



Downregulation of sphingosine-1-phosphate receptors in bronchial smooth muscle of mouse experimental asthma

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ABSTRACT

To determine whether or not sphingosine-1-phosphate (S1P) is involved in the augmented bronchial smooth muscle (BSM) contractility, one of the causes of airway hyperresponsiveness in asthmatics, the effects of S1P on BSM tone were investigated in control and repeatedly antigen-challenged mice. Both in the control and antigen-challenged animals, S1P had no effect on basal tone of the isolated BSM tissues. However, in the BSMs pre-depolarized by 60 mM K⁺, S1P caused a significant increase in tension in the control mice. The S1P-mediated contraction was abolished by JTE-013, a selective S1P receptor 2 (S1PR2) antagonist, but not by W123, a selective S1PR1 antagonist, and BML-241, a selective S1PR3 antagonist. The S1P-mediated contraction observed in BSMs of the control mice was also inhibited by Y-27632, a Rho-kinase inhibitor, suggesting that the contraction is mediated via activations of S1PR2 and probably its downstream Rho-kinase. On the other hand, interestingly, the S1P-mediated contraction was not observed at all in BSMs of the repeatedly antigen-challenged mice. A marked and significant downregulation of mRNA for S1PR2 was also observed in BSM tissues of the diseased animals. In conclusion, S1P could augment the BSM contraction via activations of its JTE-013-sensitive receptor, probably S1PR2, and the RhoA/Rho-kinase signaling in normal mice. In BSMs of the repeatedly antigen-challenged mice, the expression level of S1PR2 was much decreased, resulting in a loss of the S1P-mediated contraction.

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1. Introduction

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

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite that mediates diverse biological responses, including

smooth muscle contraction [4–8]. S1P is known to act as an extracellular ligand, activating specific G protein-coupled receptors (GPCRs) in the plasma membrane. To date, five members of the S1P receptor family, S1PR1–S1PR5, have been cloned [9,10]. The S1PR1, S1PR4 and S1PR5 are mainly coupled to G_i, while S1PR2 and S1PR3 are coupled to G_i, G_q and G_{12/13} [11]. Depending on the expression of S1P receptor subtypes and the involvement of different G proteins, S1P is able to influence a wide variety of signal transduction pathways in diverse cell types (see reviews for detail [9–11]). Among these receptor subtypes, S1PR2 is thought to contribute to contraction in various types of smooth muscles [4,5,7,8].

Recently, an involvement of S1P in allergic bronchial asthma has been suggested [12–18]. Ammit and colleagues [12] firstly demonstrated that S1P levels are elevated in the airways of individuals with asthma after segmental allergen challenge. The finding that S1P can act as a chemotactic agent for eosinophils further suggests an involvement of S1P in pathophysiology of asthma [13]. Indeed, inhalation of inhibitors for sphingosine kinase, which produces S1P directly from sphingosine, attenuated antigen-induced airway inflammation in mice [14]. In addition, S1P might have an ability to cause airway hyperresponsiveness [15–18]. Contrary to these observations, inhalation of S1P itself or FTY720, a S1P receptor agonist, prevented antigen-induced airway inflammation and hyperresponsiveness in mice [19]. Thus, the role of S1P in the

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development of asthma and/or airway hyperresponsiveness is still controversial.

The present study was designed to determine whether or not S1P is involved in the augmented bronchial smooth muscle (BSM) contractility, one of the causes of airway hyperresponsiveness in asthmatics. To identify the S1P receptor subtype(s) that contributes to changes in BSM tone, the effects of several S1P receptor antagonists on S1P-mediated contraction were also determined.

2. Materials and methods

2.1. 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 airway inflammation, which has an *in vivo* AHR [20], was performed as described previously [21–23]. In brief, BALB/c mice (8 weeks of age) were actively sensitized by intraperitoneal injections of 8 µg ovalbumin (OA; Seikagaku Co., Tokyo, Japan) with 2 mg Imject Alum (Pierce Biotechnology, Inc., Rockford, IL, USA) on Day 0 and Day 5. The sensitized mice were challenged with aerosolized OA-saline solution (5 mg/mL) for 30 min on Days 12, 16 and 20. The OA aerosol was generated with an ultrasonic nebulizer (Nihon Kohden, Tokyo, Japan) and introduced to a Plexiglas chamber box (130 mm × 200 mm, 100 mm height) in which the mice were placed.

