J. Biochem. **138**, 47–55 (2005)
DOI: 10.1093/jb/mvi100

Sphingosine 1-Phosphate–Related Metabolism in the Blood Vessel

Shinya Aoki[1,2], Yutaka Yatomi[1,*], Masato Ohta[2], Makoto Osada[2], Fuminori Kazama[2], Kaneo Satoh[2], Kazuhiko Nakahara[1] and Yukio Ozaki[2]

[1]Department of Laboratory Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655; and [2]Department of Laboratory Medicine, University of Yamanashi Faculty of Medicine, Yamanashi 409-3898

Received February 26, 2005; accepted April 11, 2005

Sphingosine 1-phosphate (Sph-1-P) is a bioactive lipid released from activated platelets and plays an important role in vascular biology. In this study, we investigated Sph-1-P–related metabolism in the blood vessel, mainly using radio-labeled Sph and Sph-1-P. Sph was metabolically stable in the plasma, while it was converted into Sph-1-P in the presence of activated platelets. When the mixture of Sph-1-P and plasma was fractionated on a gel-filtration column, all Sph-1-P co-eluted with protein fractions that coincide with lipoproteins and albumin by agarose gel electrophoresis. When evaluated by polyacrylamide gel electrophoresis, 7.2 ± 3.8%, 53.3 ± 6.4%, and 39.5 ± 7.9% of the radioactivity of Sph-1-P added to plasma was recovered in the low-density lipoprotein (LDL), high-density lipoprotein (HDL), and albumin fractions, respectively. On the other hand, 5.2 ± 3.2%, 38.4 ± 5.5%, and 56.3 ± 5.7% of the radioactivity of Sph-1-P converted from Sph in collagen-stimulated platelets and released into the plasma was recovered in the LDL, HDL, and albumin fractions, respectively. When Sph-1-P release from activated platelets was examined, a stronger response was observed in the presence of albumin than lipoproteins, suggesting efficient Sph-1-P extraction from platelets by albumin. Finally, Sph-1-P, which is stable in the plasma, was markedly degraded by an ectophosphatase activity in the presence of vascular endothelial cells or in whole blood. Although Sph-1-P is stable in the plasma, it is likely that the level of this bioactive lipid is dynamically controlled by various factors including release from platelets, distribution among plasma proteins, and degradation by ectophosphatase.

Key words: albumin, lipoproteins, plasma, platelets, sphingosine, sphingosine 1-phosphate.

Abbreviations: Sph-1-P, sphingosine 1-phosphate; EC, endothelial cell; SMC, smooth muscle cell; Sph, sphingosine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TRAP, thrombin receptor activating peptide; PRP, platelet-rich plasma; PPP, platelet-poor plasma; HUVEC, human umbilical vein EC; TLC, thin-layer chromatography; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LPP, lipid phosphate phosphatase.

Sphingosine 1-phosphate (Sph-1-P) is a bioactive lysophospholipid that induces a variety of biological responses in diverse cell types (*1–5*). Many, if not all, of these responses are mediated mainly through a family of G protein–coupled Sph-1-P receptors named $S1P_{1, 2, 3, 4, and 5}$, originally referred to as EDG-1, 5, 3, 6, and 8, respectively (*6*). The area of vascular biology is no exception in that Sph-1-P plays an important role as an intercellular messenger. Platelets themselves, vascular endothelial cells (ECs) and smooth muscle cells (SMCs) respond to this platelet-derived bioactive lipid through cell surface receptors, although Sph-1-P may act as an intracellular messenger in some cases (*1–5*).

When exogenously added, Sph-1-P induces platelet shape change and primary aggregation (which is reversible), possibly via intracellular Ca^{2+} mobilization resulting from receptor stimulation (*5*, *7*). Sph-1-P has been shown to induce more dramatic responses in ECs and SMCs, which is consistent with the presence of high affinity cell surface S1P receptors on these cells. In ECs, it is well established that Sph-1-P stimulates survival or proliferation through $S1P_1$ (*8*, *9*). Furthermore, Sph-1-P induces migration, adherens junction assembly, capillary tube formation, and the resultant angiogenesis *via* $S1P_1$ and $S1P_3$ (*9*, *10*). Sph-1-P also stimulates the production of nitric oxide, which is an important mediator in the vasculature and is dysregulated in cardiovascular disorders (*11*, *12*). Like ECs, SMCs express S1Ps and respond dramatically to Sph-1-P. However, the pattern of S1P expression is different from that in ECs; $S1P_1$, abundantly expressed on ECs, is only expressed in the fetal/intimal phenotype of SMCs, while both $S1P_2$ and $S1P_3$ receptors are expressed in adult as well as fetal/intimal phenotypes (*3*, *13*). The low ratio of $S1P_1$ to $S1P_2$ results in inhibition of migration in normal quiescent SMCs (*4*, *14*); $S1P_1$ acts as a typical chemotactic receptor while $S1P_2$ uniquely acts as a chemorepellant receptor (*4*, *15*). The roles of Sph-1-P in vasoconstriction have also been reported. Using a well-established canine isolated heart model, Sph-1-P was shown to decrease coronary blood flow and ventricular contractions; the coronary vasoconstriction effect of Sph-1-P should be related to its contractile effect on coronary SMCs, and the observed negative inotropic effect may be at least partly induced by transient myo-

*To whom correspondence should be addressed. Tel: +81-3-5800-8730, Fax: +81-3-5689-0495; E-mail: yatomiy-lab@h.u-tokyo.ac.jp

cardial ischemia (*16*). Consistent with this Sph-1-P–elicited coronary vasoconstriction *in vivo*, Sph-1-P strongly induces coronary artery SMC contraction, which can be inhibited by a specific antagonist of $S1P_2$ (*17*, *18*).

