

# Possible Involvement of Sphingosine-1-Phosphate/ $G_i$ /RhoA Pathways in Adherence of Eosinophils to Pulmonary Endothelium

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## ABSTRACT

**Background:** Sphingosine-1-phosphate (S1P), a lysophospholipid released from inflammatory cells, causes cell migration by increasing cytokines and chemokines. This study was designed to determine whether S1P causes adherence of eosinophils to pulmonary endothelial cells via enhancement of adhesion molecule expression.

**Methods:** Expression of VCAM-1 and ICAM-1 was assessed by RT-PCR and Western blot analysis in human pulmonary microvasculature endothelial cells (HPMVECs). The number of adherent eosinophils to HPMVECs was calculated according to adhesion assay.

**Results:** Pre-treatment of HPMVECs with S1P increased mRNA and protein expression of VCAM-1, in contrast, did not dramatically increase those expression of ICAM-1. The maximal expression of these adhesion molecules in mRNA and protein was observed 4 and 8 h after exposure to S1P, respectively. Pre-treatment with S1P also activated RhoA, a monomeric G protein; the ability of S1P to enhance the expression of VCAM-1 was attenuated by RhoA related inhibitors such as Y-27632, C3 exoenzyme, and GGTI-286. The effects of S1P on VCAM-1 were attenuated by pre-incubation with pertussis toxin, which catalyzes the ADP-ribosylation of  $G_i$ , a heterotrimeric G protein.

After HPMVECs were treated with S1P, adhesion of human eosinophilic leukemic cell line (EoL-1) cells to HPMVECs was enhanced in a concentration-dependent manner. Augmented adherence of EoL-1 cells by S1P was also attenuated by Y-27632 and pertussis toxin. S1P causes adherence of eosinophils to pulmonary endothelium via RhoA activation.

**Conclusions:** S1P may act as a lipid mediator in asthma. The RhoA/Rho-kinase pathway may be a therapeutic target for preventing eosinophil infiltration to the airway.

## KEY WORDS

asthma, eosinophils, lipid mediators, pertussis toxin-sensitive G protein, Rho-kinase, small G protein, VCAM-1, Y-27632

## INTRODUCTION

Sphingosine 1-phosphate (S1P), a bioactive lysophospholipid generated in the plasma membrane, is capable of wide spectrum of biological effects, including cell growth, cell differentiation, and cell migration.<sup>1</sup> A

clinical trial has demonstrated that the level of S1P is elevated in bronchoalveolar lavage (BAL) fluid from patients with asthma following allergen challenges.<sup>2</sup> S1P causes eosinophil recruitment,<sup>3,4</sup> contraction of airway smooth muscle,<sup>5</sup> augmented responsiveness to methacholine,<sup>6,7</sup> and cytokine production involving

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airway remodeling.<sup>2</sup> Therefore, S1P may play an important role in the pathophysiology of asthma.<sup>8</sup>

Inhalation of lysophosphatidylcholine (Lyso-PC), a lysophospholipid synthesized by phospholipase A<sub>2</sub>, to guinea-pigs causes eosinophil recruitment to the airways, similar to S1P.<sup>9</sup> Lyso-PC enhances expression of adhesion molecule such as vascular adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1) in human and rabbit endothelial cells.<sup>10</sup> When S1P and Lyso-PC are released from inflammatory cells, these lysophospholipids may act as novel lipid mediators in patients with asthma. However, little is currently known about mechanisms underlying eosinophil recruitment induced by S1P in detail.

Although cytokines and chemokines play an important role in the eosinophil infiltration to the airways, eosinophil adhesion to endothelial cells is a fundamental process for this phenomenon. Adhesion molecules (VCAM-1 and ICAM-1) contribute to the eosinophil recruitment from the circulation since activated endothelial cells transiently express adhesion molecules involved in the trafficking of circulating inflammatory cells across the endothelial barrier into underlying tissues.<sup>11</sup> However, involvement of S1P in the induction of the adhesion molecules remains to be elucidated.

RhoA, a small monomeric GTP-binding (G) protein, and Rho-kinase, an effector molecule of RhoA, have effects on Ca<sup>2+</sup> sensitization, cell differentiation, and cytoskeleton reorganization.<sup>12,13</sup> Y-27632 and HA-1077, Rho-kinase inhibitors, inhibit both eosinophilic inflammation and airway hyperresponsiveness induced allergen challenges in the airways.<sup>14</sup> Y-27632 also inhibits agonist-induced contraction,<sup>5,15-17</sup>  $\beta$ -adrenergic desensitization,<sup>18,19</sup> hyperresponsiveness to methacholine,<sup>6,20</sup> and cell proliferation<sup>21</sup> in airway smooth muscle. RhoA is proposed as one of the post-receptor signal transduction pathways of S1P. Therefore, relationship between S1P and the RhoA/Rho-kinase pathways may play a key role for the pathophysiology of asthma.

This study was designed to determine whether S1P is involved in the eosinophil inflammation of asthma. We examined causal relationship between S1P and expression of the adhesion molecules on the cell surface in pulmonary endothelial cells. Moreover, intracellular mechanisms of upregulation of the adhesion molecules induced by S1P were investigated in detail, focused on the RhoA/Rho-kinase processes.

## METHODS

### CELL CULTURE

Normal human pulmonary microvascular endothelial cells (HPMVECs) from multiple donors were obtained from Cambrex (Walkersville, MD, USA). The cells were maintained in culture medium (EBM-2; Cambrex), containing 5% fetal bovine serum (FBS),

hydrocortisone, hEGF, hFGF-B, VEGF, R<sup>3</sup>-IGF-1, gentamicin, amphotericinB and ascorbic acid (EGM-2MV; Cambrex) in an atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C. Cells at 5-8th passage were used for the experiments. A human eosinophilic leukemic cell line (EoL-1),<sup>22</sup> which has been considered to be an *in vitro* eosinophilic model, was obtained from RIKEN BioResource Center (Tsukuba, Japan) and maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS, penicillin and streptomycin.

