Sphingosine 1-phosphate regulates inflammation-related genes in human endothelial cells through S1P₁ and S1P₃

Chi-Iou Lin ^a, Chiung-Nien Chen ^{c,d,e}, Po-Wei Lin ^a, Hsinyu Lee ^{a,b,c,*}

^a Institute of Zoology, National Taiwan University, Taipei 106, Taiwan, ROC

^b Department of Life Science, National Taiwan University, Taipei 106, Taiwan, ROC

^c Angiogenesis Research Center, National Taiwan University, Taipei 106, Taiwan, ROC

^d Department of Surgery, National Taiwan University Hospital, Taipei 106, Taiwan, ROC

^e Division of Mechanics, Research Center for Applied Sciences, Academia Sinica, Taipei 115, Taiwan, ROC

Received 30 January 2007

Available online 20 February 2007

Abstract

Sphingosine 1-phosphate (S1P) is a bioactive lysophospholipid (LPL) ligand that binds endothelial differentiation gene (Edg) family G-protein-coupled receptors and has been implicated as an important regulator in endothelial cells during inflammation processes. In this study, we attempt to determine which S1P receptors mediating the inflammatory response in human endothelial cells. Our results indicated that introduction of siRNA against S1P₁ significantly suppressed S1P-induced ICAM-1 mRNA, total protein, and cell surface expressions in human umbilical vein endothelial cells (HUVECs). Moreover, U937 cells adhesion to S1P-treated HUVECs was profoundly reduced by knock-down of S1P₁ in HUVECs. By knock-down of S1P₁ or S1P₃ in HUVECs, S1P-enhanced IL-8, MCP-1 mRNA expression, and THP-1 cell chemotaxis toward S1P-treated HUVEC-conditioned media was profoundly reduced. These results suggested that S1P-induced inflammatory response genes expression is mediated through S1P₁ and S1P₃. Our findings suggest the possible utilization of S1P₁ or S1P₃ as drug targets to treat severe inflammation.

© 2007 Elsevier Inc. All rights reserved.

Keywords: S1P; S1P₁; S1P₃; Endothelial cells

Sphingosine 1-phosphate (S1P) is a multifunctional bioactive lipid mediator, which binds to cell surface G-protein-coupled receptors (GPCRs) [1,2]. To the present, five S1P receptors have been identified, namely S1P₁/Edg1, S1P₂/Edg5, S1P₃/Edg3, S1P₄/Edg6, and S1P₅/Edg8, which are members of the Edg (endothelial differentiation gene) receptor family [1,2]. Several signaling pathways are activated by Edg receptors via multiple G proteins, including G_{q/11}, G_{i/o}, and G_{12/13}, which induce diverse physiological functions, including cell differentiation, proliferation, intercellular communication, and wound healing [3].

S1P receptors transduce a variety of heterologous signal pathways through coupling with multiple G proteins. S1P₁

is predominantly expressed in endothelial cells and exclusively coupled to G_i, which stimulates cell growth, migration, proliferation, and tube formation [4–6]. In contrast, S1P₂ couples strongly to G_{12/13}, which negatively activates Rac but positively regulates activation of the small GTPase, RhoA [7]. A recent study reported that S1P downregulates endothelial cell morphogenesis and angiogenesis through activation of S1P₂ [8]. S1P₃ also activates RhoA under some conditions through G_{12/13} [9], and both S1P₂ and S1P₃ have been shown to stimulate phospholipase C through coupling with G_q [10]. By activation of S1P₁ and S1P₃, S1P can stimulate a Gi- and Gq-linked intracellular calcium mobilization in various cell types and also enhances endothelial cell migration through activating integrin [11]. It has been suggested that S1P₄ couples to Gi and G_{12/13} [12]. S1P₅ couples to Gi but not through Gq [13]. Our previous studies demonstrated that S1P enhances the expressions of several inflam-

* Corresponding author. Address: Department of Life Science, National Taiwan University, Taipei 106, Taiwan, ROC. Fax: +886 2 23636837.

