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Sphingosine 1-phosphate Induces Alpha-smooth Muscle Actin Expression in Lung Fibroblasts via Rho-kinase

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Transformation of fibroblasts into myofibroblasts is an important phenomenon that contributes to airway remodeling in bronchial asthma. Although several articles have recently indicated that a bioactive lysosphingolipid sphingosine 1-phosphate (S1P) plays roles in the pathogenesis of bronchial asthma, the role of S1P in the remodeling process is poorly understood. In the present study, we examined the effects of S1P on alpha-smooth muscle actin (SMA) expression and the morphology in lung fibroblasts. S1P stimulated the expression of alpha-SMA in a human lung fibroblast cell line WI38 that expresses EDG/S1P receptors. These processes were inhibited by Y-27632, but not by pertussis toxin. These results suggest that S1P induces a phenotypic change of lung fibroblasts via Rho-kinase that may lead to airway remodeling.

INTRODUCTION

Bronchial asthma is a chronic inflammatory disorder that is associated with reversible airway obstruction and bronchial hyperresponsiveness to various stimuli (19). Chronic inflammation contributes to structural changes of the asthmatic airway that are known as airway remodeling. Airway remodeling includes increased *lamina reticularis*, and the deposition of extracellular matrix produced by myofibroblasts that leads to the thickening of bronchus wall (8, 10).

Subepithelial myofibroblast hyperplasia is an important pathological feature of bronchial asthma (2, 9). Myofibroblasts are thought to be transformed from fibroblast sheath lying beneath the epithelium (2) and are characterized by high level expression of alpha-smooth muscle actin (alpha-SMA) expression (26). Transformed fibroblasts also appear during wound healing and in several fibrocontractive diseases (7). The expression of alpha-SMA suggests that the fibroblasts acquire the morphological and biochemical features of contractile cells.

Sphingosine 1-phosphate (S1P) is a bioactive lysosphingolipid implicated in cell growth, survival, mitogenesis and cytoskeletal remodeling. The roles of S1P in the pathogenesis of lung diseases are poorly understood. However, some reports have implicated S1P as an important inflammatory mediator in the pathogenesis of airway inflammation and bronchial asthma (1, 13). Ammit et al. have shown that S1P secretion in bronchoalveolar lavage fluid from the lungs of asthmatic patients is significantly increased after allergen challenge (1). S1P is indicated to stimulate histamine release from mast cells that causes the

hypercontractile state of airway smooth muscle cells associated with asthma (23). However, the effects of S1P on lung fibroblasts, which are important components of airway remodeling, remain obscure.

Here in the present study, we investigate the effects of S1P on the expression of alpha-SMA in lung fibroblast and demonstrate that S1P stimulates alpha-SMA expression via Rho-kinase in human lung fibroblast cell line WI38. Our data indicates that Rho-kinase regulates S1P-induced differentiation of lung fibroblasts into myofibroblasts which may contribute to airway wall remodeling in bronchial asthma.

MATERIALS AND METHODS

Materials

The human fetal lung fibroblast cell line, WI38, was obtained from the Riken Cell Bank (Japan). Pertussis toxin (PTX), S1P, anti-alpha-SMA monoclonal antibody and FITC-conjugated anti-mouse antibody were purchased from Sigma-Aldrich (Saint Louis, MO, USA). A Rho-kinase inhibitor Y-27632 was purchased from Calbiochem (La Jolla, CA, USA).

Cell culture

WI38 cell monolayers in 60 mm dishes maintained under a humidified 5% CO₂ atmosphere at 37°C in EMEM medium containing 10% fetal calf serum (FCS) were used between passages 3 and 8. Twenty-four hours after removing FCS from the culture media, cells were pretreated with EMEM medium containing control vehicle, PTX or Y-27632 for 24 hours. Then, cells were stimulated with control vehicle or S1P at various concentrations for 24 hours.

RNA isolation and RT-PCR analysis of EDG receptors for S1P

Total cellular RNA was extracted using ISOGEN (Nippon gene, Tokyo, Japan) as recommended by the manufacturer and then total RNA (2 µg) was reverse transcribed using a kit from Ambion (Austin, TX, USA). Amplification by PCR was proceeded using AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) under the following incubation conditions: 94 °C for 5 min and then 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by 72°C for 5 min and cooling to 4°C. Polymerase chain reaction products were size-fractionated in 1.5% agarose gels and visualized by ethidium bromide staining. The primers are listed in Table 1.

