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Effects of Repeated Antigen Exposure on the Expression of Enzymes Associated with Sphingosine-1-Phosphate Turnover

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Abstract: Increasing evidence suggests that sphingosine-1-phosphate (S1P) is involved in the pathogenesis of allergic bronchial asthma. In the present study, the changes in the expression levels of enzymes associated with S1P turnover were determined in a mouse model of allergic bronchial asthma. Male BALB/c mice were actively sensitized with ovalbumin antigen, and were repeatedly challenged with aerosolized antigen. Twenty-four hours after the last antigen, total RNAs were extracted from lungs. The RT-PCR analyses revealed that mRNAs for the S1P-producing enzymes, sphingosine kinase 1 (SphK1) and SphK2, and for the S1P-breakdown enzymes, S1P phosphatase 1 (S1PP1), S1PP2, S1P lyase, and lipid phosphate phosphatase 1a, were expressed in the lungs of mice. Among them, the mRNA levels of SphK2 and S1PP1 were significantly increased in the lungs of the repeatedly antigen-challenged mice. It is thus possible that the S1P turnover is increased in the airways of allergic bronchial asthma.

Keywords: Sphingosine-1-phosphate (S1P), sphingosine kinase (SphK), S1P phosphatase (S1PP), S1P lyase, allergic bronchial asthma.

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]. Allergic bronchial asthma is characterized by structural and functional abnormalities of the bronchial epithelium, accumulation of inflammatory cells in the bronchial mucosa, remodeling of the airway tissue structure, and airway hyperresponsiveness. Many mediators have been identified that play significant roles in the initiation and progression of the disease [*e.g.*, 2, 3], but the exact mechanism of the pathogenesis of asthma is still unclear.

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite that mediates diverse biological responses [4-8]. Recently, an involvement of S1P in allergic bronchial asthma has been suggested [9-15]. Ammit and colleagues [9] first 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 [10] further suggests an involvement of S1P in pathophysiology of asthma. Indeed, inhalation of inhibitors for sphingosine kinase (SphK), which produces S1P directly from sphingosine, attenuated antigen-induced airway inflammation in mice [11]. In addition, S1P might have an ability to cause airway hyperresponsiveness [12-15], one of the characteristic features of allergic bronchial asthma. Contrary to these observations, inhalation of S1P itself or FTY720, an S1P receptor agonist, prevented antigen-induced airway inflammation and hyperresponsive-ness in mice [16]. Thus, the role of S1P in the development of asthma and/or airway hyperresponsi-veness is still controversial.

S1P is synthesized by SphK isoenzymes, SphK1 and SphK2, both of which phosphorylate sphingosine to generate S1P, and is degraded by S1P phosphatase 1 (S1PP1), S1PP2, lipid phosphate phosphatases (LPPs), or S1P lyase [17, 18]. Various types of cells including airway structural cells [19] are known to have an ability to produce S1P. However, there is little information about the expression profile of the enzymes associated with S1P turnover in the airways. In the present study, the mRNA expressions of these enzymes were evaluated in the lungs of mice using RT-PCR. In addition, the changes in the expression levels of these enzymes were also determined in a mouse model of allergic bronchial asthma [20-23].

MATERIALS AND METHODS

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 Hoshi University (Tokyo, Japan).

Preparation of a murine model of allergic bronchial asthma, 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 two intraperitoneal injections of each 8 μg ovalbumin (OA; Seikagaku Co., Tokyo, Japan) with 2 mg Imject Alum (Pierce Biotechnology, Inc., Rockfold, 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

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chamber box (130 x 200 mm, 100 mm height) in which the mice were placed.

Measurements of mRNA Levels

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 and Aldrich, St. Louis, MO) anesthesia, and the lungs were immediately removed. Total RNA of the mouse lung 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 QuantiTectTM 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 $MgCl_2$, 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 95ºC, 2) annealing primers for 15 s at 55ºC, 3) extending the primers for 60 s at 72ºC, and the reaction was run for 30 cycles. The PCR products were subjected to electrophoresis on 1.2% agarose gel (or 4 % agarose gel for S1PP2 and LPP1a) and visualized by ethidium bromide staining. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control for data normalization. In each gene targeted, the linear range was confirmed at 20-35 PCR cycles using the method previously described [24].