E-mail address: hsinyu@ntu.edu.tw (H. Lee).

mation-related genes including ICAM-1, IL-8, and MCP-1 in HUVECs [14,15]. These results strongly suggest that S1P is an important regulator of inflammation processes. However, by which S1P receptors mediating S1P-modulated inflammatory genes expression remains unclear.

Loss-of-function genetic or pharmacologic approaches are required to determine the functions of S1P receptors *in vivo* [16]. However, S1P₁^{-/-} mice died during mid-gestational stages of embryogenesis [17]. In addition, there are no available specific pharmacologic tools appropriate for inhibiting these receptors. In this report, by utilizing RNAi to suppress S1P receptors gene expressions in HUVECs, we present evidence that S1P-upregulated ICAM-1 mRNA, total protein, cell surface expression in HUVECs, and subsequent cell adhesion of U937 cells with S1P-treated HUVECs are mediated through the activation of S1P₁. In addition, S1P-enhanced IL-8, MCP-1 mRNA expression in HUVECs, and THP-1 cell chemotaxis toward S1P-treated HUVEC-conditioned media are mediated through the activation of both S1P₁ and S1P₃. These results suggest that the binding of S1P to different S1P receptors modulates different inflammatory gene expressions, which contributes to monocyte–endothelium interaction, thus facilitating inflammation processes.

Methods

Materials. S1P, 1-oleoyl-lysophosphatidic acid (LPA), fatty acid-free bovine serum albumin (FAF-BSA), and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO). The human ICAM-1 antibody (clone M19) and FITC-conjugated mouse anti-human ICAM-1 monoclonal antibody (clone 6.5B5) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG was obtained from Boehringer Mannheim (Indianapolis, IN).

Cell culture. HUVECs were isolated from fresh umbilical cords as described previously [14] and were cultured in M199 medium supplemented with 10% FBS and 25% EGM (Cell Applications, San Diego, CA). U937 and THP-1 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS. The cells were maintained under standard cell culture condition at 37 °C in humidified air with 5% CO₂.

RT-PCR analysis. Total cellular RNA was extracted from HUVECs using the TRIzol reagent (Gibco), and a Superscript kit (Gibco) was used for the reverse-transcription (RT) synthesis of cDNA. Polymerase chain reaction (PCR) amplification was performed with Taq DNA polymerase (Geneaid, Taoyuan, Taiwan) using the set of the primers as follows: 5'-GCAAGCTCCAGTGAAATGCAAAC-3' and 5'-TGTCTACTGACC CCAACCCCTTGATG-3' (ICAM-1), 5'-ATGACTTCCAAGCTGGCCG TGGCT-3' and 5'-TCTCAGCCCTCTTCAAAAACCTCTC-3' (IL-8), 5'-TCTCAGTGCAGAGGCTCGCGA-3' and 5'-GAGTGAGTGTTCAA GTCTTCG-3' (MCP-1), 5'-ACCACAGTTCATGCCATCAC-3' and 5'-TCCACCACCTGTTGCTGTA-3' (GAPDH), 5'-GACTCTGCTGG CAAATTCAAGCGAC-3' and 5'-ACCCTTCCAGTGCATTGTTCA CAG-3' (S1P₁), 5'-CTCTCTACGCCAAGCATTATGTGCT-3' and 5'-TCAGACCACCGTGTGCCCC-3' (S1P₂), 5'-CAAAATGAGGCCT TACGACGCA-3' and 5'-TCCCATCTCTGAAGTGCTGCGTTC-3' (S1P₃), 5'-AGCCTTCTGCCCTCTACTC-3' and 5'-GTAGATGATG GGGTGTACCG-3' (S1P₄), 5'-GGAGTAGTTCCTCCGAAGGACC-3' and 5'-TCTAGAATCCACGGGGTCTG-3' (S1P₅). PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and then photographed.

Cell transfections. The effective target sequence for scrambled siRNA was: UUCUCCGAACGUGUUCACGUGdTdT and ACGUGACACGU UCGGAGAAAdTdT. S1P₁, S1P₃, S1P₄, and S1P₅ siRNA were purchased

from Santa Cruz Biotechnology. HUVECs were transfected using the optimized protocol for electroporation of HUVECs with the Nucleofector apparatus (Amaxa Biosystems, Cologne, Germany). Cells (1 × 10⁶) were trypsinized, resuspended in 100 µl of supplemented HUVEC Nucleofector™ solution (Amaxa Biosystems), and electroporated in the presence of 7.2 µg siRNA.