Western Blot Analysis

Alpha-SMA was detected by Western blotting as follows. Whole cell lysates (2.5 µg) were resolved by 10% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membrane. The blots were blocked with 5% non-fat milk in Tris-buffered saline/Tween 20 (TBS-T) and incubated with 1:2000 dilution of anti-alpha-SMA antibody in TBS-T for 1 hour at room temperature. The blots were washed and incubated with 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody for 1 hour at room temperature. The blots were repeatedly washed and incubated in detection reagents ECL Plus Western blotting detection reagents (Amersham)

Immunofluorescence staining

To evaluate alpha-SMA filament bundle formation, cultured fibroblasts spread on 2-well glass chamber slides (10,000 cells/well) were fixed in 3.0% paraformaldehyde in PBS for 20 min. After washing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. Alpha-SMA fibers were visualized by incubating with 1:400 dilution of monoclonal anti-alpha-SMA antibodies, clone 1A4 (Sigma Chemical CO., St. Louis, MO, USA) for 1 hour, followed by the incubation with 1:200 dilution of FITC-conjugated

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Table 1. Primers of EDG/S1P receptors

EDG-1 (GenBank accession number NM001400) forward 5'- GATATCATCGTCCGGCATTAC-3' (nucleotides 310-330) reverse 5'- ACCCTTCCCAGTGCATTGTTC-3' (nucleotides 1597- 1577)
EDG-3 (GenBank accession number NM005226) forward 5'- GACTGCTCTACCATCCTGCCC -3' (nucleotides 946 - 966) reverse 5' GTAGATGACAGGGTTCATGGC -3' (nucleotides 1290 - 1270)
EDG-5 (GenBank accession number AF034780) forward 5'- GCAGCTTGACTCGGAGTACCTGAAC -3' (nucleotides 5-30) reverse 5'- CGATGGCCAACAGGATGATGGAGAAG -3' (nucleotides 616-591)
EDG-6 (GenBank accession number AJ000479) forward 5'- GCCGGCTCATTGTTCTGCACTACAACC- 3' (nucleotides 87-113) reverse 5'- GCAGAAGAGGATGTAGCGCTTGGAGTAG -3' (nucleotides 646-619)
EDG-8 (GenBank accession number AF331840) forward 5'- ACTCACTTCTGAACCCCATCATCTAC -3' (nucleotides 902 - 927) reverse 5'- CTGTGGAGCCGCTGGTGTC -3' (nucleotides 1147-1129)
GAPDH (GenBank accession number BC014085) forward 5' – GGAGCCAAAAGGGTCATCATCTC -3' (nucleotides 1213-1235) reverse 5'-AGTGGGTGTCGCTGTTGAAGTC-3' (nucleotides 1744-1723)

anti-mouse antibody for 1 hour. Samples were observed using a laser scanning microscope (Axioskop, ZEISS, Germany). Twelve areas were selected at random from each well and pictures were taken at 400x magnification. The ratios of α -SMA positive fibroblasts to total cell counts were averaged for each slide.

Statistical analysis

The data were analyzed by the Wilcoxon signed-ranks test using Statcel version 2.0 (Seiunn-sya, Japan). $P < 0.05$ was considered significant. Values are expressed as means $\pm$ SE.

RESULTS

S1P initiates intracellular signaling through the specific G protein coupled receptors; EDG1/S1P1, EDG3/S1P3, EDG5/S1P2, EDG6/S1P4 and EDG8/S1P5. To determine which S1P receptors were expressed in WI38 human lung fibroblasts, total RNA was reverse transcribed and amplified by PCR. Fig. 1 shows that the mRNAs for the S1P receptors EDG1/S1P1, EDG3/S1P3 and EDG5/S1P2 were expressed in the WI38 cell line.

To explore the role of S1P in fibroblast transformation, we studied α -SMA protein expression by Western blotting and immunofluorescence microscopy. S1P induced α -SMA expression in a concentration-dependent manner in WI38 cells (Fig. 2A). As in Fig. 2B and C, immunofluorescence showed that the ratio of cells expressing α -SMA after S1P stimulation was significantly increased to $11.0 \pm 3.10\%$ (10 nM) and $12.5 \pm 3.83\%$ (100 nM) when compared to controls ($7.53 \pm 2.35\%$).