The critical requirement of Sph-1-P in vascular system development was shown in $S1P_1$-deficient mice (*19*). Homozygous $S1P_1$–/– mice reportedly die during embryonic development at 12.5–14.5 days of gestation due to hemorrhage (*19*). In the mutant embryos, vascular maturation was incomplete; disruption of the vessels, edema, and bleeding, due to an incomplete coverage of blood vessels by SMCs, were observed (*19*). When $S1P_1$ expression was disrupted solely in ECs by using the Cre/loxP system, the phenotype of the conditional mutant embryos was found to mimic that obtained in embryos globally deficient in $S1P_1$, indicating that vessel coverage by SMCs is directed by Sph-1-P interaction with $S1P_1$ receptors on ECs (*20*).

Now, there is no doubt that Sph-1-P plays important roles in vascular biology, and that the availability of Sph-1-P in the blood vessel is of clinical importance both in diagnostic and therapeutical aspects. Blood platelets store considerable amounts of Sph-1-P [possibly due to the existence of highly active sphingosine (Sph) kinase and a lack of Sph-1-P lyase], and release this bioactive lipid extracellularly upon stimulation (*5*, *7*). The fact that Sph-1-P is a normal constituent of plasma and serum, the concentration of the latter being much higher (*21*), can be best explained by Sph-1-P release from activated platelets. Although it is believed that the source of plasma Sph-1-P is platelets, not much is known about Sph-1-P–related metabolism in the blood vessel. In this study, we investigated in detail Sph-1-P–related metabolism in the blood vessel, mainly using radio-labeled Sph and Sph-1-P.

MATERIALS AND METHODS

Materials—[^{32}P]Sph-1-P was prepared by the [γ-^{32}P]ATP–dependent phosphorylation of Sph catalyzed by Sph kinase obtained from human platelets (*22*).

The following materials were obtained from the indicated suppliers: Sph-1-P (Biomol, Plymouth Meeting, PA); Sph, ceramide, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), and bovine serum albumin (essentially fatty acid–free) (Sigma, St. Louis, MO); collagen (Hormon-Chemie, Munich, Germany); the thrombin receptor activating peptide NH_2-Ser-Phe-Leu-Leu-Arg-Asn-COOH (TRAP) (Bachem Ag, Bubendorf, Switzerland); thrombin (Mochida Pharmaceutical Co., Tokyo, Japan); [3-^{3}H]Sph (20 Ci/mmol) and [3-^{3}H]Sph-1-P (20 Ci/mmol) (American Radiolabeled Chemicals, Inc., St. Louis, MO); [γ-^{32}P]ATP (3,000 Ci/mmol) and [^{14}C]sphingomyelin (40–60 mCi/mmol) (NEN™ Life Science Products, Inc., Boston, MA).

Cell and Plasma Preparation—The blood of healthy adult volunteers was drawn and anticoagulated with 3.8% sodium citrate (9 volumes of blood to 1 volume of sodium citrate). To obtain platelet-rich plasma (PRP), this whole blood sample was centrifuged at 160 × *g* for 10 min. When indicated, PRP was then centrifuged at 2,000 × *g* for 10 min to obtain platelet-poor plasma (PPP). Washed platelets (without plasma components) (*7*) and human umbilical vein ECs (HUVECs) (*10*) were prepared as described previously. All cell and PPP reactions were performed at 37°C.

Metabolism of Radiolabeled Sphingolipids—The PRP or PPP samples were incubated with 12 nM [^{3}H]Sph or [^{3}H]Sph-1-P. When indicated, PRP challenged with 5 μg/ml collagen was incubated with 12 nM [^{3}H]Sph, and then PPP was obtained by centrifugation at 12,000 × *g* for 1 min. Whole blood samples were incubated at 37°C with 1 μM [^{32}P]Sph-1-P. HUVECs were incubated with 1% FCS containing 1 μM [^{32}P]Sph-1-P, [3-^{3}H]Sph-1-P, or [3-^{3}H]Sph. The indicated reactions were terminated by the addition of ice-cold chloroform/methanol/concentrated HCl (100:200:1), and the lower chloroform phases were dried and analyzed for radiolabeled lipids by thin-layer chromatography (TLC) autoradiography as previously described (*7*). The TLC plates (silica gel 60 HPTLC plates; Merck, Darmstadt, Germany) were developed in butanol/acetic acid/water (3:1:1), and autoradiography was performed with Konica Medical Film (Konica, Tokyo, Japan) at –80°C. When ^{3}H-labeled lipids were analyzed, enhancer (EN^{3}HANCE Spray, NEN™ Life Science Products) treatment of the TLC plates was performed. Each autoradiogram shown is typical of at least three experiments. When indicated, the radioactivity of excised gel fractions was counted by liquid scintillation counting.

Gel-Filtration Chromatography of [^{3}H]Sph-1-P/PPP Mixtures—[^{3}H]Sph-1-P was incubated for 1 h with PPP as described above, and the [^{3}H]Sph-1-P/PPP mixture was fractionated in a Sephadex G25M (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) gel-filtration column equilibrated and run in phosphate-buffered saline. Fractions (0.5 ml) were collected and assayed for [^{3}H]Sph-1-P content and the protein concentration, which were determined by liquid scintillation counting and dye binding (*23*), respectively.

Agarose Gel Electrophoresis—The agarose film Titan Gel Lipokit J3045 (Helena Laboratories, Saitama, Japan) was used for agarose gel electrophoresis. The separation of lipoproteins was performed as described in the product insert. Briefly, 1 μl of plasma or serum sample was applied on each lane and electrophoresed for 1 h at 90 V. After electrophoresis, the positions of lipoproteins and albumin were confirmed with a Titan Cholesterol Supply Kit (Helena Laboratories) and ponceau, respectively. Non-stained gel samples were fractionated and analyzed for radiolabeled lipids by liquid scintillation counting.