### RT-PCR

HPMVECs were sub-cultured in 60 mm dishes in complete EBM-2 medium at a density of  $1 \times 10^5$  cells/dish, then in a serum-starved EBM-2 medium for 24 h. Total RNA was isolated after stimulation with S1P using the RNeasy mini kit (Qiagen in Japan, Tokyo, Japan) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA using a Superscript III kit (Invitrogen, Carlsbad, CA, USA). PCR amplification was performed with 30 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C. Oligonucleotide primers were generated by (Rikaken Co., Ltd., Nagoya, Japan) using the following sequences: VCAM-1, 5'-TCCGTCCTCATTGACTTGACAG-3' and 5'-TTCCAGG-GACTTCCTGTCTG-3'; ICAM-1, 5'-CATAGAGACC CCGTTGCCTA-3' and 5'-GAA-ATTGGCTCCATGGT GAT-3'; GAPDH 5'-AACGGATTTGGTCGTATTGG-3' and 5'-TGAGTCCTTCCACGATACCA-3'; very late antigen 4 (VLA-4); 5'-GAGTGCCCC-CTGATTACGA-3' and 5'-TCCAACGAGGTTTGTTCCTCA-3'; lymphocyte function associated antigen 1 (LFA-1), 5'-GCTGGGG GCTTTCTTGACCT-3' and 5'-GCCCTG-GAACTGGG GGATGA-3'. The product sizes were VCAM-1 407 bp, ICAM-1 375 bp, GAPDH 498 bp, VLA-4 581 bp, and LFA-1 876 bp. PCR products were resolved on 1.6% agarose gels, stained with ethidium bromide, and photographed. Quantification of RT-PCR were normalized to GAPDH and analyzed by using digital scanning and Scion Image software (Scion Image, Frederick, MD, USA).

### RhoA ACTIVATION ASSAY

RhoA activation was measured by Rhotekin pull-down assay. HPMVECs were grown to confluence in 100 mm dishes and then placed in serum-free medium for 24 h. Cells were stimulated with 10  $\mu$ M S1P for 10 min. After stimulation with S1P, cells were then washed with ice cold PBS. The GTP-RhoA enriched lysates was prepared using the Rho Activation Kit (Upstate Biotechnology Inc, Lake Placid, NY, USA), according to the manufacturer's instructions.

### WESTERN BLOTTING

Expression of VCAM-1, ICAM-1 and RhoA in HPMVEC were evaluated by using specific antibodies for each protein. HPMVECs were grown to con-

fluence in 60-mm dishes and then placed in serum-free medium for 24 h. Cells were stimulated with a range of concentrations (0.1-10  $\mu$ M) of S1P for various times (0-24 h). After stimulation with S1P, cells were washed with ice-cold PBS. Whole cellular lysates were prepared with lysis buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% 2-mercaptoethanol). Protein contents of cellular lysates were measured by using Bio-Rad protein assay reagent kit (Bio-Rad, Hercules, CA, USA). Equal amounts of lysates, adjusted to protein content, were resolved by SDS-PAGE using a 4-20% linear gradient running gel (Daiichi Pure Chemicals, Tokyo, Japan). Proteins were transferred to nitrocellulose membrane, and the membrane was incubated at room temperature in PBS for 1 h. Immunoblotting was performed using antibodies against VCAM-1 (sc-8304; Santa Cruz Biotechnology, Santa Cruz, CA, USA), ICAM-1 (sc-1511; Santa Cruz Biotechnology) and RhoA (RhoA [26C4]: sc-418; Santa Cruz Biotechnology). Immunodetection was accomplished using a donkey anti-rabbit secondary antibody or a rabbit anti-goat secondary antibody and the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA). The intensity was quantified by using Scion image software (Scion Image).

#### EoL-1 ADHESION ASSAY

The adhesion assays were performed as previously described with modifications.<sup>23</sup> HPMVECs were grown to confluence in 24-well plates in complete EBM-2 medium. Cells were stimulated with 1 or 10  $\mu$ M S1P for 6 h in complete medium. For experiments using inhibitors, 10  $\mu$ M Y-27632 or 300 ng/ml Pertussis toxin (PTX) were applied 30 min or 4 h before the stimulation, respectively. Before addition of human eosinophilic leukemic cells, EoL-1 cells to the endothelial cells, EoL-1 cells ( $1 \times 10^6$  cells/ml) were labeled for 40 min at 37°C with 5  $\mu$ M of fluorescence dye calcein acetomethylester (Calcein-AM; Dojindo Laboratories, Kumamoto, Japan) in RPMI1640 medium containing 10% FBS. EoL-1 cells were then washed two times with, and resuspended in RPMI 1640 medium containing 10% FBS (final concentration,  $1 \times 10^6$  cells/ml). A 1 ml aliquot of the stained cell suspension ( $1 \times 10^6$  cells/well) was added to the endothelial cells in 24-well plates from which the medium containing S1P and inhibitors were removed. The plates were incubated for 1 h at 37°C, and then washed two times with RPMI1640, and PBS (1 ml/well) was added. The relative fluorescence was measured using a fluorescence microplate reader (Fluoroskan Ascent CF; Labsystems, Helsinki, Finland), with excitation and emission wavelengths of 485 and 538 nm, respectively. The number of EoL-1 cells adhered to HPMVECs was calculated by comparing fluorescence to standard curves of Calcein-AM activity/cell.

#### EXPERIMENTAL PROTOCOLS

To determine the effects of S1P on adhesion molecules in HPMVECs, expression of VCAM-1 and ICAM-1 in response to S1P was examined using RT-PCR and Western blots. To determine whether the RhoA signal transduction pathways are involved in the upregulation of VCAM-1 and ICAM-1 expression induced by S1P, HPMVECs were treated with S1P in the presence of Y-27632, an inhibitor of Rho-kinase, exoenzyme C3, an ADP-ribosyltransferase inactivating Rho, and GGTI-286, an inhibitor of geranylgeranyl transferase-I (GGTase-I) which inhibits the activation of Rho by preventing the prenylation of these proteins and their translocations to membrane. Y-27632, exoenzyme C3, and GGTI-286 were added to the cells, 30 min prior to incubation with S1P. For the experiments using exoenzyme C3, cell permeabilization with streptolysin O was done according to previous reports.<sup>21</sup> Moreover, active form of RhoA (GTP-RhoA) was measured before and after exposure to S1P using the Rhotekin binding domain assay. To determine involvement of  $G_i$ , an inhibitory G protein of adenylyl cyclase, in the effects of S1P on these adhesion molecules, HPMVECs were treated with PTX, which catalyzes the ADP-ribosylation of the regulatory subunits of  $G_i$  protein, for 4 h prior to incubation with S1P. To determine whether S1P causes adhesion of eosinophils to endothelium, the number of adherent EoL-1 cells was measured after stimulation of HPMVECs with S1P for 8 h. To determine involvement of RhoA/Rho-kinase and  $G_i$  in adherence of EoL-1 cells to endothelial cells, the number of adherent EoL-1 cells was calculated after HPMVECs were treated with S1P, treated with S1P in the presence of Y-27632, and treated with S1P subsequent to incubation with PTX. Time-matched control cells were treated similarly to test cells, but were exposed to the serum-free medium (control) in the absence of S1P, Y-27632, exoenzyme C3, GGTI-286, and PTX.