Fig. 1

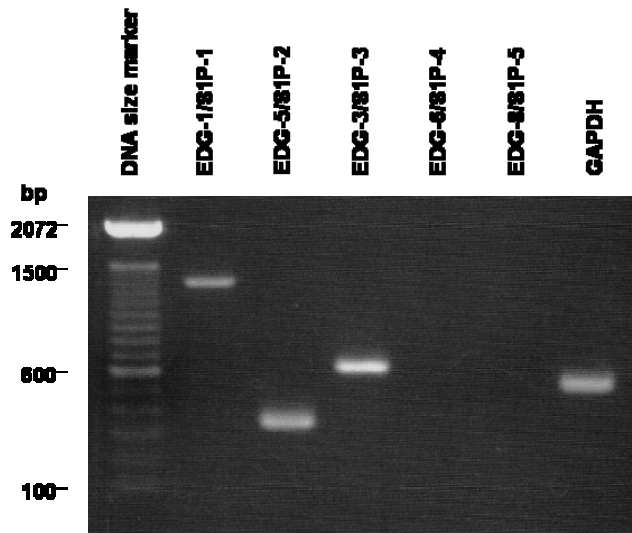


Fig. 1. Human lung fibroblasts WI38 express EDG receptors.

RT-PCR was performed to examine mRNA expression of each EDG receptor in WI38 cells. The expression of mRNA for EDG1/S1P1, EDG-5/S1P-2 and EDG-3/S1P3 was detected in WI38 cells. As a control, a housekeeping gene GAPDH mRNA expression was seen.

To investigate intracellular signaling pathways responsible for S1P-induced alpha-SMA expression in WI38 cells, we examined the involvement of Gi by the use of pertussis toxin (PTX). We found that S1P-induced alpha-SMA expression was not inhibited by the pretreatment of cells with PTX (Fig. 3A). As in Fig. 3B and C, immunofluorescence showed that the ratio of cells expressing alpha-SMA was not changed by the pretreatment of cells with PTX (control vs. PTX; $6.88 \pm 1.90\%$ vs. $6.81 \pm 2.07\%$). Significant increase of the ratio of cells expressing alpha-SMA after stimulation with 10 nM S1P was not changed by the pretreatment of cells with PTX (without PTX vs. with PTX; $10.20 \pm 2.26\%$ vs. $9.08 \pm 2.10\%$). Thus, PTX treatment was not able to block S1P-induced alpha-SMA expression in WI38 cells, suggesting that PTX-sensitive Gi is not involved in the induction of alpha-SMA expression by S1P.

The Rho/Rho-kinase-dependent signaling pathway is one of the major pathways that are associated with S1P coupled receptors. We examined the involvement of Rho-kinase in S1P-induced alpha-SMA expression in WI38 cells using a Rho-kinase inhibitor, Y-27632. Western blot analysis demonstrated that Y-27632 pretreatment decreased the basal expression of alpha-SMA compared with the control (Fig. 4A). The pretreatment of cells with Y-27632 prevented an increase in alpha-SMA expression induced by S1P in WI38 cells (Fig. 4A). Also, immunostaining with anti-alpha-SMA showed that Y-27632 decreased the ratios of alpha-SMA positive cells in both control cells ($7.50 \pm 2.33\%$ to $2.64 \pm 0.76\%$) and S1P-treated cells ($11.2 \pm 3.03\%$ to $2.39 \pm 0.83\%$)(Fig.4B). These data indicate that Rho-kinase controls basal as well as S1P-induced alpha-SMA in lung fibroblasts.