Polyacrylamide Gel Electrophoresis—Lipoprint HDL System (Quantimetrix, Redondo Beach, CA) was used for high-resolution 8.5% polyacrylamide gel electrophoresis. High-density lipoprotein (HDL) fractionation was performed as described in the product insert. Briefly, 25 μl of plasma or serum sample was mixed with 300 μl of liquid loading gel, which contained Sudan Black B dye to stain lipoproteins. The mixture was applied to the top of precast 3% polyacrylamide gel tubes. After photopolymerization at room temperature for 30 min, samples were electrophoresed for 1 h (3 mA/gel tube). The gel samples were fractionated and analyzed for radiolabeled lipids by liquid scintillation counting.

Measurement of Sph-1-P Release from Washed Platelets in the Presence of Low-Density Lipoprotein (LDL), HDL, or Albumin—LDL, HDL, and albumin were prepared from pooled serum samples by affinity columns.

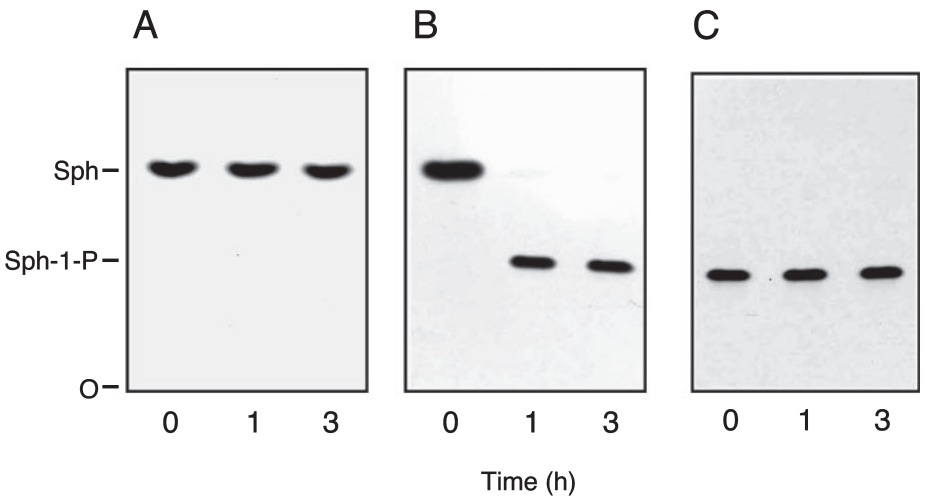

Fig. 1. **Metabolism of [^{3}H]Sph and [^{3}H]Sph-1-P in PPP or PRP.** (A) PPP was incubated with [^{3}H]Sph for 0, 1, or 3 h. (B) PRP challenged with collagen was incubated with [^{3}H]Sph for 0, 1, or 3 h, and PPP was obtained by centrifugation. (C) PPP was incubated with [^{3}H]Sph-1-P for 0, 1, or 3 h. The lipids in these PPP samples were extracted and analyzed by TLC autoradiography. The locations of the standard lipids are indicated. O, origin.

Briefly, LDL was obtained by eluting the bound fraction of a HiTrap Heparin HP column (Amersham Biosciences, Tokyo, Japan). The heparin-unbound fraction was applied to an immunoaffinity gel mixture of anti apo B-100 and apo A-I antibodies coupled to Sepharose 4B (*24*) (Japan Immunoresearch Laboratories Co., Ltd., Gunma, Japan); HDL and albumin fractions were prepared as bound and unbound fractions, respectively. LDL, HDL, and albumin were concentrated in a MiniPlus concentrator (Millipore, Billerica, MA), and their concentrations were measured by commercial kits, *i.e.*, Cholestest LDL (Daiichi Pure Chemicals CO., Ltd, Tokyo, Japan), Determiner HDL-C (Kyowa Medex Co., Ltd, Tokyo, Japan), and Clinimate ALB (Daiichi Pure Chemicals CO., Ltd, Tokyo, Japan) and Micro Albumin-HA (Wako Pure Chemicals Industries, Ltd, Osaka, Japan), respectively.

Washed platelets were adjusted to a cell density of 1×10^8/ml and incubated with 10 mg/dl of LDL, 5.3 mg/dl of HDL, or 500 mg/dl of albumin. These concentrations were selected as about one tenth of their physiological plasma concentrations. Sph-1-P release from these washed platelets was evaluated by assaying [^{3}H]Sph-1-P release from platelets labeled with [^{3}H]Sph as described previously (*7*). The percentage Sph-1-P release into the medium was calculated as 100 × ([^{3}H]Sph-1-P in medium)/(total [^{3}H]Sph-1-P in medium plus cell pellets), and stimulus-dependent Sph-1-P release as (percentage Sph-1-P release into the medium upon stimulation) – (percentage Sph-1-P release into the medium without stimulation). The data were expressed as arbitrary units; Sph-1-P release from platelets stimulated with TRAP for 15 min in the presence of albumin was designated as 100.

RNA Isolation and RT-PCR—Total RNA was prepared from HUVECs with a total RNA isolation system (Isogen, Wako Pure Chemical Industries, Ltd., Osaka, Japan), and the isolation of mRNA was performed with Oligotex™-dT30<Super> (Takara Biomedicals, Tokyo, Japan) according to the manufacturer's instructions. The isolated mRNA was reverse transcribed using a SuperScript™ Preamplification System (Gibco BRL, Life Technologies, Inc., Rockville, MD, USA). Reverse transcribed cDNA was amplified in a Perkin-Elmer 9600R thermal cycler (The Perkin-Elmer Corp., Norwalk, CT) using Takara Taq™ (rTaq DNA polymerase) (Takara Biomedicals).

Oligonucleotide primer pairs used for lipid phosphate phosphatase (LPP)-1 (PAP-2a), LPP-3 (PAP-2b), and LPP-2 (PAP-2c) were:5′-GTACGTGGCCCTCGATGT-3′ (sense) and 5′-TGGTGATTGCTCGGATAGTG-3′ (antisense) for LPP-1 (GenBank AB000888); 5′-GCAAAACTACAAGTACGACAAAGC-3′ (sense) and 5′-TGTCCACAGGTGAAAGGATTT-3′ (antisense) for LPP-3 (GenBank AB000889); 5′-CTCGACGTGCTGTGCTTACT-3′ (sense) and 5′-GTGCGGGTATCCATAGTGGT-3′ (antisense) for LPP-2 (GenBank AF056083).