#### REAGENTS

S1P was obtained from BIOMOL (Plymouth Meeting, PA, USA). PTX was obtained from Sigma (St. Louis, MO, USA). Y-27632 was obtained from Wako (Osaka, Japan). GGTI-286 and NSC23766 were obtained from EMD Biosciences (San Diego, CA, USA). C3 exoenzyme was obtained from List Biological Laboratories, Incorporated (Campbell, CA, USA).

#### STATISTICAL ANALYSIS

All of the experiments were performed in triplicate. The results of multiple observations are presented as mean  $\pm$  S.D., and compared using analysis of variance followed by Bonferroni-Dunn *post hoc* test.  $P < 0.05$  was considered to be a significant difference.

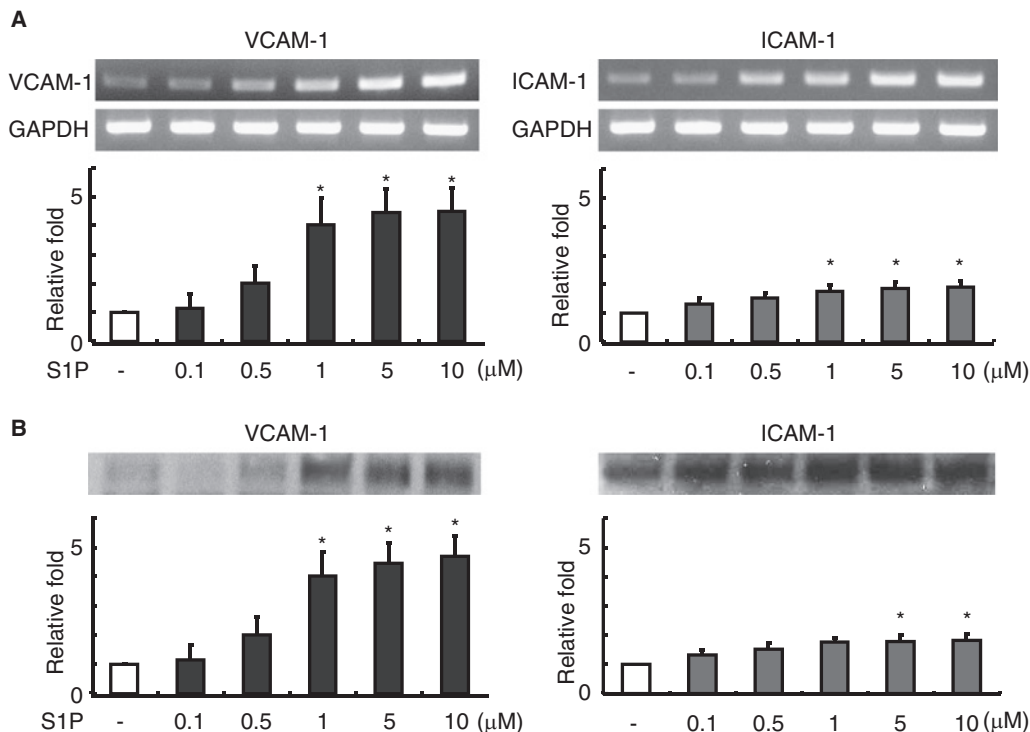


**Fig. 1** Involvement of S1P in the expression of adhesion molecules in HPMVECs. mRNA (**A**) and protein (**B**) expression of adhesion molecules (VCAM-1, ICAM-1) were increased in S1P-treated HPMVECs in a concentration-dependent manner. The expression of GAPDH was used to normalize the expression of adhesion molecules in RT-PCR analysis. In histograms, data were shown by the relative expression compared with the expression in control. -, control. \* $P < 0.05$  vs control.

## RESULTS

### ROLE OF S1P IN EXPRESSION OF ADHESION MOLECULES IN HPMVECs

The expression of VCAM-1 and ICAM-1 was examined by RT-PCR in HPMVECs incubated with S1P between 0.1 and 10  $\mu$ M for 4 h, and was examined by Western blotting analysis in HPMVECs with S1P at equi-molars incubated for 8 h. Exposure to S1P markedly upregulated both the mRNA and protein expression of VCAM-1 in a concentration-dependent manner (Fig. 1A, B). S1P ( $>1$   $\mu$ M) increased the mRNA and protein expression of VCAM-1 by  $4.5 \pm 0.8$  ( $n = 3$ ,  $P < 0.05$ ) and  $4.7 \pm 0.7$  ( $n = 3$ ,  $P < 0.05$ ) fold, respectively. S1P also upregulated these expression of ICAM-1 in a concentration-dependent manner (Fig. 1A, B). S1P ( $>1$   $\mu$ M) increased these expression of ICAM-1 by  $1.9 \pm 0.2$  ( $n = 3$ ,  $P < 0.05$ ) and  $1.8 \pm 0.2$  ( $n = 3$ ,  $P < 0.05$ ) fold, respectively.

Next, the mRNA and protein expression of VCAM-1 in HPMVECs were examined after incubation with 10  $\mu$ M S1P for up to 24 h. The mRNA expression of VCAM-1 was maximally increased at 4 h after application of S1P, but when the treatment with S1P was prolonged, the expression was decreased (Fig. 2A). In contrast, the protein expression of VCAM-1 in re-

sponse to S1P was maximally increased at 8 h after application of S1P (Fig. 2B).

### ROLE OF S1P IN ACTIVATION OF RhoA

After HPMVECs were exposed to serum-free medium in the absence (control) and presence of 10  $\mu$ M S1P for 10 min, relative proportion of GTP-RhoA to total RhoA (GTP-RhoA/total RhoA) was measured using the Rhotekin binding domain assay. The values of GTP-RhoA/total RhoA was increased to  $3.2 \pm 0.4$  fold ( $n = 3$ ,  $P < 0.05$ ) by application of 10  $\mu$ M S1P, compared with the control (Fig. 3).