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Fig. 2

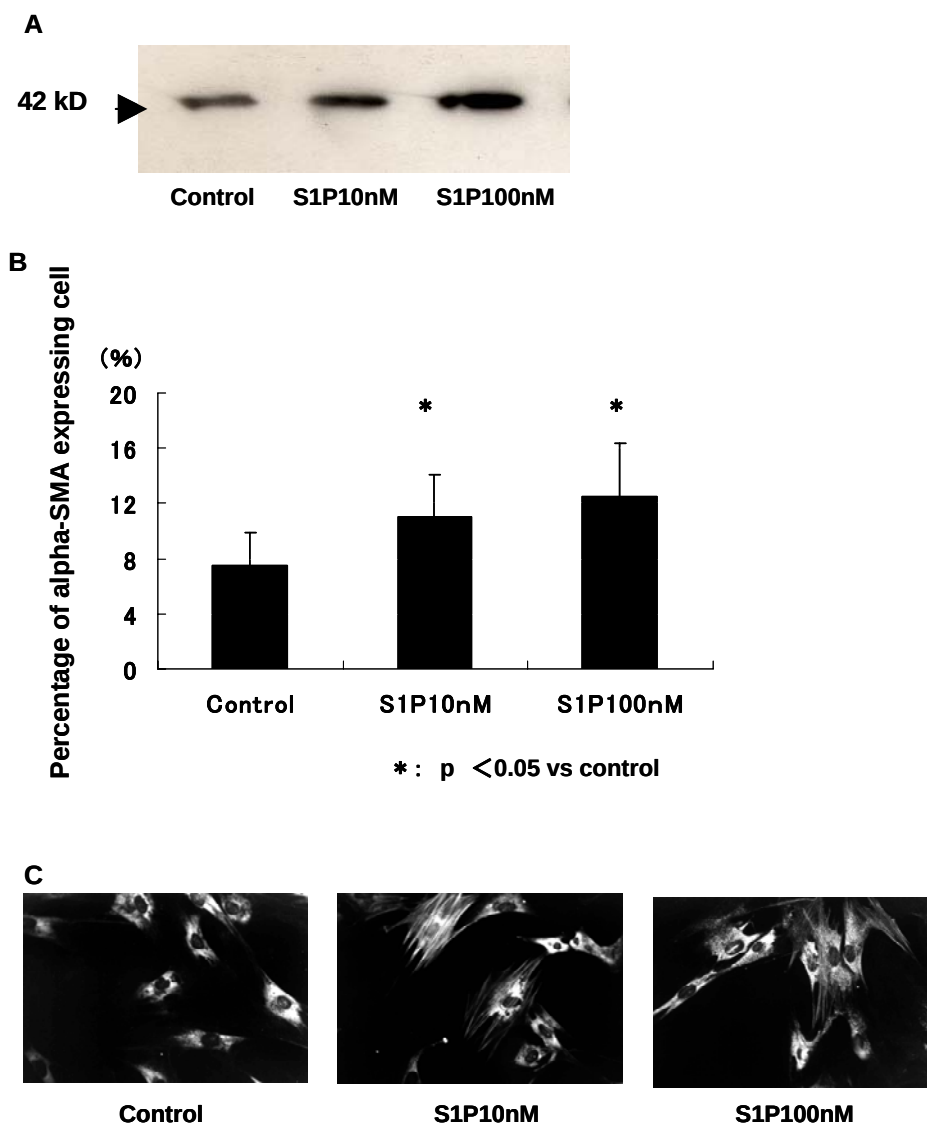


Fig. 2. S1P stimulates alpha-SMA expression stimulated in lung fibroblasts.

(A) Western blot analysis showed that the treatment of WI38 cells with 10 and 100 nM S1P significantly increased alpha-SMA protein expression. (B) The ratios of alpha-SMA positive WI38 cells to total cell counts were statistically analysed. The ratio of cells expressing alpha-SMA was significantly increased by S1P stimulation. (C) Representative photos of WI38 cells from each group immunostained with anti-alpha SMA are shown.