Amplification was conducted with 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. Absence of contaminating DNA was confirmed by control reactions with RNA that had not been reverse transcribed. PCR prod-

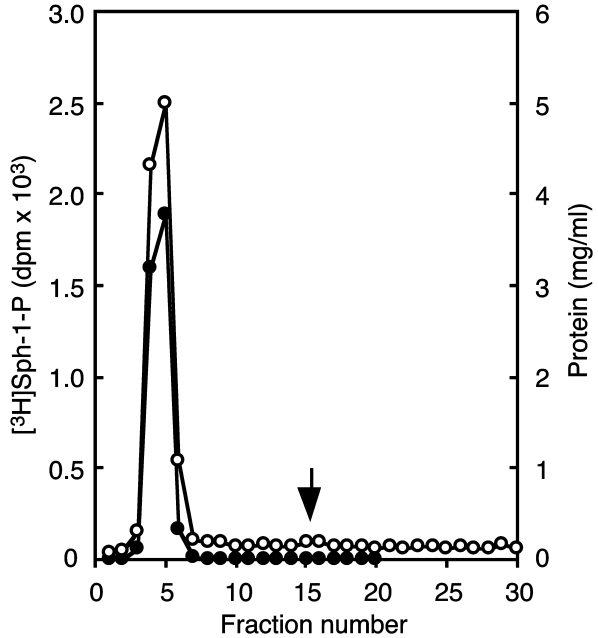

Fig. 2. **The absence of free [^{3}H]Sph-1-P in PPP**. PPP was incubated with [^{3}H]Sph-1-P, and the mixture was fractionated in a gel-filtration column. The fractions were collected and assayed for [^{3}H]Sph-1-P radioactivity (open circles) and protein concentration (closed circles). The arrow indicates the location of free [^{3}H]Sph-1-P (in the absence of protein).

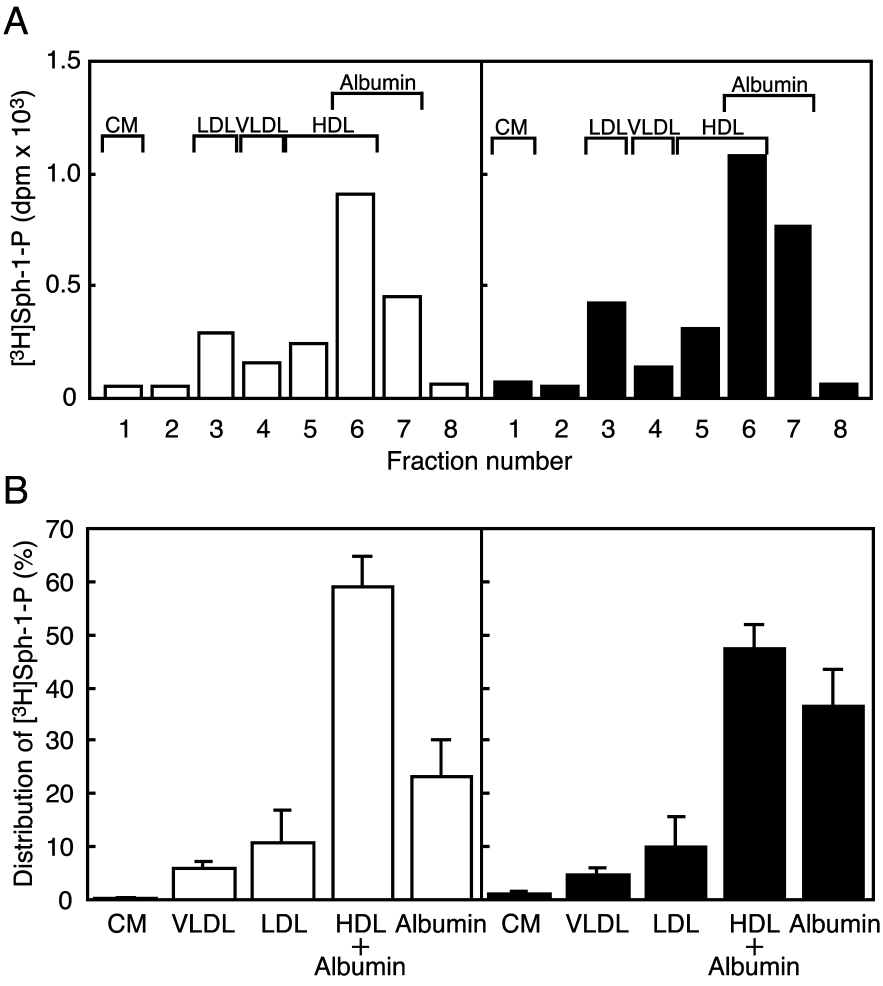

Fig. 3. **Distribution of [³H]Sph-1-P radioactivities in the plasma as analyzed by agarose gel electrophoresis.** A: (Left Panel) PPP was incubated with [³H]Sph-1-P for 1 h (open columns). (Right Panel) PRP challenged with collagen was incubated with [³H]Sph for 1 h, and PPP was obtained by centrifugation (solid columns). The PPP samples were subjected to agarose gel electrophoresis, and [³H]Sph-1-P radioactivities in the fractionated samples were measured by liquid scintillation counting. B: The percent distribution of the [³H]Sph-1-P radioactivity in each protein fraction was calculated. CM, chylomicron; VLDL, very low-density lipoprotein.

ucts (5 μl) were resolved by electrophoresis on a 2% agarose gel in TBE buffer (90 mM Tris-Borate, 2 mM EDTA, pH 8.3) and stained with ethidium bromide. The PCR products were cut from gels, solubilized, and sequenced with Dye Terminator Cycle Sequencing FS Ready Reaction Kits (The Perkin-Elmer Corp.), and analyzed using an ABI PRISM 310 Genetic Analyzer (The Perkin-Elmer Corp.) according to the supplied protocols (data not shown).