2.2. Functional studies

Twenty-four hours after the last OA challenge, mice were sacrificed by exsanguination from abdominal aorta under urethane (1.6 g/kg, i.p.; Sigma–Aldrich, St. Louis, MO) 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 [24]. 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 (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 acetylcholine (ACh; 10^{−7} to 10^{−3} M in final concentration) was constructed cumulatively. After the measurement of bronchial smooth muscle (BSM) responsiveness to ACh, the tissues were frequently washed out and re-equilibrated. Then the tissues were depolarized by isotonic high K⁺ (60 mM) solution in the presence of atropine and indomethacin (both 10^{−6} M). When the plateau response to 60 mM K⁺ was observed, sphingosine-1-phosphate (S1P; Cayman Chemical, Co., Ann Arbor, MI, USA; 10^{−6} and 10^{−5} M in final concentration) or its vehicle (methanol; 1% or less in final concentration) was applied. In some experiments, W123 (a selective S1P1 receptor antagonist [25,26]; Cayman Chemical, Co.), JTE-013 (a selective S1P2 receptor antagonist [25,27]; Cayman Chemical, Co.), BML-241 (a selective S1P3 receptors antagonist [15,25,27]; Enzo Biochem, New York, NY, USA), Y-27632 (a selective Rho-kinase inhibitor [28,29] or their vehicle DMSO; 0.2% or less in final concentration) was also applied when the plateau response to S1P was obtained.

2.3. Measurements of mRNA levels

After the exsanguinations, the left main bronchus of each animal was isolated, and the epithelium was removed as much as possible by the method previously described [24]. Total RNA of the mouse bronchial smooth muscle tissue was isolated with a one-step guanidium–phenol–chloroform extraction procedure using 1 mL of TRI Reagent (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The cDNAs were prepared from the total RNA (10 ng) using QuantiTect™ reverse transcription kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions.

An aliquot of resultant cDNA solution (1 µL) was subjected to PCR (0.1 µM forward and reverse primers, 0.025 U/L Taq DNA polymerase, 2 mM MgCl₂, 0.2 mM dNTPs) in a final volume of 11 µL. The PCR primer sets used are shown in Table 1, which were designed from published sequences. The thermal cycle profile used was (1) denaturing for 15 s at 95 °C, (2) annealing primers for 15 s at 55 °C, and (3) extending the primers for 60 s at 72 °C, and the reaction was run for 30 cycles. The PCR products were subjected to electrophoresis on 1.2% agarose gel and visualized by ethidium bromide staining. The intensity of bands was quantified by a densitometer (Atto Densitograph; Atto Co., Tokyo, Japan). The ratios of intensities of respective S1PRs to the corresponding GAPDH bands were calculated. In each target gene, data were expressed as % change from control animals.

The mRNA levels were also quantified by real-time RT-PCR. An aliquot of cDNA solution (1 µL) was subjected to real-time

Table 1
Primer sequences for RT-PCR used in the present study.

Gene name	Accession no.		Sequence	Amplicon size
Mouse S1PR1	NM.007901	Sense	5'-GATATCATCGTCCGGCAITAC-3'	1286 bp
		Antisense	5'-ACCCTTCCAGTGCAITGTTC-3'	
Mouse S1PR2	NM.010333	Sense	5'-TATCGTGGCTCTGTACGTCC-3'	261 bp
		Antisense	5'-CGCCACGTATAGATGACAGG-3'	
Mouse S1PR3	NM.010101	Sense	5'-GACTGCTCTACCATCTGCCCC-3'	342 bp
		Antisense	5'-GTAGATGACAGGGTTCATGGC-3'	
Mouse S1PR4	NM.010102	Sense	5'-AAGACCAGCCGTGTGTATGG-3'	292 bp
		Antisense	5'-TCAGCACGGTGTGAGTAGC-3'	
Mouse S1PR5	NM.053190	Sense	5'-ACTCACTTCTGAACCCCATCATCTAC-3'	252 bp
		Antisense	5'-CTGTGGAGCCGCTGGTGTTC-3'	
Mouse GAPDH	NM.008084	Sense	5'-ACCACAGTCCATGCCATCAC-3'	452 bp
		Antisense	5'-TCCACCACCTGTGCTGT-3'	