### ROLE OF THE RhoA SIGNAL TRANSDUCTION PATHWAY IN S1P-INDUCED EXPRESSION OF ADHESION MOLECULES IN HPMVECs

#### Involvement of RhoA

When HPMVECs were exposed to 10  $\mu$ M S1P in the presence of exoenzyme C3 (10-30 ng/ml), levels of mRNA and protein expression in VCAM-1 were also attenuated by exoenzyme C3 in a concentration-dependent manner. Exoenzyme C3 (30 ng/ml) decreased the values of relative fold of mRNA and protein expression in VCAM-1 to  $1.4 \pm 0.2$  ( $n = 3$ ,  $P < 0.05$ ) and  $1.6 \pm 0.4$  ( $n = 3$ ,  $P < 0.05$ ) fold, respectively (Fig. 4A, B).

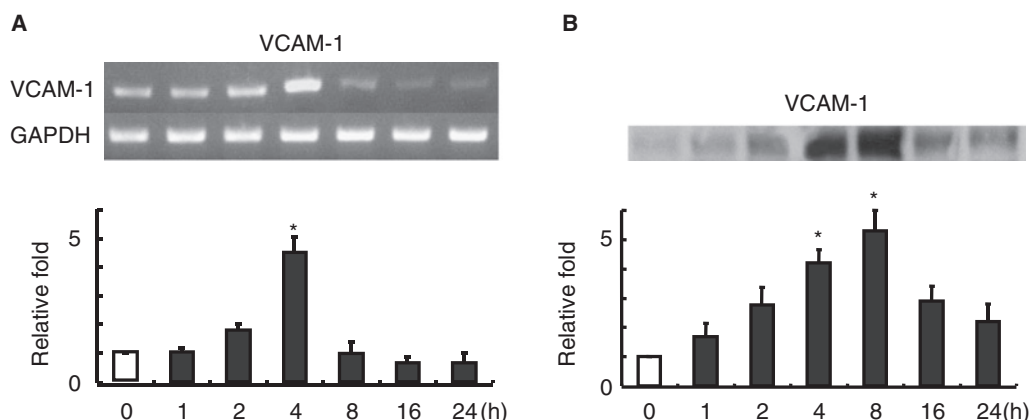


**Fig. 2** Time-dependent effects of S1P on the expression of adhesion molecules in HPMVECs. mRNA (A) and protein (B) expression of VCAM-1 were increased in 10  $\mu$ M S1P-treated HPMVECs in a time-dependent manner. The expression of GAPDH was used to normalize the expression of adhesion molecules in RT-PCR analysis. In histograms, data were expressed as shown in Figure 1. \* $P < 0.05$  vs control.

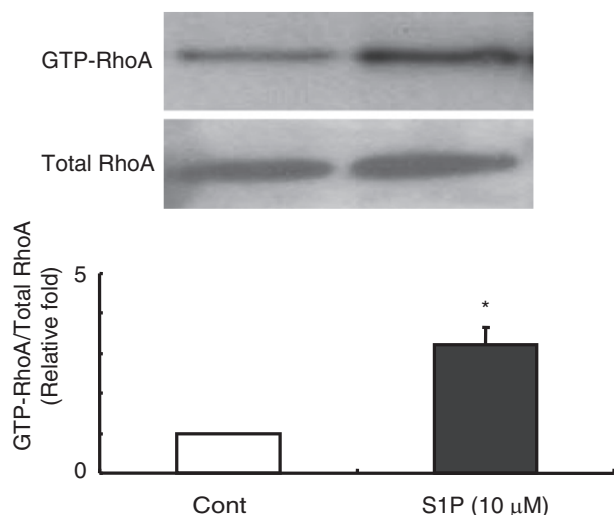


**Fig. 3** Involvement of S1P in RhoA activity in HPMVECs. Activated forms of RhoA (GTP-RhoA) was pulled down from the cell lysates with Rhotekin-binding conjugated beads, and analyzed by Western blotting using anti-RhoA. The identical cell lysates were used to determine total RhoA (top trace). The value of GTP-RhoA/total RhoA induced by S1P was calculated relative to that for control (bottom trace). \* $P < 0.05$  vs control.

#### Involvement of Geranylgeranyl Transferase-I

Furthermore, when HPMVECs were exposed to an equi-molar of S1P in the presence of GGTI-286 (1-10  $\mu$ M), levels of mRNA and protein expression in VCAM-1 were attenuated by GGTI-286 in a concentration-dependent manner. GGTI-286 (10  $\mu$ M) decreased the values of relative fold of mRNA and protein expression in VCAM-1 to  $1.9 \pm 0.2$  ( $n = 3$ ,  $P <$

0.05) and  $1.7 \pm 0.2$  ( $n = 3$ ,  $P < 0.05$ ) fold, respectively (Fig. 4C, D).

#### Involvement of Rho-kinase

Next, when HPMVECs were exposed to an equi-molar of S1P in the presence of Y-27632 (0.1-10  $\mu$ M), levels of mRNA and protein expression in VCAM-1 were attenuated by Y-27632 in a concentration-dependent manner. Y-27632 (10  $\mu$ M) decreased the values of relative fold of mRNA and protein expression in VCAM-1 to  $1.8 \pm 0.2$  ( $n = 3$ ,  $P < 0.05$ ) and  $1.2 \pm 0.5$  ( $n = 3$ ,  $P < 0.05$ ) fold, respectively (Fig. 5A, B).

#### ROLE OF $G_i$ IN S1P-INDUCED EXPRESSION OF ADHESION MOLECULES IN HPMVECs

HPMVECs were exposed to 10  $\mu$ M S1P in the absence and presence of PTX (10-300 ng/ml) for 4 h. Pre-treatment with PTX suppressed markedly the augmented levels of mRNA and protein expression in VCAM-1 induced by 10  $\mu$ M S1P in a concentration-dependent manner. After treatment with 300 ng/ml PTX, the values of relative fold of mRNA and protein expression in VCAM-1 were decreased to  $1.9 \pm 0.3$  ( $n = 3$ ,  $P < 0.05$ ) and  $1.2 \pm 0.3$  ( $n = 3$ ,  $P < 0.05$ ) fold, respectively (Fig. 6A, B).

