced by an activation of STAT6 in BSMs themselves is involved in the IL-13-induced up-regulation of RhoA.

The current findings suggest that IL-13 is capable of up-regulating RhoA via an activation of STAT6 in hBSCs. In

addition to the cytokine receptor-mediated activation of STATs such as STAT6 activation by IL-13 observed in the present study, Pelletier and coworkers (51) have previously reported that Rho family of proteins themselves play an essential role in induction of STAT transcriptional activity mediated by G protein-coupled receptors in vascular smooth muscle cells. To determine the role of RhoA itself in the IL-13-induced up-regulation of RhoA, the effect of Y-27632 (1 μ M), a selective inhibitor of RhoA downstream Rho-kinase, was examined. As shown in Figure 1D, Y-27632 had no inhibitory effect on the IL-13-induced up-regulation of RhoA. It is thus unlikely that the RhoA/Rho-kinase signaling itself is involved in the RhoA up-regulation induced by IL-13 in hBSMC.

In conclusion, the *in vivo* treatment with AS1517499 ameliorated the antigen-induced BSM hyperresponsiveness by inhibiting the RhoA up-regulation in BSMs and, at least in part, by reducing the IL-13 production in the airways in mice. Both the direct and indirect effects of STAT6 inhibitory agents, such as AS1517499, on BSMs might be useful for the treatment of allergic bronchial asthma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