Western blotting for ICAM-1. Transfected HUVECs cultures were plated in 6-well plates and treated with 5 µM of LPA or S1P for 8 h. Treated cells were lysed with RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) containing a protease inhibitor cocktail (Sigma) and 2 mM Na vanadate. Equal amounts of cell lysates were separated by 4–10% SDS-PAGE and electrophoretically transferred to Immobilon® membranes (Millipore, Bedford, MA). The transferred membranes were blocked with blocking buffer (PBS + 0.1% Tween 20 with 1% BSA) at room temperature for 1 h. The membranes were immunoblotted with an anti-human ICAM-1 monoclonal antibody for 2 h, washed, and re-incubated for 1 h with secondary antibody. Membranes were washed and developed using an ECL system (Amersham Biosciences, Piscataway, NJ).

FACSscan. Transfected HUVEC cultures were serum starved for 16 h and incubated with LPA or S1P for 8 h at 37 °C. Suspensions of 10⁶ HUVECs in PBS (200 µl) with 0.1% fatty acid-free BSA received 2 µl of FITC-conjugated human anti-ICAM-1 and were incubated for 30 min at 4 °C. Antibody binding of HUVECs with and without stimulation was determined by CyFlow® SL (Partec, Münster, Germany) and analyzed by WinMDI version 2.8 software.

In vitro adhesion assay. Transfected HUVECs were cultured in 6-well plates and stimulated with 5 µM LPA or S1P for 8 h. After washing with M199 medium, U937 monocytes (1 ml, 5 × 10⁶ cells/ml) were added to the cultures, and then incubated at 37 °C for 1 h. Cultures were washed by M199 medium until no visible suspension of U937 cells was observed and then imaged by microscopy. The number of monocytes per culture was counted.

Chemotaxis assay. The trans-well migration of human THP-1 cells toward conditioned media was studied in a 48-well microchamber (Neuro Probe, Cabin John, MD) as described previously [15]. Cells migrating across the filter onto the lower surface were photographed using light microscopy. Quantification of the migration results was determined by the color intensity of the spots solubilized by solubilization buffer, and the absorbance was determined at 540 nm.

Statistical analysis. Significant differences between control and treatment groups were determined by ANOVA. Each experiment was repeated at least three times. The results are expressed as means ± SE. A value of *p* < 0.05 was considered statistically significant.

Results

S1P-induced ICAM-1 mRNA expression is mediated through S1P₁, while IL-8 and MCP-1 mRNA expression is mediated through S1P₁ and S1P₃ in HUVECs

We first determined the S1P receptor expression profiles in HUVECs by RT-PCR (Fig. 1A). Our results indicated that at least S1P₁, S1P₃, S1P₄, and S1P₅ were expressed in HUVECs. Therefore, we first investigated these S1P receptors' mediation of S1P-induced ICAM-1, IL-8, and MCP-1 mRNA expressions. RNA interference was used to knock-down the expression level of a given S1P receptors. HUVECs were transfected with scrambled- or S1P₁-, S1P₃-, S1P₄-, S1P₅-, and S1P₁ + S1P₃-siRNA duplex. Here, we use LPA, a structural and functional homologous of S1P, as a control in the following experiments. HUVECs transfected with siRNAs specific for each S1P receptors for 48 h showed significantly decreased each S1P receptors

Fig. 1. Effects of S1P receptor siRNAs on ICAM-1, IL-8, and MCP-1 mRNA expressions in HUVECs. (A) cDNA of HUVECs was analyzed by RT-PCR to determine the S1P receptor expression profiles. The expected PCR products were 352, 512, 701, 339, and 236 bp for S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅. HUVECs were transfected with scrambled or S1P₁ (B), S1P₃ (C), S1P₄ (D), S1P₅ (E), S1P₁ + S1P₃ (F) siRNA duplex. Twenty-four hours after transfection, HUVECs were starved and treated with 5 μ M LPA or S1P for 4 h. ICAM-1, IL-8, and MCP-1 mRNA levels were assessed by RT-PCR. The expression level of S1P₁, S1P₃, S1P₄ or S1P₅ in each panel was used to evaluate the efficiency of each siRNA. The amount of GAPDH was used as a loading control in each panel. C represents the untreated control.