Data Presentation and Statistics—The data (other than those for TLC autoradiography) are presented as the means ± SD or representative of at least 3 separate experiments. When indicated, the statistical significance of the difference between the two groups was determined by means of Student's t test. $P < 0.05$ was considered significant.

RESULTS

Metabolism of Sph and Sph-1-P in the Plasma—We first checked the metabolic fate of Sph, the substrate of Sph kinase for the production of Sph-1-P, in the plasma. [³H]Sph was metabolically stable in plasma and remained unchanged for at least 3 h (Fig. 1A). However, [³H]Sph was converted into [³H]Sph-1-P in PRP (Fig. 1B), possibly due to [³H]Sph incorporation into platelets and the subsequent conversion to [³H]Sph-1-P and its release (*5*, *7*). [³H]Sph-1-P added to plasma was metabolically stable and remained unchanged for at least 3 h. These results confirm the importance of platelets in the blood as the converter of Sph to Sph-1-P.

Distribution of Sph-1-P in the Plasma—When a mixture of [³H]Sph-1-P and PPP was fractionated in a gel-filtration column, entire Sph-1-P co-eluted with the protein fractions (Fig. 2). To examine further the distribution of Sph-1-P in the protein fractions, the mixture of [³H]Sph-1-P and PPP was fractionated by agarose gel electrophoresis. As expected from previous reports (*25*, *26*), [³H]Sph-1-P seemed to bind mainly to HDL and albumin fractions (Fig. 3, left panels). When the distribution of [³H]Sph-1-P, which was converted from [³H]Sph in and released from collagen-stimulated platelets, was examined, a similar result was obtained except that the percentage of [³H]Sph-1-P bound to albumin was higher (Fig. 3, right panels).

Since the separation of HDL and albumin was not sufficient by agarose gel electrophoresis, we next employed polyacrylamide gel electrophoresis to fractionate the plasma samples. By this method, which enabled complete separation of HDL and albumin, it was found that 7.2 ± 3.8%, 53.3 ± 6.4%, and 39.5 ± 7.9% of the radioactivity of [³H]Sph-1-P added to plasma was recovered in the LDL, HDL, and albumin fractions, respectively (Fig. 4, left panels). When the distribution of [³H]Sph-1-P, which was

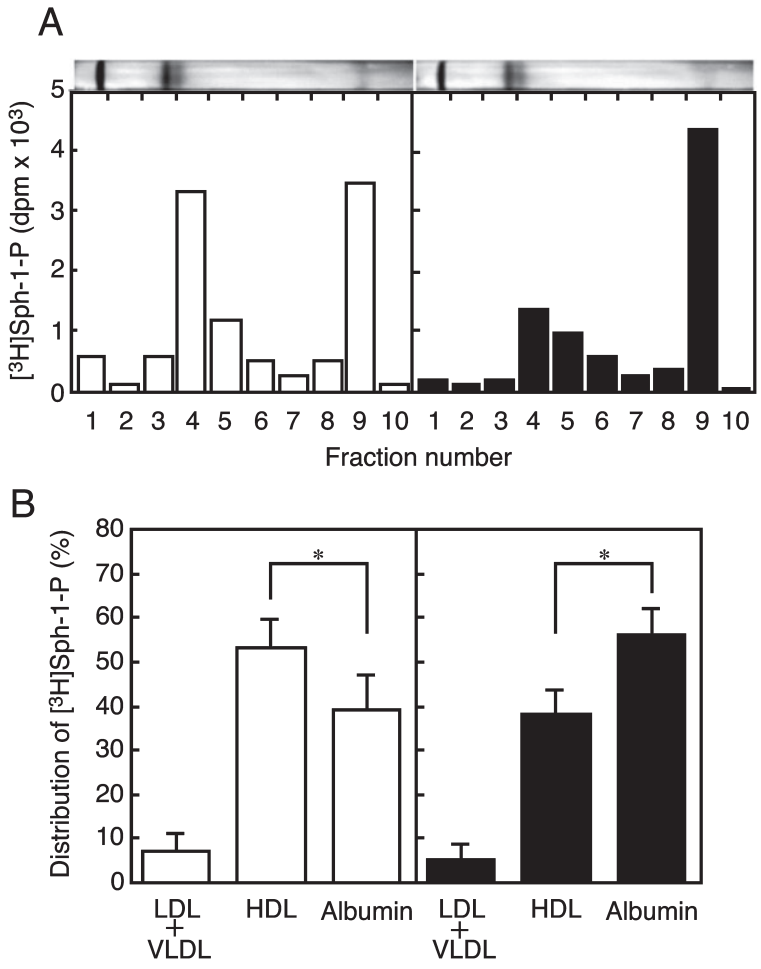

Fig. 4. **Distribution of [³H]Sph-1-P radioactivities in the plasma as analyzed by polyacrylamide gel electrophoresis.** A: (Left Panel) PPP was incubated with [³H]Sph-1-P for 1 h (open columns). (Right Panel) PRP challenged with collagen was incubated with [³H]Sph for 1 h, and PPP was obtained by centrifugation (solid columns). The PPP samples were subjected to polyacrylamide gel electrophoresis, and [³H]Sph-1-P radioactivities in the fractionated samples were measured by liquid scintillation counting. A typical electrophoretogram is shown at the top. B: The percent distribution of the [³H]Sph-1-P radioactivity in each protein fraction was calculated. *The difference is statistically significant.

converted from [³H]Sph in and released from collagen-stimulated platelets, was examined, however, 5.2 ± 3.2%, 38.4 ± 5.5%, and 56.3 ± 5.7% of the radioactivity was recovered in the LDL, HDL, and albumin fractions, respectively (Fig. 4, right panels).