- Boushey HA, Holtzman MJ, Sheller JR, Nadel JA. Bronchial hyperactivity. *Am Rev Respir Dis* 1980;121:389–413.
- Jeffery PK. Microscopic structure of airway secretory cells: variation in hypersecretory disease and effects of drugs. In: Takishima T, Shimura S, editors. *Airway secretion: physiological basis for the control of mucus hypersecretion*. New York, NY: Marcel Dekker; 1993. pp. 149–215.
- Wiggs BR, Moreno R, Hogg JC, Hilliam C, Pare PD. A model of the mechanics of airway narrowing. *J Appl Physiol* 1990;69:849–860.
- Seow CY, Schellenberg RR, Pare PD. Structural and functional changes in the airway smooth muscle of asthmatic subjects. *Am J Respir Crit Care Med* 1998;158:S179–S186.
- Martin JG, Duguet A, Eidelman DH. The contribution of airway smooth muscle to airway narrowing and airway hyperresponsiveness in disease. *Eur Respir J* 2000;16:349–354.
- Yoshii A, Iizuka K, Dobashi K, Horie T, Harada T, Nakazawa T, Mori M. Relaxation of contracted rabbit tracheal and human bronchial smooth muscle by Y-27632 through inhibition of Ca^{2+} sensitization. *Am J Respir Cell Mol Biol* 1999;20:1190–1200.
- Chiba Y, Takada Y, Miyamoto S, Mitsui-Saito M, Karaki H, Misawa M. Augmented acetylcholine-induced, Rho-mediated Ca^{2+} sensitization of bronchial smooth muscle contraction in antigen-induced airway hyperresponsive rats. *Br J Pharmacol* 1999;127:597–600.
- Chiba Y, Ueno A, Shinozaki K, Takeyama H, Nakazawa S, Sakai H, Misawa M. Involvement of RhoA-mediated Ca^{2+} sensitization in antigen-induced bronchial smooth muscle hyperresponsiveness in mice. *Respir Res* 2005;6:4.
- Chiba Y, Sakai H, Wachi H, Sugitani H, Seyama Y, Misawa M. Upregulation of rhoA mRNA in bronchial smooth muscle of antigen-induced airway hyperresponsive rats. *J Smooth Muscle Res* 2003;39:221–228.
- Gosens R, Schaafsma D, Nelemans SA, Halayko AJ. Rho-kinase as a drug target for the treatment of airway hyperresponsiveness in asthma. *Mini Rev Med Chem* 2006;6:339–348.
- Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science* 1998;282:2258–2261.
- Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, *et al.* Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;282:2261–2263.
- Walter DM, McIntire JJ, Berry G, McKenzie AN, Donaldson DD, DeKruyff RH, Umetsu DT. Critical role for IL-13 in the development of allergen-induced airway hyperactivity. *J Immunol* 2001;167:4668–4675.
- Wynn TA. IL-13 effector functions. *Annu Rev Immunol* 2003;21:425–456.
- Zhu Z, Zheng T, Homer RJ, Kim YK, Chen NY, Cohn L, Hamid Q, Elias JA. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science* 2004;304:1678–1682.
- Wills-Karp M. Interleukin-13 in asthma pathogenesis. *Immunol Rev* 2004;202:175–190.
- Yang G, Volk A, Petley T, Emmell E, Giles-Komar J, Shang X, Li J, Das AM, Shealy D, Griswold DE, *et al.* Anti-IL-13 monoclonal antibody inhibits airway hyperresponsiveness, inflammation and airway remodeling. *Cytokine* 2004;28:224–232.
- Yang G, Li L, Volk A, Emmell E, Petley T, Giles-Komar J, Rafferty P, Lakshminarayanan M, Griswold DE, Bugelski PJ, *et al.* Therapeutic dosing with anti-interleukin-13 monoclonal antibody inhibits asthma progression in mice. *J Pharmacol Exp Ther* 2005;313:8–15.
- Eum SY, Maghni K, Tolloczko B, Eidelman DH, Martin JG. IL-13 may mediate allergen-induced hyperresponsiveness independently of IL-5 or eotaxin by effects on airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L576–L584.
- Berry MA, Parker D, Neale N, Woodman L, Morgan A, Monk P, Bradding P, Wardlaw AJ, Pavord ID, Brightling CE. Sputum and bronchial submucosal IL-13 expression in asthma and eosinophilic bronchitis. *J Allergy Clin Immunol* 2004;114:1106–1109.
- Saha SK, Berry MA, Parker D, Siddiqui S, Morgan A, May R, Monk P, Bradding P, Wardlaw AJ, Pavord ID, *et al.* Increased sputum and bronchial biopsy IL-13 expression in severe asthma. *J Allergy Clin Immunol* 2008;121:685–691.
- Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999;103:779–788.
- Yang M, Hogan SP, Henry PJ, Matthei KI, McKenzie AN, Young IG, Rothenberg ME, Foster PS. Interleukin-13 mediates airways hyperactivity through the IL-4 receptor- α chain and STAT-6 independently of IL-5 and eotaxin. *Am J Respir Cell Mol Biol* 2001;25:522–530.
- Bryce PJ, Mathias CB, Harrison KL, Watanabe T, Geha RS, Oettgen HC. The H1 histamine receptor regulates allergic lung responses. *J Clin Invest* 2006;116:1624–1632.
- Laporte JC, Moore PE, Baraldo S, Jouvin MH, Church TL, Schwartzman IN, Panettieri RA Jr, Kinet JP, Shore SA. Direct effects of interleukin-13 on signaling pathways for physiological responses in cultured human airway smooth muscle cells. *Am J Respir Crit Care Med* 2001;164:141–148.
- Chiba Y, Nakazawa S, Todoroki M, Shinozaki K, Sakai H, Misawa M. Interleukin-13 augments bronchial smooth muscle contractility with an up-regulation of RhoA Protein. *Am J Respir Cell Mol Biol* 2009;40:159–167.
- Zhao A, McDermott J, Urban JF Jr, Gause W, Madden KB, Yeung KA, Morris SC, Finkelman FD, Shea-Donohue T. Dependence of IL-4, IL-13, and nematode-induced alterations in murine small intestinal smooth muscle contractility on Stat6 and enteric nerves. *J Immunol* 2003;171:948–954.
- Akiho H, Blennerhassett P, Deng Y, Collins SM. Role of IL-4, IL-13, and STAT6 in inflammation-induced hypercontractility of murine smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol* 2002;282:G226–G232.
- Lee JH, Kaminski N, Dolganov G, Grunig G, Koth L, Solomon C, Erle DJ, Sheppard D. Interleukin-13 induces dramatically different transcriptional programs in three human airway cell types. *Am J Respir Cell Mol Biol* 2001;25:474–485.
- Akiho H, Lovato P, Deng Y, Ceponis PJ, Blennerhassett P, Collins SM. Interleukin-4- and -13-induced hypercontractility of human intestinal muscle cells-implication for motility changes in Crohn's disease. *Am J Physiol Gastrointest Liver Physiol* 2005;288:G609–G615.
- Akimoto T, Numata F, Tamura M, Takata Y, Higashida N, Takashi T, Takeda K, Akira S. Abrogation of bronchial eosinophilic inflammation and airway hyperactivity in signal transducers and activators of transcription (STAT)6-deficient mice. *J Exp Med* 1998;187:1537–1542.
- Kuperman D, Schofield B, Wills-Karp M, Grusby MJ. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J Exp Med* 1998;187:939–948.
- Tomkinson A, Kanehiro A, Rabinovitch N, Joetham A, Cieslewicz G, Gelfand EW. The failure of STAT6-deficient mice to develop airway eosinophilia and airway hyperresponsiveness is overcome by interleukin-5. *Am J Respir Crit Care Med* 1999;160:1283–1291.