PCR analyses using iQTM SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The thermal cycle profile used was (1) denaturing for 15 s at 95 °C, (2) annealing primers for 30 s at 55 °C, and (3) extending the primers for 30 s at 72 °C. The PCR amplification was performed at 40 cycles with monitoring fluorescence. The primer sets used were: QuantiTect Primer Assay, QT0019388 for mouse GAPDH (Qiagen Inc.), 5'-GCCATCGCCATCGAGAGA-3' and 5'-TGTCAGTCCGTAGAGCTTGA-3' for mouse S1P receptor 2 (S1PR2), and 5'-TGGCTGTGTGTGCCTTCATT-3' and 5'-GCGGACCA-GACCAAGAG-3' for mouse S1PR5. The comparative threshold cycle (C_T) method was used for relative quantification of the target genes. Differences in the C_T values (ΔC_T) between the tested gene and the GAPDH were calculated to determine the relative expression levels, using the following formula: $\Delta \Delta C_T = (\Delta C_T \text{ of the treated sample}) - (\Delta C_T \text{ of the control sample})$. The relative expression level between the samples was calculated according to the equation $2^{-\Delta \Delta C_T}$.

2.4. Statistical analyses

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

3. Results

3.1. Effects of repeated antigen exposure on BSM responsiveness

Fig. 1A shows the acetylcholine (ACh) responsiveness of bronchial smooth muscles (BSMs) isolated from nonsensitized normal control and repeatedly antigen-challenged mice. The nonsensitized normal control animals were used as controls

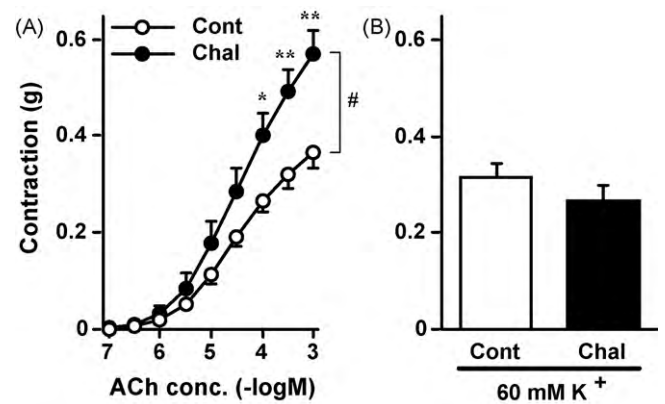


Fig. 1. Effects of repeated antigen exposure on bronchial smooth muscle responsiveness to acetylcholine (ACh; A) and isotonic 60 mM K^+ depolarization (in the presence of atropine and indomethacin, both 10^{-6} M; B) in mice. Smooth muscle preparations were isolated from the left main bronchi of the repeatedly antigen-challenged (Chal) and control animals (Cont). Each value represents the mean with SEM from 6 (Cont) and 9 (Chal) animals. * $P < 0.05$ and ** $P < 0.001$ vs. Cont by Bonferroni/Dunn's test. # $P < 0.05$ by two-way ANOVA.

because our previous studies demonstrated that the sensitization procedure alone has no effect on the BSM contractility [24,30]. Application of ACh (10^{-7} to 10^{-3} M) to isolated BSMs elicited a concentration-dependent contraction in all animals used. The contractile response to ACh of BSMs from the repeatedly antigen-challenged mice was markedly augmented, i.e., BSM hyperresponsiveness, compared with those from the control animals: the ACh concentration–response curve was significantly shifted upward by the antigen exposure ($P < 0.05$ by two-way ANOVA). On the other hand, no significant change in the contractile response induced by 60 mM K^+ -depolarization was observed between the groups (Fig. 1B). These findings are consistent with our previous report [24] that demonstrates an augmented