#### EoL-1 CELLS ADHESION TO S1P-TREATED HPMVECs

RT-PCR analysis demonstrated that VLA-4 and LFA-1 are expressed in EoL-1 cells (Fig. 7A). After EoL-1 cells were treated with the medium in the absence and presence of S1P (1-10  $\mu$ M) for 8 h, EoL-1 cells were incubated with HPMVECs monolayers for 1 h. Adherence of EoL-1 cells to HPMVECs was increased by pre-exposure to S1P in a concentration-dependent manner (Fig. 7B). S1P (10  $\mu$ M) caused a marked increase in the number of adherent cells

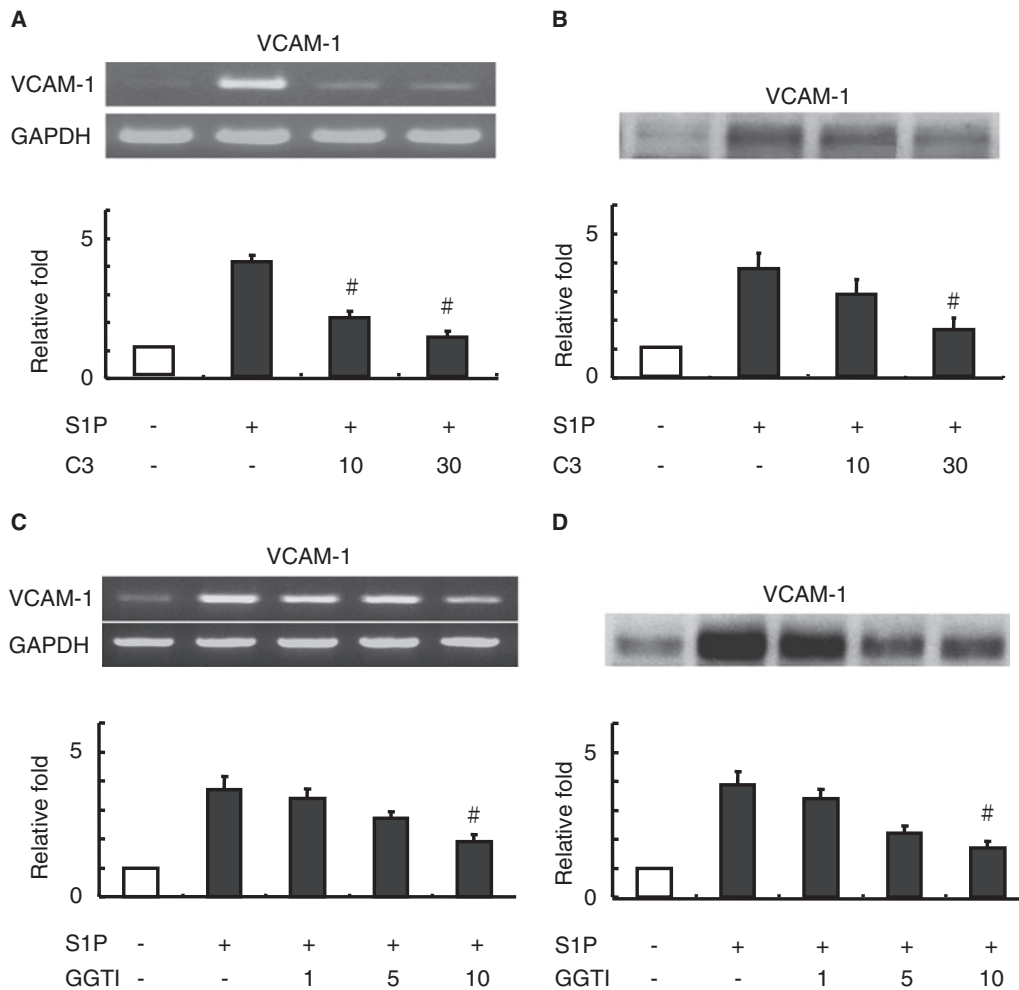


**Fig. 4** Involvement of RhoA in the expression of adhesion molecules by S1P in HPMVECs. mRNA (A) and protein (B) expression of VCAM-1 after exposure to 10  $\mu$ M S1P in the absence and presence of C3 exoenzyme (10-30 ng/ml). mRNA (C) and protein (D) expression of VCAM-1 after exposure to 10  $\mu$ M S1P in the absence and presence of GGTI-286 (1-10  $\mu$ M). The expression of GAPDH was used to normalize the expression of adhesion molecules in RT-PCR analysis. In histograms, data were expressed as shown in Figure 1. C3, C3 exoenzyme. <sup>#</sup> $P < 0.05$  vs 10  $\mu$ M S1P stimulation.

from  $0.6 (\pm 0.1) \times 10^5$  to  $1.4 (\pm 0.3) \times 10^5$  ( $n = 4$ ,  $P < 0.01$ ) (Fig. 7C). When EoL-1 cells were treated with 10  $\mu$ M S1P in the presence of 10  $\mu$ M Y-27632, augmented adherence of EoL-1 cells to HPMVECs by S1P was markedly suppressed by Y-27632 (Fig. 7B). Y-27632 decreased the number of adherent cells to  $0.7 (\pm 0.2) \times 10^5$  ( $n = 4$ ,  $P < 0.01$ ) (Fig. 7C). When EoL-1 cells were treated with 10  $\mu$ M S1P subsequent to incubation with 300 ng/ml PTX, adherence of EoL-1 cells to HPMVECs was also markedly suppressed (Fig. 7B). PTX decreased the number of adherent cells to  $0.8 (\pm 0.1) \times 10^5$  ( $n = 4$ ,  $P < 0.01$ ) (Fig. 7C).

## DISCUSSION

We have demonstrated for the first time that S1P induces the expression of VCAM-1 in the endothelium of human pulmonary vessels such as HPMVECs,

similar to human umbilical vein endothelial cells (HUVECs).<sup>24,25</sup> As shown in Figure 1, 2, after exposure to S1P ( $>1 \mu$ M) for 4 h, the expression of VCAM-1 was markedly enhanced, in contrast, an increase in the expression of ICAM-1 induced by S1P was significant, but not so drastic. Therefore, its response may not be meaningful for the physiological effect.