Fig.3

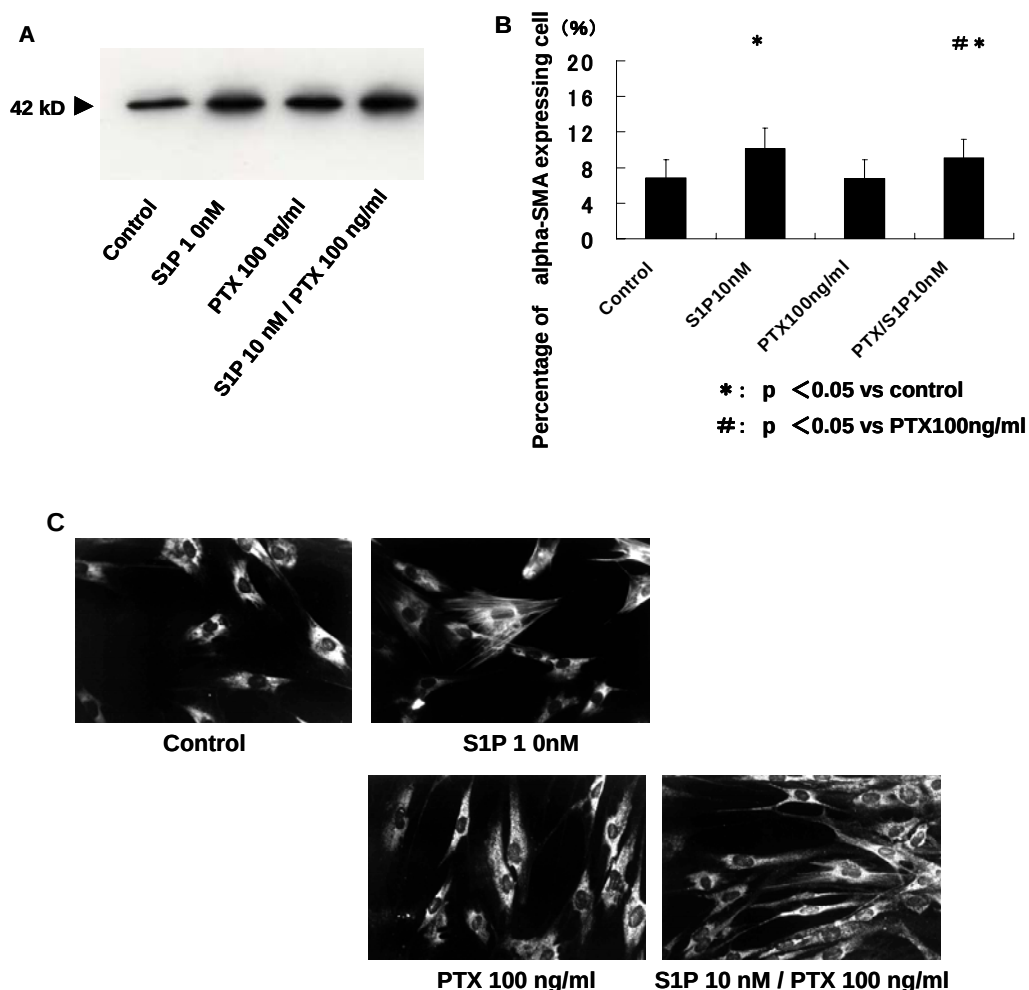


Fig. 3. PTX-sensitive Gi is not involved in S1P-induced alpha-SMA expression in lung fibroblasts. (A) Western blot analysis showed that the pretreatment of cells with PTX (100 ng/ml) did not inhibit alpha-SMA expression stimulated by S1P. (B) After immunofluorescence of alpha-SMA, the ratios of alpha-SMA positive WI38 cells to total cell counts were statistically analysed. The ratio of cells expressing alpha-SMA was significantly increased by S1P stimulation. This increase was not blocked by the pretreatment of cells with PTX. (C) Representative photos of WI38 cells from each group immunostained with anti-alpha SMA are shown.