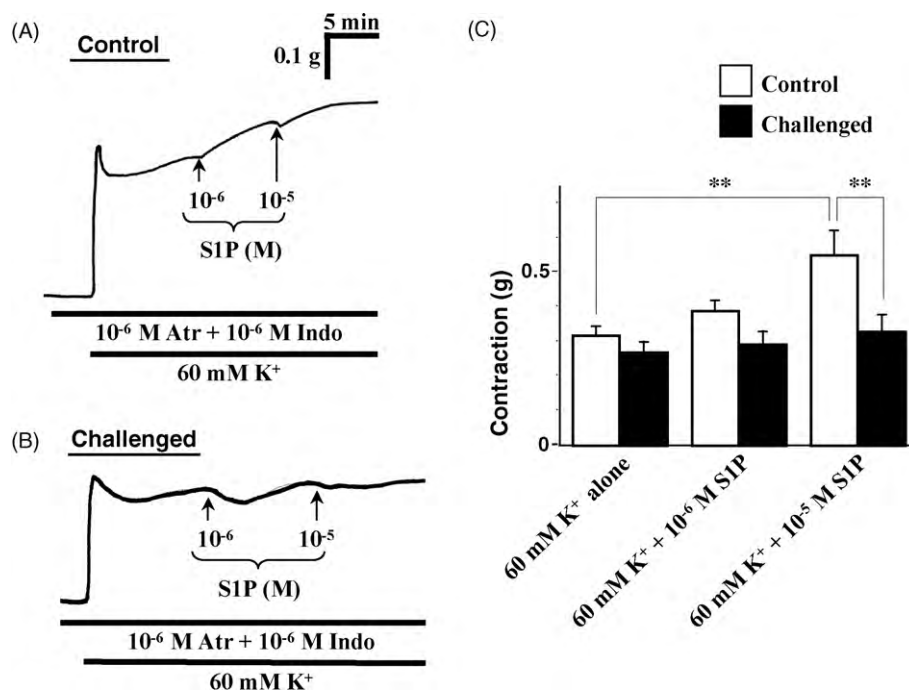


Fig. 2. Effects of sphingosine-1-phosphate (S1P) on bronchial smooth muscle responsiveness to high K^+ depolarization in control and repeatedly antigen-challenged mice. Smooth muscle preparations were isolated from the left main bronchi of the repeatedly antigen-challenged (Challenged) and control animals (Control). After the contraction induced by isotonic 60 mM K^+ depolarization (in the presence of atropine and indomethacin, both 10^{-6} M) reached to a plateau, S1P (10^{-6} and 10^{-5} M) was applied cumulatively. Typical recordings are shown in A (Control) and B (Challenged), and data are summarized in C. Each value represents the mean with SEM from 6 (Control) and 9 (Challenged) animals. ** $P < 0.01$ vs. Control by Bonferroni/Dunn's test.

agonist-induced Ca^{2+} sensitization of contraction in the repeatedly antigen-challenged mice.

3.2. Effects of S1P on BSM tone

Both in the control and repeatedly antigen-challenged groups, S1P ($\sim 10^{-5}$ M) had no effect on basal BSM tone under the experimental condition currently used (data not shown). In the control mice, however, application of S1P (10^{-6} and 10^{-5} M) caused a significant increase in tension in an S1P concentration-dependent manner when the BSM tissue was pre-depolarized by 60 mM K^+ (Fig. 2, $P < 0.05$), whereas its vehicle had no effect on the 60 mM K^+ -induced contraction (data not shown). On the other hand, interestingly, S1P did not cause any contractile response in the pre-depolarized BSM tissues of the repeatedly antigen-challenged mice (Fig. 2).

3.3. Effects of S1P receptor antagonists and a Rho-kinase inhibitor on S1P-induced BSM contraction

To identify the S1P receptor subtype(s) that contributes to the S1P-mediated contraction observed in the BSMs of control mice,