It is generally considered that S1P is related to recruitment of eosinophils to the airway wall mediated by migration of cultured human eosinophils and upregulation of CCR3, which is the fundamental chemokine receptor involved in the eosinophil function.<sup>3</sup> Since S1P augments expression of adhesion molecules (mainly VCAM-1) in HPMVECs (Fig. 1, 2), this present study demonstrate that S1P also acts on not only chemotaxis but also the first step (adhesion to endothelium) in the recruitment of eosinophils.

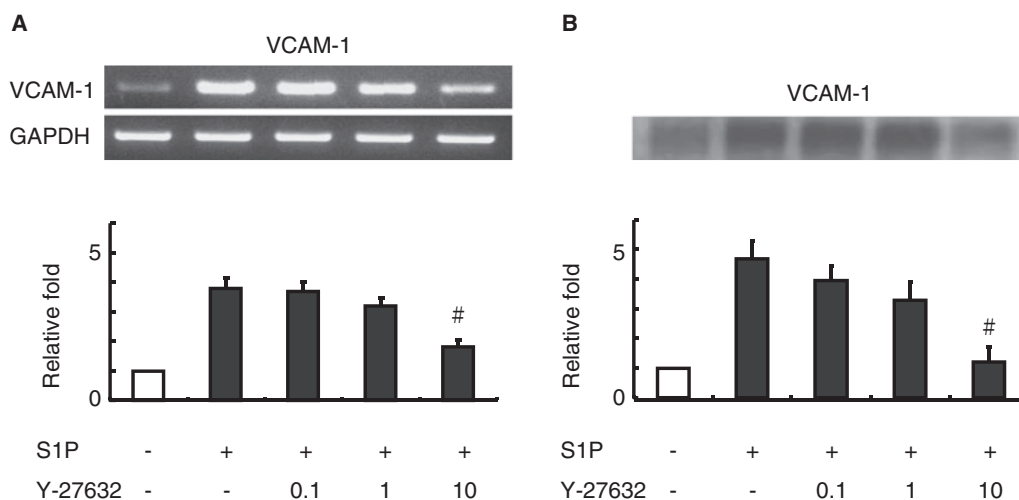


**Fig. 5** Involvement of Rho-kinase in the expression of adhesion molecules by S1P in HPMVECs. mRNA (A) and protein (B) expression of VCAM-1 after exposure to 10  $\mu$ M S1P in the absence and presence of Y-27632 (0.1-10  $\mu$ M). The expression of GAPDH was used to normalize the expression of adhesion molecules in RT-PCR analysis. In histograms, data were expressed as shown in Figure 1. <sup>#</sup> $P < 0.05$  vs 10  $\mu$ M S1P stimulation.

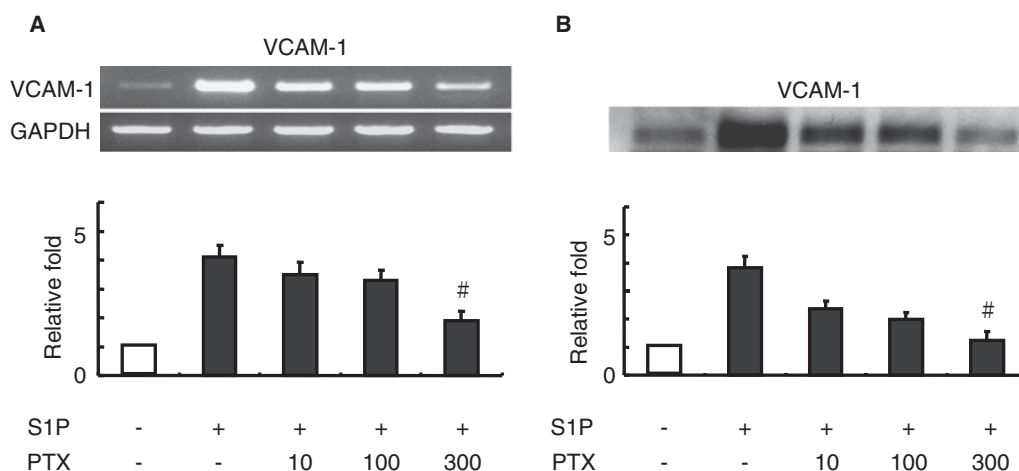


**Fig. 6** Involvement of  $G_i$  in the expression of adhesion molecules by S1P in HPMVECs. mRNA (A) and protein (B) expression of VCAM-1 after exposure to 10  $\mu$ M S1P subsequent to incubation with the serum-free medium and PTX (10-300 ng/ml). The expression of GAPDH was used to normalize the expression of adhesion molecules in RT-PCR analysis. In histograms, data were expressed as shown in Figure 1. <sup>#</sup> $P < 0.05$  vs 10  $\mu$ M S1P stimulation.

The maximal effect on expression of VCAM-1 was observed 4-8 h after exposure to S1P (Fig. 2). This time lag is roughly consistent with that lag which the late asthmatic response occurs mediated by eosinophilic inflammation after patients with asthma inhale allergens. The concentrations of S1P in BAL are increased from approximate 3 to 13 nM after allergen challenges in patients with asthma.<sup>1</sup> These observations indicate that S1P, which is released from mast cells via the IgE-mediated allergic reaction,<sup>1,26</sup> might serve as a lipid mediator for the processes of infiltra-

tion of eosinophils to the airways implicated in this disease.