mRNA expression in LPA- and S1P-treated and untreated samples (Figs. 1B–F). The expression patterns of GAPDH, which were used as the loading control, did not significantly differ in LPA- or S1P-treated, or untreated samples. Decreases in S1P- but not LPA-induced ICAM-1 mRNA expression were observed in HUVECs transfected with S1P₁ siRNA but not in those transfected with scrambled siRNA (Fig. 1B). Moreover, S1P- but not LPA-induced IL-8 and MCP-1 mRNA expression in HUVECs was significantly suppressed by transfecting with S1P₁ (Fig. 1B) or S1P₃ (Fig. 1C) siRNA. However, the treatment of S1P₄ (Fig. 1D) or S1P₅ (Fig. 1E) showed no suppressive effects. Co-transfection of S1P₁ and S1P₃ siRNAs also showed significant suppressive effects on S1P- but not LPA-enhanced IL-8 and MCP-1 mRNA expression as well as ICAM-1 in HUVECs (Fig. 1F). These results demonstrated that S1P-induced ICAM-1 mRNA expression is mediated through S1P₁, while IL-8, and MCP-1 mRNA expressions are mediated through S1P₁ and S1P₃ in HUVECs.

S1P-induced ICAM-1 protein expression is mediated through S1P₁ in HUVECs

Since S1P₁'s mediation of S1P-induced ICAM-1 mRNA expression was examined, we further investigated if

the S1P-elevated mRNA levels of ICAM-1 mediated through S1P₁ were also correlated to protein expression levels. Total ICAM-1 protein levels were detected by Western blotting. Decreases in S1P- but not LPA-induced ICAM-1 total protein expression were observed in HUVECs treated with S1P₁ siRNA (Fig. 2A, left panel) but not those treated with the S1P₃ siRNA (Fig. 2B, left panel). The expression patterns of β -actin, which were used as the loading control, did not significantly differ in LPA- or S1P-treated, or untreated samples. By FACScan analysis, which detects the antigenic activities of proteins on cell surfaces, we also observed a significant decrease in S1P- but not LPA-induced ICAM-1 cell surface expressions in HUVECs treated with S1P₁ siRNA (Fig. 2A, right panel) and but not those treated with the S1P₃ siRNA (Fig. 2B, right panel). These results indicated that the enhancement effects of S1P on ICAM-1 expression mediated through S1P₁ also occurred with total protein and cell surface expression in HUVECs.

S1P-enhanced adhesive properties of HUVECs toward U937 cells are mediated through S1P₁

In a previous study, we demonstrated that LPLs' enhancement of monocyte adherence to endothelial cells is

Fig. 2. SIP₁ siRNAs suppressed total and cell surface ICAM-1 protein expressions in HUVECs. HUVECs were transfected with scrambled or SIP₁(A), SIP₃ (B) siRNA duplexes. Transfected HUVECs were starved and treated with 5 μ M LPA or SIP for 8 h. Total cell lysates were isolated, and ICAM-1 protein expression levels were monitored by Western blotting (left panel) using a human monoclonal anti-ICAM-1 antibody. A human β -actin antibody was used as the loading control. Treated cells were dissociated by trypsinization and incubated with an FITC-labeled mouse anti-human ICAM-1 monoclonal antibody for 30 min and analyzed by FACScan (right panel). C represents the untreated control.