Release of Sph-1-P from Activated Platelets—Previously, we reported that platelet Sph-1-P can be released extracellularly upon stimulation, which may be mediated by protein kinase C activation (*5*, *7*). Since the distribution pattern of [³H]Sph-1-P converted from [³H]Sph in and released from activated platelets was different from that obtained in the mere mixture of plasma and [³H]Sph-1-P, we next examined Sph-1-P release from platelets in the presence of albumin and lipoproteins. As reported previously for BSA (*25*), the extracellular release of Sph-1-P into the medium was much higher in the presence of extracellular human albumin (Fig. 5). In the presence of HDL or LDL, the release was significantly weaker (Fig. 5). We assume that albumin extracts Sph-1-P from platelets more efficiently than lipoproteins.

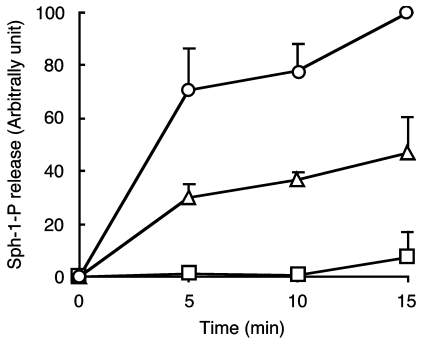

Fig. 5. **Sph-1-P release from platelets.** [³H]Sph-1-P release from washed platelets loaded with [³H]Sph and stimulated with 100 μg/ml of TRAP for the indicated durations was examined in the presence of albumin (circles), HDL (triangles), or LDL (squares).

Metabolism of Sph-1-P in the Presence of HUVECs or in Whole Blood—As described above, Sph-1-P was metabolically stable in the plasma (Fig. 1C). In an *in vivo* situation, however, plasma Sph-1-P can always interact with endothelial cells and blood cells. Accordingly, we examined the metabolism of radiolabeled Sph-1-P in the presence of these cells. [³²P]Sph-1-P underwent extensive degradation when added extracellularly to HUVECs (Fig. 6, upper panels). The radioactivity of [³²P]Sph-1-P must disappear when [³²P]P_i is removed from [³²P]Sph-1-P by an ectophosphatase; [³²P]P_i was recovered in the extracellular medium (data not shown). The radioactivity of [3-³H]Sph-1-P, treated similarly, also decreased very rapidly, with [³H]Sph, [³H]ceramide, and [³H]sphingomyelin emerging inside the cells (Fig. 6, middle panels). These results can be best explained by the incorporation of nonpolar [3-³H]Sph, formed from polar [3-³H]Sph-1-P by an ectophosphatase activity, into HUVECs and its conver-

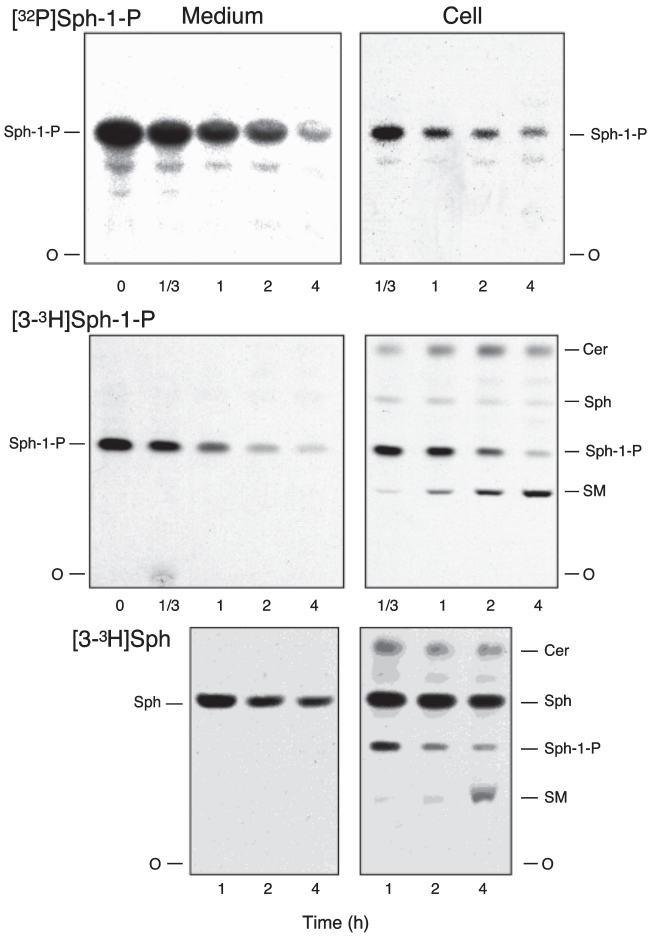

Fig. 6. **Metabolic fates of [32P]Sph-1-P, [3-3H]Sph-1-P, or [3-3H]Sph added exogenously to HUVECs.** HUVECs were cultured in the presence of [32P]Sph-1-P (upper), [3-3H]Sph-1-P (middle), or [3-3H]Sph (lower) for the indicated durations. Lipids were then extracted from the media (left) or cells (right), and analyzed for the formation of [3H]sphingolipids by TLC autoradiography. Locations of standard lipids are indicated. Cer, ceramide; SM, sphingomyelin.