Statistical Analyses

All the data were expressed as the mean with S.E. Statistical significance of difference was determined by Bonferroni/Dunn (StatView for Macintosh ver. 5.0, SAS Institute, Inc., NC). A value of $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

Sphingosine-1-phosphate (S1P) is normally contained in the plasma at concentrations ranging from 0.2-0.5 µM, and the level of S1P is tightly controlled by its producing and breakdown enzymes. Sphingosine is phosphorylated by sphingosine kinase 1 (SphK1) and SphK2 to generate S1P. S1P can be degraded either by reversible dephosphorylation to sphingosine by a variety of phosphohydrolases, such as S1P phosphatase 1 (S1PP1), S1PP2, and lipid phosphate phosphatase 1a (LPP1a), or by irreversible cleavage to ethanolamine and hexadecenal by S1P lyase [17]. To identify the expression of enzymes associated with S1P turnover in the lungs of mice, RT-PCR analyses were carried out using total RNAs extracted from the lungs. Total RNAs of mouse whole brain were used as positive controls (Fig. **1**). As shown in Fig. (**1**), all the target genes, SphK1, SphK2, S1P lyase, S1PP1, S1PP2, and LPP1a, were expressed in the lungs of mice. It is thus indicated that the S1P metabolic pathways are present in the airways of mice.

Although physiological and/or pathophysiological role of S1P in the airways is not fully understood to date, increasing evidence suggests an involvement of S1P in the pathogenesis of allergic bronchial asthma [9-15]. Indeed, an increase in the level of S1P was reported in the airways of asthmatics [9], suggesting that the SphKs might be upregulated in the

Table 1. Primer Sequences for RT-PCR Used in the Present Study

Gene name	Gene ID		Sequence	Amplicon Size
mouse SphK1	20698	Sense	5'-CTTCTGGGCTGCGGCTCTATTCTG-3'	
		Antisense	5'-GGAAAGCAACCACGCGCACA-3'	507 bp
mouse SphK2	56632	Sense	5'-TTGCCCTCACCCTCACAACACAAG-3'	
		Antisense	5'-CCTCGTAAAGCAGCCCGTCTCCA-3'	564 bp
mouse S1PP1	81535	Sense	5'-CAACTTGCCGCTCTACTACCT-3'	
		Antisense	5'-GAAGCCCGATGATGATGA-3'	524 bp
mouse S1PP2	433323	Sense	5'-GCCTTTGGTGAGCCTTCTCA-3'	
		Antisense	5'-TGCCGGTGCTGCTCTGT-3'	57 bp
mouse LPP1a	30038764	Sense	5'-CCACACACGACTCTCCATGAA-3'	
		Antisense	5'-GGCTCGTGATTGGTTGAGTAGTT-3'	59 bp
mouse S1P lyase	20397	Sense	5'-TACCGGGACTTGGCGTTAG-3'	
		Antisense	5'-TGCTTGGAGATGCGTAGACAC-3'	514 bp
mouse GAPDH	14433	Sense	5'-ACCACAGTCCATGCCATCAC-3'	
		Antisense	5'-TCCACCACCCTGTTGCTGT-3'	452 bp

SphK: sphingosine kinase, S1PP: sphingosine-1-phosphate (S1P) phosphatase, LPP: lipid phosphate phosphatase, and GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