To determine the mechanisms of pre-treatment with S1P in expression of VCAM-1, involvement of RhoA was examined in HPMVECs. As shown in Figure 3, S1P activates RhoA in HPMVECs, similar to human airway smooth muscle cells.<sup>6</sup> Furthermore, as shown in Figure 4, 5, the expression of VCAM-1 induced by S1P is attenuated by RhoA inhibitors such as exoenzyme C3 and GGTI-286. These results indicate that S1P upregulates VCAM-1 mediated by

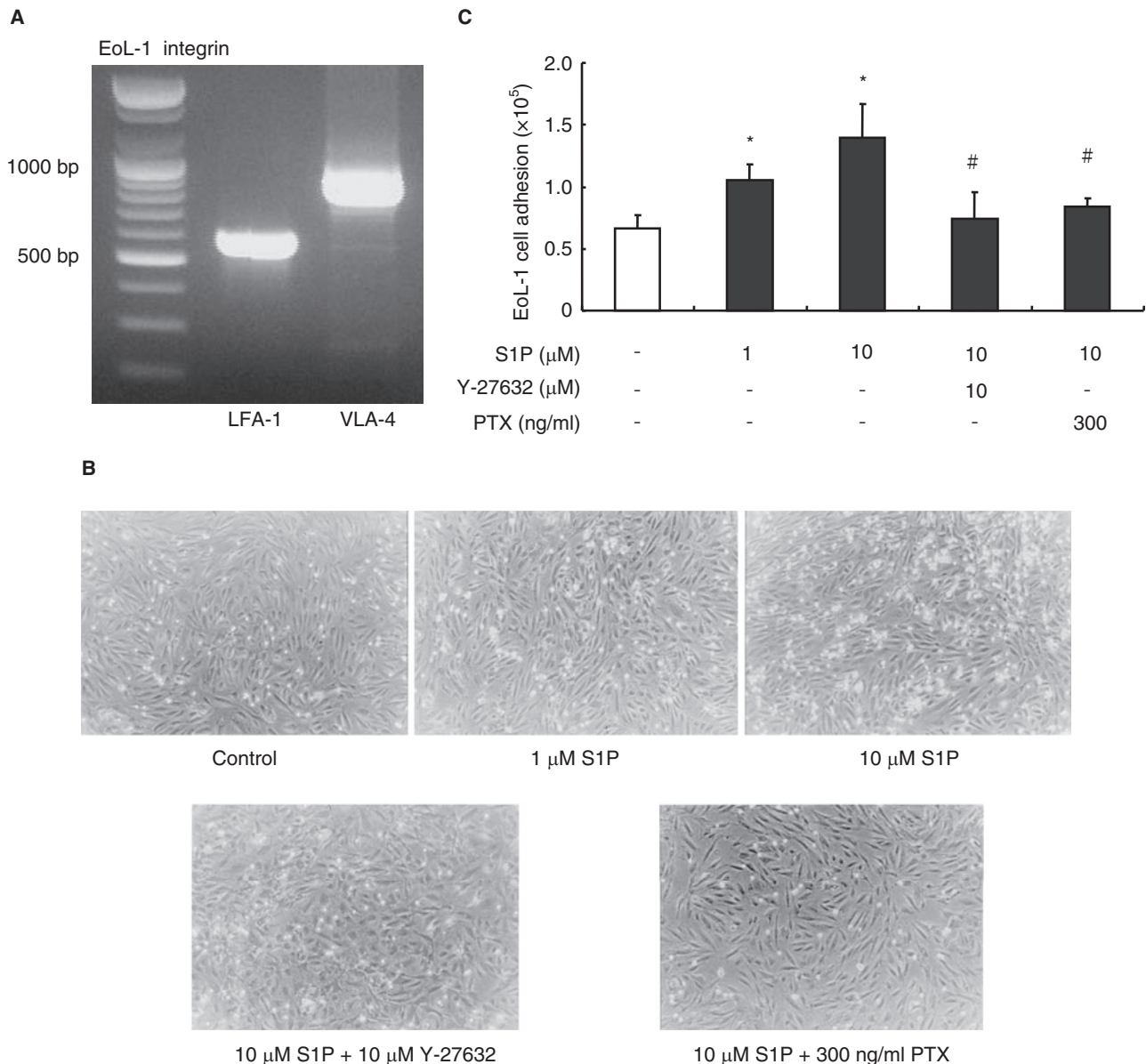


**Fig. 7** Involvement of S1P on EoL-1 cell adhesion to HPMVECs. **(A)** mRNA expression of LFA-1 and VLA-4 in EoL-1 cells. **(B)** Microphotograph of adherence of EoL-1 to HPMVECs treated with the serum-free medium (control), treated with S1P (1-10  $\mu$ M), treated with 10  $\mu$ M S1P in the presence of 10  $\mu$ M Y-27632, and treated with 10  $\mu$ M S1P subsequent to incubation with 300 ng/ml PTX. **(C)** The number of adhered EoL-1 cells to HPMVECs under each experimental condition shown in **(B)**. \* $P$  < 0.05 vs control. # $P$  < 0.05 vs 10  $\mu$ M S1P stimulation.

RhoA activation. Although exoenzyme C3 and GGTI-286 may inhibit RhoA, RhoB, and RhoC nonselectively, the effects of these agents are at least mediated by RhoA in this study (Fig. 3). The three Rho isoforms are structurally highly homologous, however have different effects on cell function.<sup>27</sup> The fundamental roles of RhoA, RhoB, and RhoC are actomyosin contractility, cytokine trafficking and cell survival, and cell locomotion, respectively.

It is well known that the effector protein of RhoA is Rho-kinase. Y-27632, a selective inhibitor of Rho-

kinase, inhibits an increase in expression of VCAM-1 induced by S1P in HPMVECs (Fig. 5). Therefore, RhoA/Rho-kinase processes contribute to this phenomenon. Previous reports have shown that RhoA/Rho-kinase processes are involved in the expression of VCAM-1 induced by UTP or ATP in coronary artery endothelial cells,<sup>28</sup> and that these processes are also involved in the expression of ICAM-1 induced by thrombin in HUVECs.<sup>29</sup> In HPMVECs, Y-27632 (<1  $\mu$ M) did not significantly reduce the expression of VCAM-1 induced by S1P (Fig. 5). The values of IC<sub>50</sub>