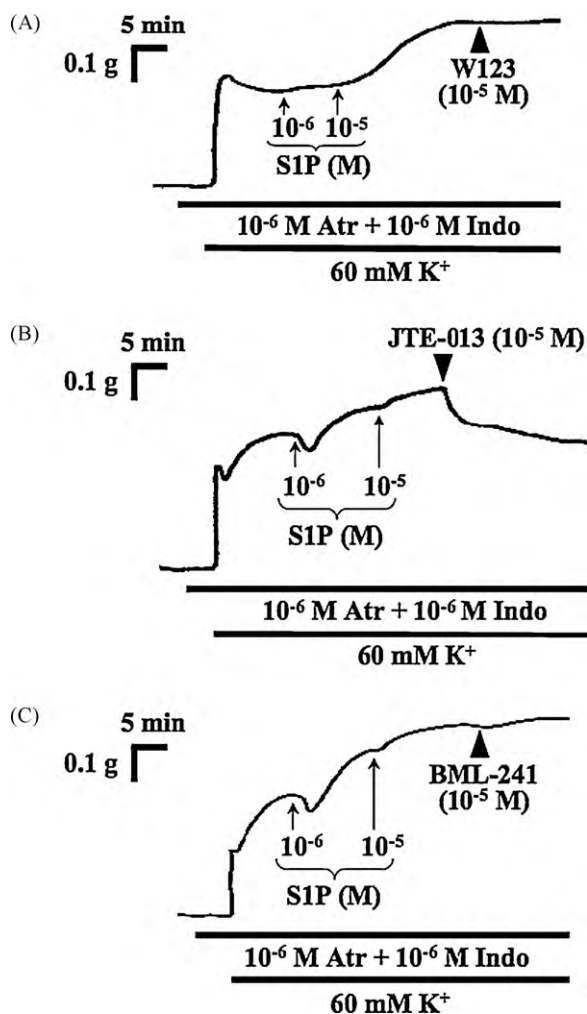


Fig. 3. Effects of sphingosine-1-phosphate receptor (S1PR) antagonists on the S1P-mediated contraction of bronchial smooth muscle in control mice. After the contraction induced by 10^{-5} M S1P (pre-depolarized with isotonic 60 mM K^+ in the presence of atropine and indomethacin, both 10^{-6} M) reached to a plateau, relatively high concentration (10^{-5} M) of respective S1P receptor antagonist was applied. Typical recordings of effects of a selective S1PR1 antagonist W123 (A), a selective S1PR2 antagonist JTE-013 (B), and a selective S1PR3 antagonist BML-241 (C) are shown.

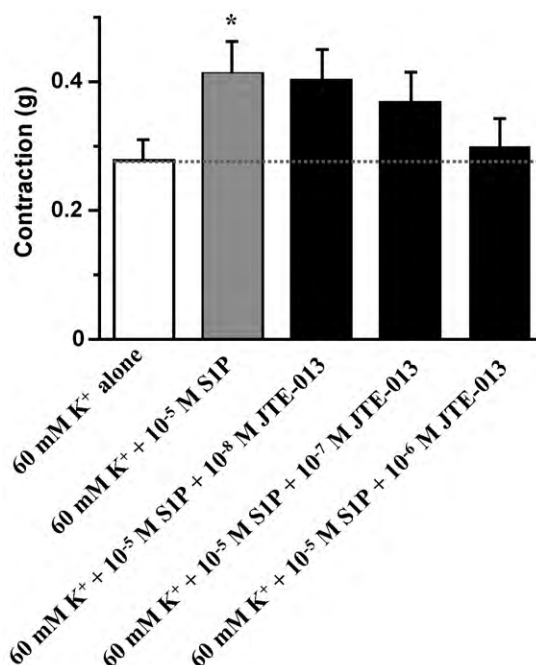


Fig. 4. Effects of a sphingosine-1-phosphate receptor 2 (S1PR2) antagonist JTE-013 on the S1P-mediated contraction of bronchial smooth muscle in control mice. After the contraction induced by 10^{-5} M S1P (pre-depolarized with isotonic 60 mM K^+ in the presence of atropine and indomethacin, both 10^{-6} M) reached to a plateau, JTE-013 (10^{-8} to 10^{-6} M) was applied cumulatively. Each value represents the mean with SEM from 5 different animals. * $P < 0.05$ vs. just before the application of S1P (60 mM K^+ alone) by Bonferroni/Dunn's test.

the effects of various S1P receptor antagonists were examined. As shown in Fig. 3, JTE-013 (10^{-5} M; a selective S1PR2 antagonist [25,27]) inhibited the S1P-mediated contraction almost completely (Fig. 3B), whereas W123 (10^{-5} M; a selective S1PR1 antagonist [25,26]) and BML-241 (a selective S1PR3 antagonist [15,25,27]) had no effect (Fig. 3A and C). The S1P-mediated contraction was inhibited by JTE-013, concentration-dependently: no significant difference was observed between the remaining contraction in the presence of 10^{-6} M JTE-013 and the contraction induced by 60 mM K^+ itself (Fig. 4). In addition, the S1P-mediated contraction of the pre-depolarized BSMs of control mice was also inhibited by Y-27632, a Rho-kinase inhibitor [28,29]: almost complete inhibition was observed when 10^{-6} M of Y-27632 was applied; no significant difference was observed between the remaining contraction in the presence of 10^{-6} M Y-27632 and the contraction induced by 60 mM K^+ itself (Fig. 5). Both JTE-013 and Y-27632 had no effect on the 60 mM K^+ -induced contraction itself at the concentrations used (data not shown).