Fig. 3. SIP₁ siRNAs suppressed SIP- but not LPA-enhanced ICAM-1-mediated U937/HUVEC adhesion. (A) HUVECs were transfected with scrambled, SIP₁ or SIP₃ siRNA duplex. Transfected cells were treated with medium, LPA (5 μ M), or SIP (5 μ M) for 8 h and then co-incubated with for 1 h 1 μ g/ml LPS-stimulated U937 cells. (B) Histograms represent quantification of adherent U937 cell counts (* p < 0.05).

mediated through upregulating ICAM-1 expression in endothelial cells [14]. In the present study, we found that the enhancement of ICAM-1 expression by SIP in HUVECs was mediated through SIP₁ (Figs. 1 and 2). We next attempted to investigate if SIP-induced monocytes adhesion toward endothelial cells is mediated through SIP₁. By *in vitro* adhesion assay, treatment of scrambled siRNA showed no effects on LPS-activated U937 cells adherence to LPA- and SIP-treated HUVECs. However, the treatment of SIP₁ but not SIP₃ siRNA significantly inhibited U937 cells adherence to SIP- but not LPA-treated HUVECs (Fig. 3). These results illustrate that the enhancement effect of SIP on the adhesive property of endothelial cells toward monocytes is only mediated through the activa-

tion of SIP₁. Our results suggest that SIP₁ might mediate SIP-induced ICAM-1 expression in HUVECs and subsequently monocytes–endothelium adhesion.

SIP-enhanced chemotactic activity of HUVECs toward U937 cells is mediated through SIP₁ and SIP₃

Our previous study indicated that the enhancement effect of SIP on chemotactic activity of endothelial cell is mediated through upregulation of the expressions of IL-8 and MCP-1 [15]. In addition, SIP-enhanced IL-8 and MCP-1 mRNA expressions were mediated through SIP₁ and SIP₃ (Fig. 1). Therefore, we further tested the hypothesis that SIP's enhancement of the chemotactic activity in

Fig. 4. SIP₁ and SIP₃ siRNAs inhibit the chemotactic activity of conditioned media from SIP- but not LPA-treated HUVECs. (A) HUVECs were transfected with scrambled, SIP₁, SIP₃ or SIP₁ + SIP₃ siRNA duplex, followed by medium, LPA (5 μ M), or SIP (5 μ M) treatment for 24 h. The chemotactic activity was determined using a 48-well micro chemotaxis chamber. THP-1 cells were added to the upper chambers and allowed to migrate for 4 h through 8- μ m porous membranes toward the lower chambers which contained the harvested conditioned media which had been added as indicated. Cells migrated to the lower chambers were fixed, stained, and photographed. (B) Histograms represent quantification results of migrating THP-1 cells (* p < 0.05). All data are relative multiples of expression compared to untreated cells.

endothelial cells is mediated through SIP₁ and SIP₃. By a chemotaxis assay, treatment with scrambled siRNA showed no effects on THP-1 cells trans-well migration toward conditioned media of LPA- and SIP-treated HUVECs. However, conditioned media collected from SIP₁, SIP₃ or SIP₁ + SIP₃ siRNA-transfected HUVECs significantly inhibited SIP- but not LPA-enhanced THP-1 cell trans-well migration (Fig. 4). These results imply that the enhancement effects of SIP on the chemotactic activity of endothelial cell are mediated through SIP₁ and SIP₃ activation. Our results suggest that SIP₁ and SIP₃ might mediate SIP-induced IL-8 and MCP-1 expression in HUVECs and subsequently monocytes chemoattracting toward endothelium.

Discussion

By RT-PCR, we found that not all SIP receptors are expressed in HUVECs. SIP₂ is not expressed in HUVECs, which is consistent with previous reports [3]. A previous study reported that long-term silencing of SIP₁ profoundly alters various gene expressions in a cultured human SIP₁-knock-down angiosarcoma cell line [18]. In the present study, we further confirmed that SIP₁ regulates inflammation-related gene expressions of ICAM-1, IL-8, and MCP-1 in HUVECs (Fig. 1). Recent evidence indicates that SIP₁ and SIP₃ are the most abundant SIP receptors in HUVECs [5,6,19]. Through activation of SIP₁ and SIP₃, SIP induces endothelial cell migration, and these effects are exclusively Gi-dependent [6,19]. Our previous study demonstrated that SIP-induced ICAM-1 expression in HUVECs is Gi-dependent and independent of Rho [14], whereas IL-8

and MCP-1 expression are both Gi- and Rho-dependent [15]. These observations are consistent with the facts that SIP₁ exclusively couples to Gi [4] and SIP₃ could activate G_{12/13}-Rho pathway [7]. Through the *in vitro* evidences presented in this report, we suggest that SIP₁ expressed on endothelial cells plays a key role on regulating monocytes adhesion and chemotaxis to endothelial cells, whereas SIP₃ is only essential for endothelial cells to chemoattract monocytes.