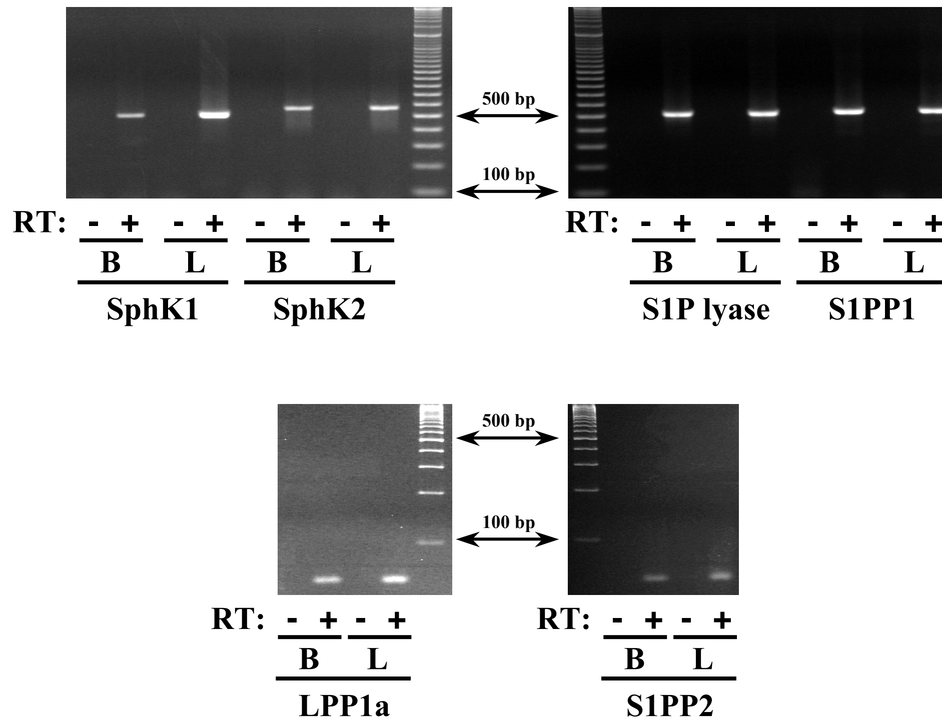

Fig. (1). Expression of mRNAs for the sphingosine-1-phosphate (S1P)-producing enzymes, sphingosine kinase 1 (SphK1) and SphK2, and for the S1P-breakdown enzymes, S1P phosphatase 1 (S1PP1), S1PP2, S1P lyase, and lipid phosphate phosphatase 1a (LPP1a), in the lungs of mice. Reverse transcription (RT) reactions were carried out using total RNAs extracted from the lungs (L) and whole brain (B: as positive controls) as described in MATERIALS AND METHODS, and polymerase chain reactions (PCR) were conducted using the primer sets specific for respective genes as shown in Table **1**. The resultant PCR products were detected by 4% (for S1PP2 and LPP1a) or 1.2% (for the other genes) agarose gel electrophoresis. The bands shown are representative of 3 independent experiments, respectively. RT-: without RT reaction.

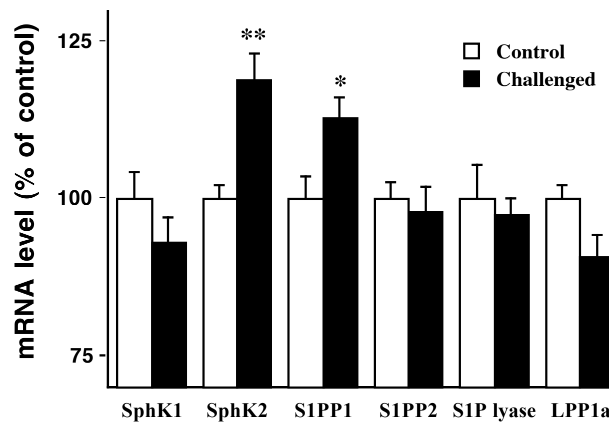

Fig. (2). Changes in the mRNA levels of sphingosine kinase 1 (SphK1) and SphK2, sphingosine-1-phosphate (S1P) phosphatase 1 (S1PP1), S1PP2, S1P lyase, and lipid phosphate phosphatase 1a (LPP1a), in the lungs of repeatedly antigen-challenged mice. The RT-PCR analyses were conducted as shown in Fig. 1, and the band densities of the target genes were normalized by corresponding GAPDH bands. Data were expressed as % change from control animals, respectively. Each value represents the mean ± SEM from 6 animals. $^{**}P < 0.01$ vs. Control by Bonferroni/Dunn's test.

airways of the disease. To test the hypothesis, the mRNA levels of SphK1 and SphK2 in the lungs were compared between the repeatedly antigen-challenged and control mice. In mice with lipopolysaccharide (LPS)-induced lung injury, the respective genes showed that the expression levels of proteins were reflected almost by those of mRNAs [25]. As shown in Fig. (**2**), the mRNA level of SphK2 was significantly increased in the repeatedly antigen-challenged mice, whereas the SphK1 expression was control level. Since S1P levels are tightly regulated by SphKs, the upregulation of SphK2 might cause an increase in S1P in the airways and be involved in the pathogenesis of allergic bronchial asthma. The mechanism of upregulation of SphK2 should be solved in future studies.

The current study also revealed a slight but statistically significant increase in the mRNA level of S1PP1 in the lungs of the repeatedly antigen-challenged mice (Fig. **2**). S1PP1 catalyzes the degradation of S1P via salvage and recycling of sphingosine into long-chain ceramides [17, 18]. Although the exact mechanism is not known here, an elevation of S1P by the upregulation of SphK2 might be a cause of the upregulation of S1PP1.

In summary, SphK1, SphK2, S1P lyase, S1PP1, S1PP2, and LPP1a, were expressed in the lungs of mice. Among them, up-regulations of SphK2 and S1PP1 were observed in the inflamed lungs induced by repeated antigen exposure. It is thus possible that the S1P turnover is increased in the airways of allergic bronchial asthma.

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Received: September 26, 2010 Revised: October 06, 2010 Accepted: October 07, 2010

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