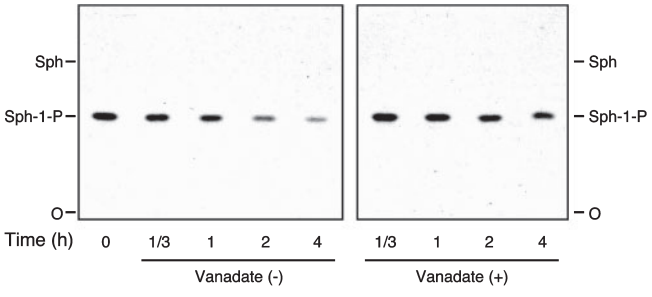

Fig. 7. **Effects of vanadate on the metabolism of [3H]Sph-1-P added to HUVECs.** HUVECs were incubated with [3H]Sph-1-P in the absence (left) or presence (right) of 1 mM vanadate for the indicated durations. Lipids were then extracted from the media, and analyzed for the metabolism of [3H]Sph-1-P by TLC autoradiography. Locations of standard lipids are indicated.

sion to [3H]ceramide (and then to [3H]sphingomyelin); Sph (but not Sph-1-P) is hydrophobic and easily passes the lipid bilayer. In fact, [3-3H]Sph, added exogenously, was rapidly incorporated into HUVECs and converted to

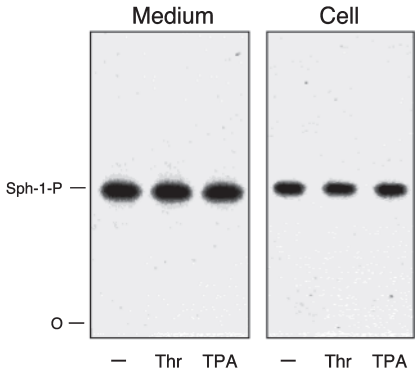

Fig. 8. **Effects of thrombin or TPA on the metabolism of [32P]Sph-1-P added to HUVECs.** HUVECs were incubated with [32P]Sph-1-P for 20 min under simultaneous stimulation with 0.1 U/ml of thrombin (Thr), 1 μM TPA, or without stimulation (–). Lipids were then extracted from the media (left) or cells (right), and analyzed for the metabolism of [32P]Sph-1-P by TLC autoradiography.

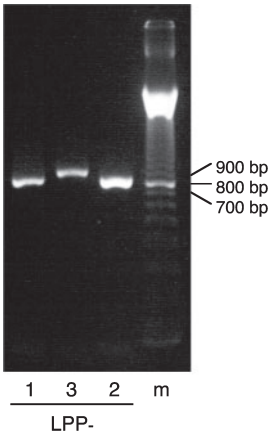

Fig. 9. **RT-PCR detection of the expression of the mRNAs for LPP-1, 2, and 3 in HUVECs.** Amplified products for LPP-1 (1), 3 (3), and 2 (2) were resolved in a 2% agarose gel. Lane m contained the size standards [base pairs (bp)] as indicated. The specific amplified products for LPP-1, 3, and 2 are 825 bp, 899 bp, and 828 bp, respectively.

[3-3H]ceramide (and then to [3H]sphingomyelin) (Fig. 6, lower panels). [3-3H]Sph was also converted to [3-3H]Sph-1-P, but transiently (Fig. 6, lower panels). Furthermore, the Sph-1-P degradation was strongly inhibited in the presence of the phosphatase inhibitor vanadate (Fig. 7), confirming the involvement of an ectophosphatase activity.

We also checked the effect of thrombin and TPA on Sph-1-P ectophosphatase activity. Dephosphorylation of [32P]Sph-1-P (exogenously added) was not affected by treating HUVECs with any of these stimuli (Fig. 8). In fact, there have been no reports that ectophosphatase activity toward Sph-1-P is affected by cell activation.

Several isoenzymes of mammalian LPPs have been cloned, and they are believed to act at the outer leaflet of the cell surface bilayer and can account for the ecto-lipid phosphate phosphatase activities (including that for Sph-1-P) previously described (*27–29*). Accordingly, we employed RT-PCR to check the expression of LPPs, which may be responsible for Sph-1-P degradation. RNA from

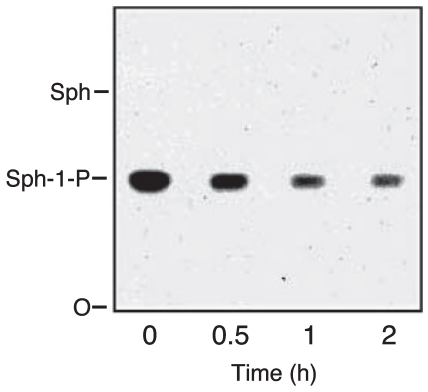

Fig. 10. **Metabolic fates of [^{32}P]Sph-1-P added exogenously to whole blood.** Whole blood samples were incubated with [^{32}P]Sph-1-P for the indicated durations. The lipids were then extracted and analyzed for the metabolism of [^{32}P]Sph-1-P by TLC autoradiography.

HUVECs was prepared and reverse transcribed, and then specific transcripts were amplified by PCR. As demonstrated in Fig. 9, HUVECs were found to express mRNA transcripts for LPP-1, 2, and 3; the sequences of these PCR products were confirmed (data not shown). Of these LPPs, LPP-1 and -3 have been shown to utilize Sph-1-P as a good substrate (*27*, *28*).

Finally, Sph-1-P metabolism in whole blood was examined. As expected from the fact that blood cells express LPPs, [^{3}H]Sph-1-P underwent rapid degradation when added to whole blood samples (Fig. 10).

DISCUSSION

Sph-1-P Metabolism in the Blood—Both Sph-1-P and its precursor Sph are metabolically stable in plasma, while platelets convert Sph to Sph-1-P, a finding consistent with the idea that platelets are the main source for Sph-1-P in the blood (*5*, *21*). However, the possibility cannot be ruled out that Sph-1-P is released from other cell types or formed by unknown mechanism(s). In this context, it was demonstrated that a small but significant fraction of Sph kinase is secreted extracellularly from HUVECs (*30*). This secretion has been reported to be constitutive and independent of cell stimulation (*30*). It was also shown that the secreted Sph kinase is enzymatically active and produces Sph-1-P; extracellular Sph seems to be a rate-limiting factor (*30*). However, Sph-1-P was not formed in plasma incubated with Sph in the present study, and hence the significance of this route of Sph-1-P formation in the blood stream remains to be determined. A limitation of our present study is that pathways for Sph-1-P formation other than Sph kinase were not detected. Hydrolysis of sphingosylphosphorylcholine to Sph-1-P or deacylation of ceramide 1-phosphate to Sph-1-P is also theoretically possible. However, the significance of these pathways for Sph-1-P production *in vivo* remains to be clarified.