It has been reported that SIP₁ might mediate both stimulatory and inhibitory signals for monocyte-endothelial cell interaction [20]. In our study, SIP (5 μ M) enhances monocytes adhesion to endothelial cells through upregulating the ICAM-1 expression of endothelial cells [14], and these enhancement effects are mediated through activation of SIP₁. However, recent study also reported that low concentration of SIP (0.1 μ M) prevents monocytes adhesion to endothelial cells extracted from diabetic NOD mice through activation of SIP₁, and these suppressive effects are due to decrease of ICAM-1 and VCAM-1 expression [21]. In addition, SIP (0.1 μ M) also prevented TNF- α -induced monocyte adhesion to endothelial cells through SIP₁ [22]. Moreover, moderate concentration (1 μ M) of HDL-composed SIP positively or negatively regulates ICAM-1 expression in endothelial cells and monocytes-endothelium adhesion through SIP₁ and SIP₃, respectively [23,24]. These observations suggest that upon binding with SIP₁, different concentrations of SIP may play diverse roles in mediating monocyte-endothelium adhesion.

Accumulating evidence indicates that SIP₁ is important for SIP's regulation of various cellular behaviors in endothelial cells such as cell migration [6], tube formation

[16,17], and cell adherent junction morphogenesis [5], which suggests that the activation of S1P₁ is essential for S1P's promotion of angiogenesis [17,25]. Our findings further suggest that S1P₁ and S1P₃ are required for S1P's upregulation of the adhesive property for the tight adherence with monocytes and the chemotactic activity of endothelial cells, which play important roles in S1P-regulated inflammation processes. Recent studies proposed that S1P might be a target for establishing a therapeutic strategy for controlling cardiovascular disease [26]. Moreover, S1P₁ and S1P₃ are key targets in new drug development for regulating angiogenesis [27]. Herein, our findings were focused on modulating the functions of S1P₁ and S1P₃, which may further offer a novel perspective for anti-inflammation drug discoveries.

In summary, our results clearly indicate that S1P increases ICAM-1 mRNA, total protein, and cell surface expression in HUVECs are mediated through the activation of S1P₁. In addition, S1P increases IL-8 and MCP-1 mRNA expression in HUVECs is mediated through S1P₁ and S1P₃. Moreover, S1P₁ expressed on endothelial cells mediates the positive regulation on monocytes adhesion and chemotaxis toward endothelium, whereas S1P₃ is only critical for endothelial cells chemoattract monocytes. Our findings suggest that S1P₁ and S1P₃ might be essential receptors for S1P on modulating monocyte–endothelial cell interactions, which provides valid information necessary for developing new therapeutic drugs against the inflammation process and atherosclerosis formation.

Acknowledgments

We thank Dr. S.M. Hsieh at National Taiwan University Hospital provided umbilical cords for endothelial cell preparation. This work was supported by Grants 95-2311-B-002-018-MY2 (H. Lee), and NSC 95-2314-B002-151 (C. Chen) from the National Science Council.