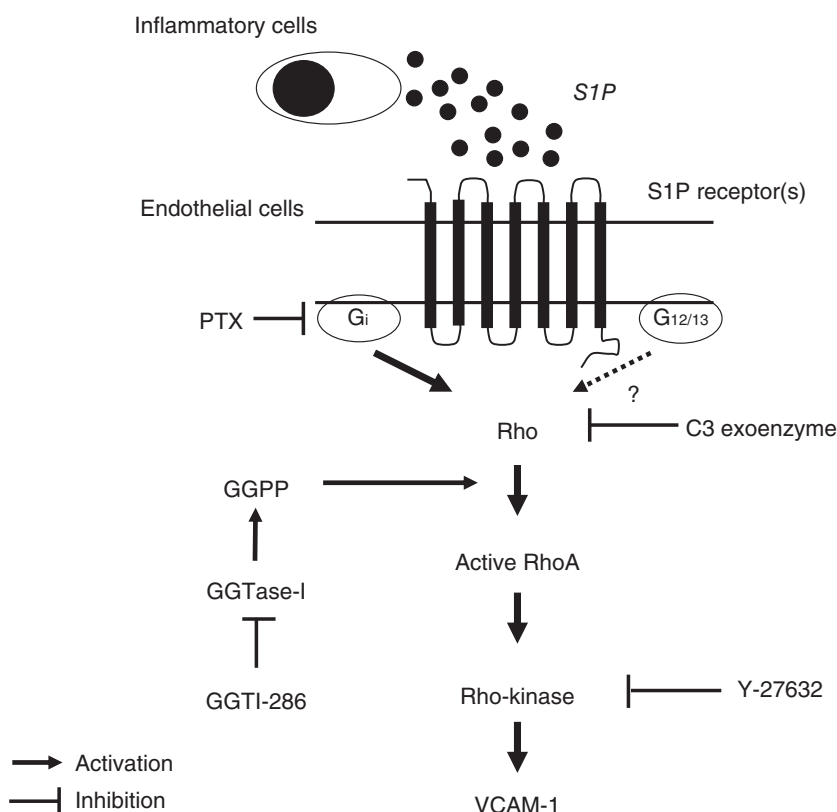


**Fig. 8** The intracellular mechanisms underlying the upregulation of adhesion molecules induced by S1P. S1P acts on pulmonary endothelial cells as a lipid mediator. In the endothelium, the expression of VCAM-1 is markedly augmented via activation of  $G_i$  and RhoA, leading to enhancement of adherence of eosinophils. GGPP, geranylgeranylpyrophosphate.

for the effects of Y-27632 on this adhesion molecule were approximately 3  $\mu\text{M}$  in HPMVECs (Data not shown). 10  $\mu\text{M}$  Y-27632 is also applied to suppress enhancement of VCAM-1 in coronary artery endothelial cells,<sup>28</sup> and HUVECs,<sup>29</sup> respectively. On the other hand, the  $K_i$  and  $\text{IC}_{50}$  values for Y-27632 for inhibiting Rho-kinase are 0.14<sup>30</sup> and 0.5 - 0.7  $\mu\text{M}$ <sup>30,31</sup> in smooth muscle. S1P-induced contraction is completely suppressed by 1  $\mu\text{M}$  Y-27632 in guinea pig tracheal smooth muscle.<sup>6</sup> However, approximately 10 fold higher concentrations of Y-27632 were needed to observe a marked inhibition in the expression of VCAM-1 in vascular endothelial cells. This discrepancy may be due to difference in species and tissues.

Next, we sought to determine signal transduction pathways between S1P and RhoA in the upregulation of VCAM-1. There are five specific receptors for S1P (S1P<sub>1-5</sub>), which belong to a family of specific GTP (G)-protein coupled receptors. These receptors are able to couple to different G-proteins to elicit various cellular responses.<sup>32</sup> S1P-induced airway hyperresponsiveness is attenuated by incubation of tracheal tissues with 1000 ng/ml PTX for 6 h.<sup>6</sup> A loss of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel activity by muscarinic stimula-

tion is reversed by incubation of tracheal smooth muscle cells with 100 ng/ml PTX for 4 h.<sup>33</sup> According to the results shown in these previous reports, HPMVECs were treated with 300 ng/ml PTX for 4 h (Fig. 6). Since pre-treatment with PTX results in a functional uncoupling of  $G_i$ , the inhibitory heterotrimeric G protein of adenylyl cyclase, from its associated receptor via the ADP ribosylation,  $G_i$  may be involved in the ability of S1P to elevate the levels of expression of VCAM-1. Our results suggest that relationship between  $G_i$  and RhoA plays an essential role for this S1P-induced action as the post-receptor signal transduction pathways (Fig. 8), similar to airway smooth muscle cells.<sup>6</sup> It is well known that effector proteins of  $G_i$  are Rac, a Rho family, and Ras, another monomeric G protein.<sup>34</sup> When HPMVECs were treated with S1P in the presence of NSC23766, an inhibitor of Rac, the ability of S1P to enhance the expression of VCAM-1 was not markedly reduced (Data not shown). Since Ras stimulates mitogen-activated protein kinase, HPMVECs were exposed to S1P in the presence of PD98059, an inhibitor of mitogen-activated protein kinase. The effects of S1P on adhesion molecules were also not significantly affected (Data not

shown). Moreover, when HPMVECs were treated with S1P in the presence of bisindolymaleimide, an inhibitor of protein kinase C, the ability of S1P to enhance the expression of VCAM-1 was not markedly reduced (Data not shown). Rac, Ras and protein kinase C may not contribute to S1P-induced enhancement of the expression of VCAM-1 in HPMVECs, different from results shown in previous reports using HUVECs.<sup>35</sup>

Finally, we examined whether S1P, which causes the expression of VCAM-1, leads to an increase in the adherence of eosinophils to endothelial cells in pulmonary vessels. Human blood eosinophils have a limited life-span, and purification of these cells from patients with asthma is not so easy because the percentages of eosinophils are not always high in circulating leucocytes. Hence, in this study, EoL-1, a human eosinophilic leukemic cell line, was used to investigate the adhesion of eosinophils in response to S1P in vitro. In EoL-1 cells, pre-treatment with S1P increased the number of adherent cells to the HPMVECs in a concentration-dependent manner (Fig. 7B, C). Since VLA-4 is expressed in the EoL-1 cells (Fig. 7A), these results prove that S1P enhances adhesion of eosinophils to endothelial cells in pulmonary vessels via upregulation of VCAM-1. Enhanced adherence of EoL-1 cells by S1P was attenuated by Y-27632 and PTX (Fig. 7B, C), supporting the idea that G<sub>i</sub>/RhoA/Rho-kinase processes play a key role for this S1P-induced adherence, although this signal transduction pathway has never demonstrated in molecular biological technique.<sup>36</sup>

In conclusions, S1P may be involved in the eosinophil recruitment via enhancing adhesion to pulmonary endothelial cells. This adhesive action is regulated by the G<sub>i</sub>/RhoA/Rho-kinase processes in the respiratory systems. Although clinical relevance of these results may remain unknown, our observations may provide that the Rho-kinase may be a therapeutic target in the eosinophilic inflammation of asthma.<sup>37,38</sup>

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