3.4. Effects of repeated antigen exposure on mRNA expression of S1P receptors

The results of functional studies reminded us of the decreases in S1P receptor(s) in BSMs of the repeatedly antigen-challenged mice. So in the present study, the steady-state mRNA levels of S1P receptor subtypes in BSMs were measured and compared between the control and repeatedly antigen-challenged mice. As shown in Fig. 6A, all the S1P receptor subtypes were expressed in mouse BSM. Semi-quantitative analyses using the band densities of amplicons revealed that the mRNA levels of S1PR2 and S1PR5 were significantly decreased in BSMs of the repeatedly antigen-challenged mice as compared to those of control animals (Fig. 6B). Quantitative real-time RT-PCR analyses also revealed the downregulation of these two receptor subtypes (Fig. 7). In contrast, no significant

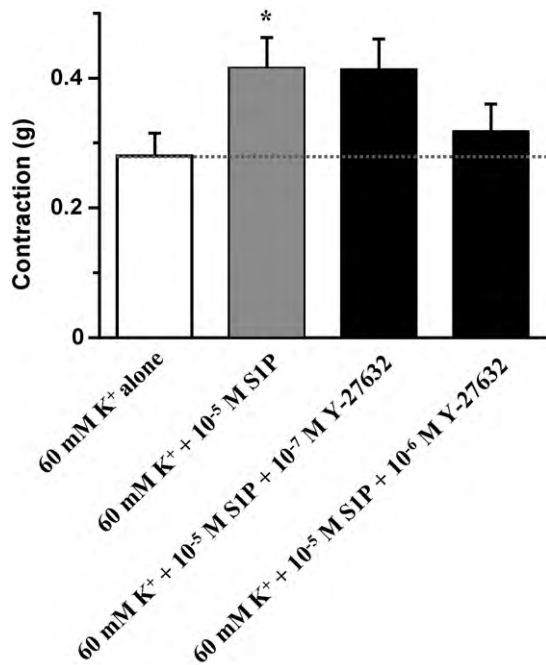


Fig. 5. Effects of a Rho-kinase inhibitor Y-27632 on the S1P-mediated contraction of bronchial smooth muscle in control mice. After the contraction induced by 10^{-5} M S1P (pre-depolarized with isotonic 60 mM K^+ in the presence of atropine and indomethacin, both 10^{-6} M) reached to a plateau, Y-27632 (10^{-7} and 10^{-6} M) was applied cumulatively. Each value represents the mean with SEM from 5 different animals. * $P < 0.05$ vs. just before the application of S1P (Pre) by Bonferroni/Dunn's test.

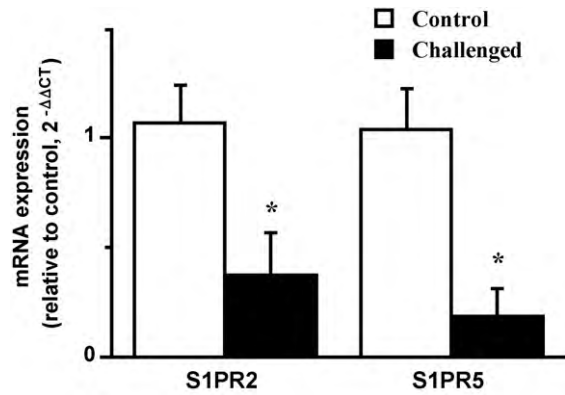


Fig. 7. Effects of repeated antigen exposure on mRNA expression of sphingosine-1-phosphate receptors 2 (S1PR2) and S1PR5 in bronchial smooth muscle tissues of mice. Expression levels of mRNAs for S1PR2 and S1PR5 were determined by quantitative real-time RT-PCR. The relative gene expressions of S1PR2 and S1PR5 to GAPDH mRNAs were calculated by the $2^{-\Delta\Delta CT}$ methods as described in METHODS section. Each column represents the mean with SEM from 5 different animals. * $P < 0.05$ vs. respective control by Bonferroni/Dunn's test.

change in the mRNA expression of S1PR1, S1PR3, or S1PR4 was observed between the groups (Fig. 6B).