34. McCusker CT, Wang Y, Shan J, Kinyanjui MW, Villeneuve A, Michael H, Fixman ED. Inhibition of experimental allergic airways disease by local application of a cell-penetrating dominant-negative STAT-6 peptide. *J Immunol* 2007;179:2556–2564.
35. Li J, Si HF, Lü X, Guo A, Jiang H. Suppressive effects of leflunomide on leptin-induced TIMP-1 production involves on hepatic stellate cell proliferation and apoptosis. *Eur J Pharmacol* 2008;580:63–69.
36. Fernandez D, Sargent J, Sachse FB, Sanguinetti MC. Structural basis for ether-a-go-go-related gene K⁺ channel subtype-dependent activation by niflumic acid. *Mol Pharmacol* 2008;73:1159–1167.
37. Nagashima S, Yokota M, Nakai E, Kuromitsu S, Ohga K, Takeuchi M, Tsukamoto S, Ohta M. Synthesis and evaluation of 2-[[2-(4-hydroxyphenyl)-ethyl]amino]pyrimidine-5-carboxamide derivatives as novel STAT6 inhibitors. *Bioorg Med Chem* 2007;15:1044–1055.
38. Kato Y, Manabe T, Tanaka Y, Mochizuki H. Effect of an orally active Th1/Th2 balance modulator, M50367, on IgE production, eosinophilia, and airway hyperresponsiveness in mice. *J Immunol* 1999;162:7470–7479.
39. Chiba Y, Kurotani R, Kusakabe T, Miura T, Link B, Misawa M, Kimura S. UGRP1 expression suppresses allergic airway inflammation in mice. *Am J Respir Crit Care Med* 2006;173:958–964.
40. Chiba Y, Kusakabe T, Kimura S. Decreased expression of uteroglobin-related protein 1 in inflamed mouse airways is mediated by IL-9. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L1193–L1198.
41. Amrani Y, Panettieri RA Jr. Modulation of calcium homeostasis as a mechanism for altering smooth muscle responsiveness in asthma. *Curr Opin Allergy Clin Immunol* 2002;2:39–45.
42. Chiba Y, Ueno A, Sakai H, Misawa M. Hyperresponsiveness of bronchial but not tracheal smooth muscle in a murine model of allergic bronchial asthma. *Inflamm Res* 2004;53:636–642.
43. Somlyo AP, Somlyo AV. Ca²⁺ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 2003;83:1325–1358.
44. Chiba Y, Misawa M. The role of RhoA-mediated Ca²⁺ sensitization of bronchial smooth muscle contraction in airway hyperresponsiveness. *J Smooth Muscle Res* 2004;40:155–167.
45. Sakurai M, Nishio M, Yamamoto K, Okuda T, Kawano K, Ohnuki T. TMC-264, a novel antiallergic heptaketide produced by the fungus *Phoma* sp. TC 1674. *Org Lett* 2003;5:1083–1085.
46. Sakurai M, Nishio M, Yamamoto K, Okuda T, Kawano K, Ohnuki T. TMC-264, a novel inhibitor of STAT6 activation produced by *Phoma* sp. TC 1674. *J Antibiot (Tokyo)* 2003;56:513–519.
47. Chiba Y, Sakai H, Misawa M. Augmented acetylcholine-induced translocation of RhoA in bronchial smooth muscle from antigen-induced airway hyperresponsive rats. *Br J Pharmacol* 2001;133:886–890.
48. Chiba Y, Sakai H, Yu Y, Misawa M. Effects of repeated antigen exposure on endothelin-1-induced bronchial smooth muscle contraction and activation of RhoA in sensitized rats. *J Biochem* 2005;137:751–756.
49. Chiba Y, Arima J, Sakai H, Misawa M. Lovastatin inhibits bronchial hyperresponsiveness by reducing RhoA signaling in rat allergic asthma. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L705–L713.
50. Naoe Y, Setoguchi R, Akiyama K, Muroi S, Kuroda M, Hatam F, Littman DR, Taniuchi I. Repression of interleukin-4 in T helper type 1 cells by Runx/Cbfbeta binding to the Il4 silencer. *J Exp Med* 2007;204:1749–1755.
51. Pelletier S, Duhamel F, Coulombe P, Popoff MR, Meloche S. Rho family GTPases are required for activation of Jak/STAT signaling by G protein-coupled receptors. *Mol Cell Biol* 2003;23:1316–1333.