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Fig.4

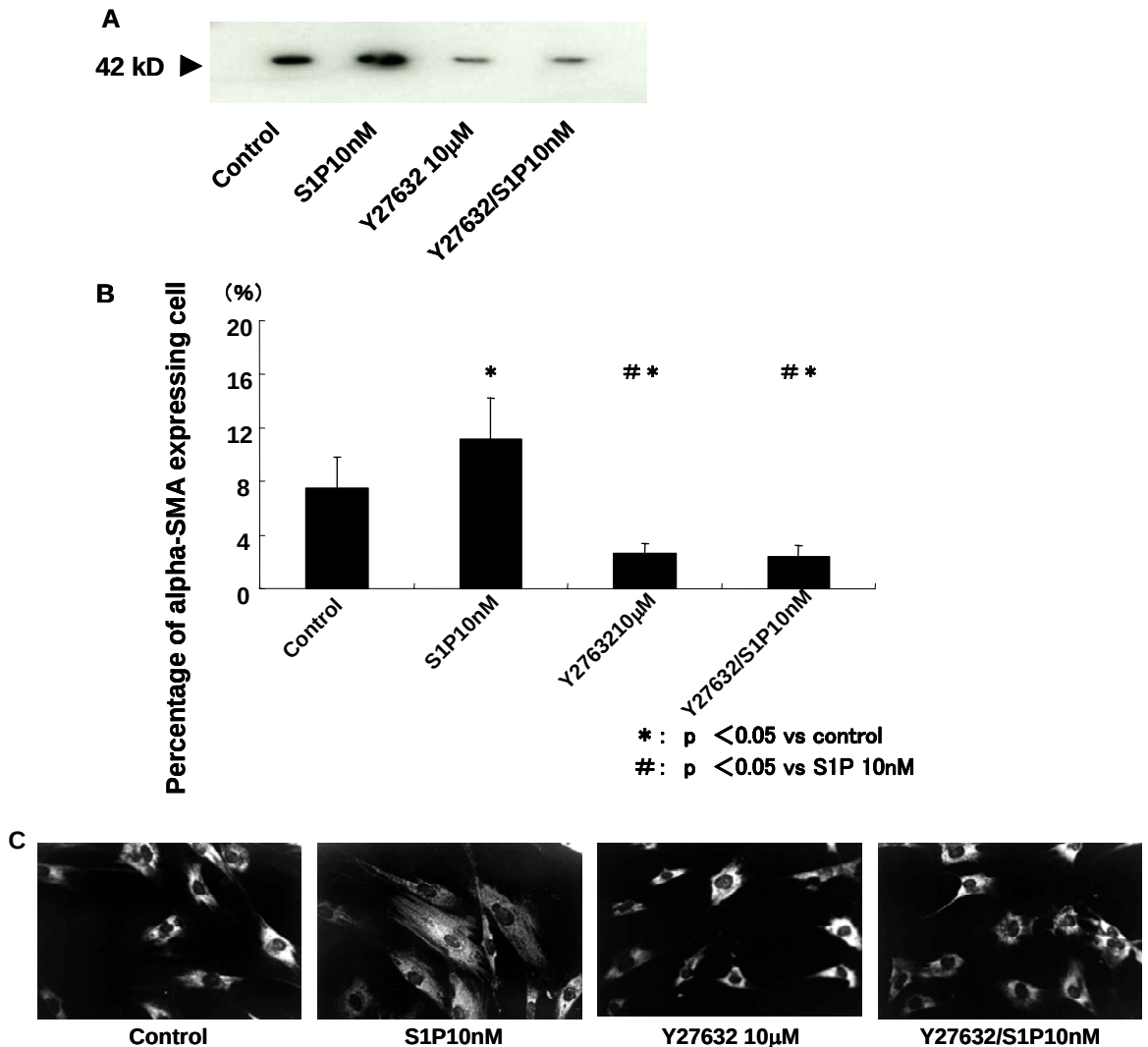


Fig. 4. Y-27632 inhibited S1P-induced fibroblast transformation.

(A) Western blots of anti- α -SMA. The pretreatment of cells with Y-27632 (10 μ M) significantly inhibited basal and S1P-induced α -SMA expression in WI38 cells. (B) The ratios of α -SMA positive WI38 cells to total cell counts were statistically analysed. The ratio of cells expressing α -SMA was significantly increased by Y-27632. (C) Representative photos of WI38 cells from each group immunostained with anti- α -SMA are shown.

DISCUSSION

Fibroblasts produce collagens and play important structural roles in the connective tissues of various organs. Fibroblasts undergo transformation to myofibroblasts during

wound healing (4), as well as in inflammatory diseases such as hepatitis and nephropathy (7). Myofibroblasts that are characterized by high expression levels of alpha-SMA secrete more collagens at inflammatory interstitial sites during inflammation.

Electron microscopy has shown that the numbers of cells with the ultrastructural features of myofibroblasts and smooth muscle cells are increased in asthmatics following bronchial allergen challenge (9). Carroll *et al.* reported that myofibroblast-like cells expressing SMA were detected in bronchi from lung of a patient with fatal asthma (3). Elevated contents of TGF-beta and EGF in bronchoalveolar lavage fluid from asthmatic airways are considered to potentially promote fibroblast transformation into myofibroblasts (29). Thus, fibroblast transformation is a key step in airway remodeling, but the precise mechanisms remain obscure.