References

- [1] S. An, E.J. Goetzl, H. Lee, Signaling mechanisms and molecular characteristics of G protein-coupled receptors for lysophosphatidic acid and sphingosine 1-phosphate, *J. Cell. Biochem.* 30–31 (1998) 147–157.
- [2] W.H. Moolenaar, Bioactive lysophospholipids and their G protein-coupled receptors, *Exp. Cell Res.* 253 (1999) 230–238.
- [3] H. Lee, E.J. Goetzl, S. An, Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing, *Am. J. Physiol. Cell Physiol.* 278 (2000) C612–C618.
- [4] H. Okamoto, N. Takuwa, K. Gonda, H. Okazaki, K. Chang, Y. Yatomi, H. Shigematsu, Y. Takuwa, EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways, including phospholipase C activation, Ca²⁺ mobilization, Ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition, *J. Biol. Chem.* 273 (1998) 27104–27110.
- [5] M.J. Lee, S. Thangada, K.P. Claffey, N. Ancellin, C.H. Liu, M. Kluk, M. Volpi, R.I. Sha'afi, T. Hla, Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate, *Cell* 99 (1999) 301–312.
- [6] J.H. Paik, S. Chae, M.J. Lee, S. Thangada, T. Hla, Sphingosine 1-phosphate-induced endothelial cell migration requires the expression of EDG-1 and EDG-3 receptors and Rho-dependent activation of alpha vbeta3- and beta 1-containing integrins, *J. Biol. Chem.* 276 (2001) 11830–11837.
- [7] N. Sugimoto, N. Takuwa, H. Okamoto, S. Sakurada, Y. Takuwa, Inhibitory and stimulatory regulation of Rac and cell motility by the G_{12/13}-Rho and Gi pathways integrated downstream of a single G protein-coupled sphingosine 1-phosphate receptor isoform, *Mol. Cell. Biol.* 23 (2003) 1534–1545.
- [8] I. Inoki, N. Takuwa, N. Sugimoto, K. Yoshioka, S. Takata, S. Kaneko, Y. Takuwa, Negative regulation of endothelial morphogenesis and angiogenesis by S1P₂ receptor, *Biochem. Biophys. Res. Commun.* 346 (2006) 293–300.
- [9] I. Ishii, X. Ye, B. Friedman, S. Kawamura, J.J. Contos, M.A. Kingsbury, A.H. Yang, G. Zhang, J.H. Brown, J. Chun, Marked perinatal lethality and cellular signaling deficits in mice null for the two sphingosine 1-phosphate (S1P) receptors, S1P(2)/LP(B2)/EDG-5 and S1P(3)/LP(B3)/EDG-3, *J. Biol. Chem.* 277 (2002) 25152–25159.
- [10] T. Hla, M.J. Lee, N. Ancellin, J.H. Paik, M.J. Kluk, Lysophospholipids—receptor revelations, *Science* 294 (2001) 1875–1878.
- [11] S. An, T. Bleu, Y. Zheng, Transduction of intracellular calcium signals through G protein-mediated activation of phospholipase C by recombinant sphingosine 1-phosphate receptors, *Mol. Pharmacol.* 55 (1999) 787–794.
- [12] M.H. Graler, R. Grosse, A. Kusch, E. Kremmer, T. Gudermann, M. Lipp, The sphingosine 1-phosphate receptor S1P₄ regulates cell shape and motility via coupling to Gi and G_{12/13}, *J. Cell. Biochem.* 89 (2003) 507–519.
- [13] D.S. Im, C.E. Heise, N. Ancellin, B.F. O'Dowd, G.J. Shei, R.P. Heavens, M.R. Rigby, T. Hla, S. Mandala, G. McAllister, S.R. George, K.R. Lynch, Characterization of a novel sphingosine 1-phosphate receptor, Edg-8, *J. Biol. Chem.* 275 (2000) 14281–14286.
- [14] H. Lee, C.I. Lin, J.J. Liao, Y.W. Lee, H.Y. Yang, C.Y. Lee, H.Y. Hsu, H.L. Wu, Lysophospholipids increase ICAM-1 expression in HUVEC through a Gi- and NF-kappaB-dependent mechanism, *Am. J. Physiol. Cell Physiol.* 287 (2004) C1657–C1666.
- [15] C.I. Lin, C.N. Chen, J.H. Chen, H. Lee, Lysophospholipids increase IL-8 and MCP-1 expressions in human umbilical cord vein endothelial cells through an IL-1-dependent mechanism, *J. Cell. Biochem.* 99 (2006) 1216–1232.
- [16] S.S. Chae, J.H. Paik, H. Furneaux, T. Hla, Requirement for sphingosine 1-phosphate receptor-1 in tumor angiogenesis demonstrated by in vivo RNA interference, *J. Clin. Invest.* 114 (2004) 1082–1089.
- [17] Y. Liu, R. Wada, T. Yamashita, Y. Mi, C.X. Deng, J.P. Hobson, H.M. Rosenfeldt, V.E. Nava, S.S. Chae, M.J. Lee, C.H. Liu, T. Hla, S. Spiegel, R.L. Proia, Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation, *J. Clin. Invest.* 106 (2000) 951–961.
- [18] V. Krump-Konvalinkova, S. Yasuda, T. Rubic, N. Makarova, J. Mages, W. Erl, C. Vosseler, C.J. Kirkpatrick, G. Tigyi, W. Siess, Stable knock-down of the sphingosine 1-phosphate receptor S1P₁ influences multiple functions of human endothelial cells, *Arterioscler. Thromb. Vasc. Biol.* 25 (2005) 546–552.
- [19] T. Kimura, T. Watanabe, K. Sato, J. Kon, H. Tomura, K. Tamama, A. Kuwabara, T. Kanda, I. Kobayashi, H. Ohta, M. Ui, F. Okajima, Sphingosine 1-phosphate stimulates proliferation and migration of human endothelial cells possibly through the lipid receptors, Edg-1 and Edg-3, *Biochem. J.* 348 (2000) 71–76.
- [20] T. Kimura, H. Tomura, C. Mogi, A. Kuwabara, M. Ishiura, K. Shibasawa, K. Sato, S. Ohwada, D.S. Im, H. Kurose, T. Ishizuka, M. Murakami, F. Okajima, Sphingosine 1-phosphate receptors mediate stimulatory and inhibitory signalings for expression of adhesion molecules in endothelial cells, *Cell. Signal.* 18 (2006) 841–850.
- [21] A.M. Whetzel, D.T. Bolick, S. Srinivasan, T.L. Macdonald, M.A. Morris, K. Ley, C.C. Hedrick, Sphingosine-1-phosphate prevents monocyte/endothelial interactions in type 1 diabetic NOD mice