4. Discussion

The current study clearly showed that S1P caused an increase in tension in 60 mM K^+ -depolarized BSMs of control mice, whereas it did not affect basal tone. The S1P-mediated contraction was abolished by JTE-013 (Figs. 3 and 4) or Y-27632 (Fig. 5), suggesting that the contraction is mediated via activations of S1PR2 and

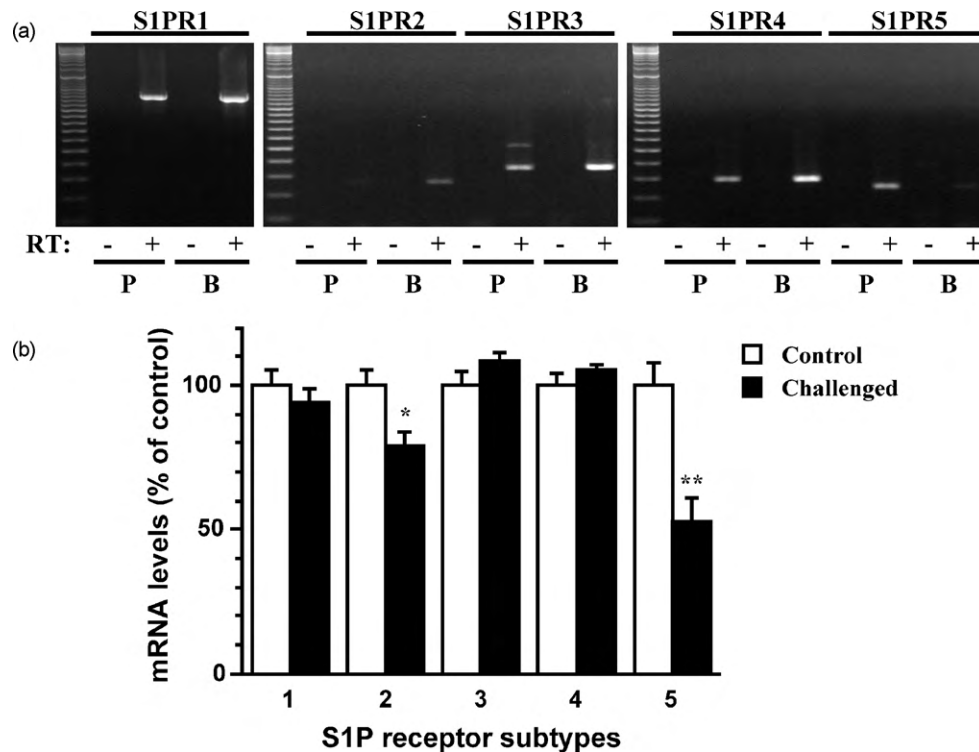


Fig. 6. (A) Expression of mRNAs for sphingosine-1-phosphate receptors (S1PRs) in bronchial smooth muscle (BSM) tissues of mice. Total RNAs extracted from whole brains (positive control; P) and bronchi (B) of the nonsensitized normal control mice were subjected to the RT-PCR analyses. RT–: without RT reaction. The bands shown are representative of 5 independent experiments, respectively. (B) Effects of repeated antigen exposure on mRNA expression of S1PRs in BSM tissues of mice. Total RNAs extracted from BSMs of nonsensitized normal control (Control) and repeatedly antigen-challenged mice (Challenged) were subjected to the RT-PCR analyses. The band densities of the target genes were normalized by corresponding GAPDH bands, and the data were expressed as % change from control animals. Each column represents the mean with SEM from 5 different animals. * $P < 0.05$ and ** $P < 0.01$ vs. respective control by Bonferroni/Dunn's test.

probably its downstream Rho-kinase. Interestingly, however, the S1P-mediated contraction was not observed at all in BSMs of the repeatedly antigen-challenged mice (Fig. 2). This is probably due to the downregulation of S1PR2 in BSMs (Figs. 6 and 7). These findings suggest that S1P itself does not contribute to bronchoconstriction at least at the stage where the antigen-induced BSM hyperresponsiveness has been developed (Fig. 1).