The bioactive sphingolipid, S1P, is a powerful inflammatory mediator that induces airway smooth muscle cell growth, contraction and cytokine secretion (1, 25). Nanomolar amounts of S1P stimulate the growth of airway smooth muscle cell and further enhance DNA synthesis (1). However, few studies have demonstrated a role of S1P in the cell components involved in airway remodeling. It is reported that the concentration of S1P is increased to about 10 nM in bronchoalveolar lavage fluid and to nanomolar range in serum from asthmatics (1, 20). Receptors for EDG appear to be functional at nanomolar concentrations. For instance, the reported dissociation constant (Kd) for EDG1 and the EC50 of S1P are 2-30 nM and 1.5 nM, respectively (14, 15, 22, 28). Therefore, it is suggested that S1P in bronchoalveolar lavage fluid and serum is a potential mediator of airway inflammation.

S1P is produced ubiquitously by many types of cells including activated platelets, mast cells and monocytes, and acts as both an extracellular and an intracellular signal. Whereas extracellular S1P acts as a ligand for EDG/S1P receptors and activates intracellular signaling pathways (22), intracellular S1P functions as a second messenger. Davaille *et al.* suggested that S1P of a higher (micromolar) concentration tends to induce antiproliferative properties and apoptosis whereas S1P of nanomolar amounts passes through the membrane and exert intracellular effects (5). Since S1P of sub-micromolar concentrations activates EDG receptors (6), we used 10 and 100 nM S1P, which is considered to be appropriate for studying the function of S1P at endogenous levels.

The known members of the S1P receptor family are EDG1/S1P1, EDG5/S1P2, EDG3/S1P3, EDG6/S1P4 and EDG8/S1P5 (27). All S1P receptor subtypes are coupled to several heterotrimeric G proteins (24). EDG1/S1P1 is singularly coupled to Gi, whereas EDG5/S1P2 and EDG3/S1P3 receptors bind not only to Gi but also to Gq and G_{12/13} (27). G_{12/13} is shown to be implicated in the control of cell shape, gene expression and cell growth. These responses result, at least in part, from activation of the small G protein Rho (11,16). Ishii *et al.* recently generated mice that are null for S1P2 and for both S1P2 and S1P3, and found that Rho is activated (via G_{12/13}) through S1P2/EDG5 and S1P3/EDG3 receptors in mouse embryonic fibroblasts (12).

Rho proteins which are subfamily of Ras superfamily interacts with several effectors, and one of the best characterized Rho effectors is Rho-kinase (18). Rho-kinase is a serine-threonine kinase, which mediates many of the cytoskeletal effects of Rho including stress fiber formation. The regulation of alpha-SMA expression is studied recently, and the roles of Rho kinase and cytoskeleton have been revealing in several types of mesenchymal cell. Mack *et al.* have shown that the rat alpha-SMA promoter is regulated by actin polymerization in rat smooth muscle cells (17). In mesangial cells, it was demonstrated that

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the actin cytoskeleton regulated alpha-SMA promoter activity and alpha-SMA expression (21).

We found abundant EDG1/S1P1, EDG3/S1P3 and EDG5/S1P2 expression in the WI38 cell line. In this study, we examined the involvement of Gi and Rho-kinase in S1P effects on lung fibroblasts by pharmacological inhibition using PTX and Y-27632, respectively. Our results showed that S1P induced alpha-SMA expression in lung fibroblasts by a pathway that was dependent on Rho-kinase, but was independent of PTX-sensitive Gi. These data indicate that Rho-kinase plays important role in the phenotypic change of lung fibroblasts into myofibroblast-like cells. Interestingly, Rho-kinase seems to control basal expression of alpha-SMA in lung fibroblast (Fig. 4), suggesting that Rho-kinase may be implicated in the maintenance of fibroblastic phenotype. Thus, we provide novel evidence that S1P stimulates alpha-SMA expression via Rho-kinase leading to the transformation of lung fibroblasts into myofibroblast-like cells which is an important step of airway remodeling in asthma.

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