- through activation of the S1P1 receptor, *Circ. Res.* 99 (2006) 731–739.
- [22] D.T. Bolick, S. Srinivasan, K.W. Kim, M.E. Hatley, J.J. Clemens, A. Whetzel, N. Ferger, T.L. Macdonald, M.D. Davis, P.S. Tsao, K.R. Lynch, C.C. Hedrick, Sphingosine-1-phosphate prevents tumor necrosis factor- α -mediated monocyte adhesion to aortic endothelium in mice, *Arterioscler. Thromb. Vasc. Biol.* 25 (2005) 976–981.
- [23] T. Kimura, K. Sato, E. Malchinkhuu, H. Tomura, K. Tamama, A. Kuwabara, M. Murakami, F. Okajima, High-density lipoprotein stimulates endothelial cell migration and survival through sphingosine 1-phosphate and its receptors, *Arterioscler. Thromb. Vasc. Biol.* 23 (2003) 1283–1288.
- [24] T. Kimura, H. Tomura, C. Mogi, A. Kuwabara, A. Damirin, T. Ishizuka, A. Sekiguchi, M. Ishiwara, D.S. Im, K. Sato, M. Murakami, F. Okajima, Role of scavenger receptor class B type I and sphingosine 1-phosphate receptors in high-density lipoprotein-induced inhibition of adhesion molecule expression in endothelial cells, *J. Biol. Chem.* 281 (2006) 37457–37467.
- [25] H.M. Rosenfeldt, J.P. Hobson, M. Maceyka, A. Olivera, V.E. Nava, S. Miltien, S. Spiegel, EDG-1 links the PDGF receptor to Src and focal adhesion kinase activation leading to lamellipodia formation and cell migration, *FASEB J.* 15 (2001) 2649–2659.
- [26] Y. Yatomi, Sphingosine 1-phosphate in vascular biology: possible therapeutic strategies to control vascular diseases, *Curr. Pharm. Des.* 12 (2006) 575–587.
- [27] C. Waeber, N. Blondeau, S. Salomone, Vascular sphingosine-1-phosphate S1P₁ and S1P₃ receptors, *Drug News Perspect.* 17 (2004) 365–382.