Recent evidence has indicated that S1P acts as an intercellular mediator, interacting with the S1P receptor family of GPCRs, S1PR1–S1PR5 [9–11]. Among these receptor subtypes, S1PR2 is ubiquitously expressed and might be the predominant receptor in smooth muscle cells. In human coronary artery smooth muscle cells, S1P induced a contraction, which was inhibited by JTE-013 or Y-27632 [4]. In permeabilized cat esophagus smooth muscle cells, the S1P-induced contraction was inhibited by treatment with antibody against S1PR2 [7]. In rabbit gastric smooth muscle cells, S1P induced a sustained Ca^{2+} -independent, RhoA/Rho-kinase-mediated contraction, and the S1P-induced activation of Rho-kinase was abolished by S1PR2 siRNA [8]. Thus, S1PR2 is the main receptor subtype that is involved in the contraction of various types of smooth muscles, and Rho-kinase is a key intermediate in the S1PR2-mediated contraction. However, the effect of S1P on airway smooth muscle is still controversial. Consistent with the current results, Roviezzo and colleagues [15] also revealed that, in the BSMs isolated from normal control BALB/c mice, S1P itself had no significant effect on basal tone. On the other hand, Kume and colleagues [16] reported a significant contraction from basal tone induced by S1P itself in tracheal smooth muscles isolated from guinea pigs. Some species and/or regional difference may be involved in the difference in S1P response in airway smooth muscles.

In the present study, although S1P itself did not develop any tension, it caused a significant increase in tension in BSMs predepolarized with 60 mM K^+ in control mice (Fig. 2). The results might also be consistent with the report of Roviezzo and colleagues [15], where S1P augmented the contraction induced by ACh in mouse BSMs. To identify the S1P receptor subtype(s) that contributes to the S1P-mediated contraction observed, W123 [25,26], JTE-013 [25,27] and BML-241 [15,25,27] were used as selective antagonists for S1PR1, S1PR2 and S1PR3, respectively. Unfortunately, specific antagonists for S1PR4 and S1PR5 are not commercially available to date. The S1P-mediated contractile component was abolished by JTE-013, but not by W123 or BML-241 (Figs. 3 and 4), indicating that S1PR2 is responsible for inducing the contraction. In addition, the S1P-mediated contraction observed in the pre-depolarized BSMs was also inhibited by a Rho-kinase inhibitor Y-27632 [28,29] almost completely (Fig. 5). Our previous study demonstrated that the high K^+ stimulation did not activate RhoA, an upstream of Rho-kinase, in the BSMs [31]. Y-27632 was insensitive to the contraction induced by the high K^+ depolarization itself (see Section 3). Taken together, these findings suggest that S1P activates the RhoA/Rho-kinase signaling via an activation of S1PR2, resulting in an augmentation of the contraction induced by high K^+ depolarization in the BSMs of the control mice.

It was postulated that the S1P-mediated contraction would be augmented in BSMs of the repeatedly antigen-challenged mice, because an augmentation of RhoA/Rho-kinase signaling has been suggested in animal models of allergic airway inflammation [24,32]. Contrary to our expectation, the S1P-mediated contractile response was not observed far from augmenting at all (Fig. 2). An upregulation of S1P level has been demonstrated in the airways of asthmatics after allergen challenge [12]. Similarly, the level of mRNA for sphingosine kinase 2 (SphK2), one of the main enzymes that convert sphingosine to S1P [33], was upregulated in the airways of the repeatedly antigen-challenged mice (our personal observations), also indicating an increased biosynthesis of

S1P in the airways of these animals. It is thus possible that the loss of the S1P-mediated BSM contraction in the diseased mice might be explained by a downregulation of S1P receptors responsible for the contraction, probably S1PR2 as described above. To test the hypothesis, the levels of mRNAs for S1P receptor subtypes in BSMs were measured. As expected, the current study revealed a significant decrease in the mRNA level of S1PR2 in BSMs of the repeatedly antigen-challenged mice (Figs. 6 and 7). Interestingly, a marked and significant decrease in the level of S1PR5 was also observed in BSMs of the diseased animals (Figs. 6 and 7). Although the physiological role(s) of S1PR5 in the airways is unclear now, it has been suggested that the S1PR5 is also coupled to $\text{G}_{12/13}$ [34], a heterotrimeric G protein that has an ability to activate RhoA/Rho-kinase signaling [35]. Further studies are needed to make clear the role of S1PR5 in smooth muscle contraction.

In conclusion, S1P could augment the BSM contraction via activations of its JTE-013-sensitive receptor, probably S1PR2, and the RhoA/Rho-kinase signaling in normal mice. In BSMs of the repeatedly antigen-challenged mice, the expression level of S1PR2 was much decreased, resulting in a loss of the S1P-mediated contraction. Thus, S1P itself does not contribute to bronchoconstriction in an asthmatic attack and the airway hyperresponsiveness.

Conflicts of interest

None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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