Sph-1-P Carriers in the Plasma—Sph-1-P in the plasma, which is metabolically stable, was first thought to be bound to albumin (*25*). It was recently reported by Okajima *et al*., however, that when expressed as the per unit amount of protein, Sph-1-P is concentrated in lipoprotein fractions with a rank order of HDL > LDL > very low-density lipoprotein > lipoprotein-deficient plasma (mainly albumin), among plasma and serum components (*26*). Our present study analyzing the mixture of radiolabeled Sph-1-P and plasma is consistent with previous data based on separation by density gradient centrifugation of plasma samples, followed by the measurement of Sph-1-P in each fraction by a radioreceptor assay (*26*). Although the affinity of Sph-1-P for albumin is much lower than for HDL, about 40% of Sph-1-P was bound to albumin because its plasma levels are very high. The interaction of Sph-1-P with lipoproteins was reported to reduce its bioactivity and active concentration (*26*). The authors speculated that this might be an important protection mechanism from full activation of S1P receptors; Sph-1-P concentrations in plasma and serum are much higher than the Sph-1-P K_d values for its receptors. On the other hand, albumin does not affect Sph-1-P bioactivities (*25*). Furthermore, it was also suggested that Sph-1-P, by binding to lipoproteins, may be protected from degradation by ectoenzymes such as LPPs or by uptake into the cells; the half-life of HDL-associated Sph-1-P is reportedly about four times longer than that of Sph-1-P in the presence of 0.1% albumin (but not HDL) when examined in HUVECs (*8*). Accordingly, it was postulated that compared with free Sph-1-P (without HDL), HDL-associated Sph-1-P may exert a much weaker response in short-term reactions (such as intracellular Ca^{2+} mobilization) but a sustained response in long-term reactions (such as survival and proliferation) (*8*, *26*). Several lines of evidence have shown that the HDL-induced cytoprotective actions are mediated by the activation of $S1P_1$ and $S1P_3$ receptors by Sph-1-P, and it seems likely that the anti-atherogenic characteristics of HDL can be ascribed at least in part to Sph-1-P associated with it (*8*, *31*, *32*). Accordingly, whether Sph-1-P is bound to albumin or HDL is a very important matter; the concentrations of HDL and albumin may affect the bioactivity of Sph-1-P *in vivo*.

Another important finding in this study is that Sph-1-P released from activated platelets is bound more efficiently to albumin than HDL, at least immediately after its release. In support of this, Sph-1-P extraction from activated platelets is enhanced in the presence of albumin, as suggested in a previous study (*25*). This is closely related to the mechanism by which activated platelets release Sph-1-P, which is mostly unknown except for the importance of the involvement of protein kinase C (*7*, *25*). This must require the function of specific transporter(s) since Sph-1-P possesses the polar nature of the head group. Albumin may extract Sph-1-P stored in platelets when the Sph-1-P location inside cells shifts upon protein kinase C activation. Since Sph-1-P stored in platelets becomes susceptible to depletion by albumin when platelet protein kinase C is activated, it may be possible to consider that the route of Sph-1-P externalization is enhanced by transbilayer movement across the plasma membrane, as a result of modifications in the physical properties of the membrane (accompanying protein kinase C activation). It has been demonstrated that the ABC transporters may transport phospholipids (*33*, *34*). ABCA7, which has been reported to be expressed preferentially in platelets and suggested to play an important

role there (*35*), might be responsible for Sph-1-P release, but this remains to be determined. Since the Sph-1-P distribution pattern *in vivo* differs somewhat from the pattern obtained for Sph-1-P extracted from platelets, there must be an unknown mechanism that shifts Sph-1-P from albumin to HDL. We checked for the conversion of the albumin-bound Sph-1-P radioactivity (extracted from platelets) to other fractions; however, we could not detect any significant shift in Sph-1-P radioactivity, at least within 3 hours (data not shown). It is important to address this problem in the future.

Regulation of Plasma Sph-1-P Levels, Possibly by LPPs—Sph-1-P is stable when incubated with plasma or serum, indicating a negligible enzymatic activity for Sph-1-P degradation there. However, when the fate of radioactive Sph-1-P was analyzed in the presence of HUVECs or in whole blood, this bioactive lipid was found to undergo marked degradation. Instead, radioactive Sph, ceramide, and sphingomyelin were formed in the cells. Probably, non-polar Sph, formed from polar Sph-1-P by ectophosphatase activity, is incorporated into ECs and then converted to ceramide (and then to sphingomyelin) intracellularly; Sph (but not Sph-1-P) is hydrophobic and easily passes through the lipid bilayer.

Recently, several isoenzymes of mammalian LPP, that is, type 2 phosphatidic acid phosphatase, have been cloned, and they are believed to act at the outer leaflet of the cell surface bilayer, accounting for the ecto-lipid phosphate phosphatase activities (toward Sph-1-P, lysophosphatidic acid, or phosphatidic acid) previously described (*27–29*). In fact, HUVECs were found to express mRNA transcripts for LPP-1, 2, and 3. Sph-1-P degradation on the surface of these vascular cells may be important when the *in vivo* effects of Sph-1-P are evaluated. Based on the previous results, K_ds for S1P receptors are much lower than the concentrations of Sph-1-P in the plasma; the K_d of S1P for these receptors is within the range of 2 to 30 nM (*1–4*). Probably, due to Sph-1-P dephosphorylation at the cell surface, the concentrations of Sph-1-P interacting with S1P receptors may be lower than the plasma Sph-1-P levels. This is also consistent with a recent *in vitro* transfection study showing that LPPs may limit the bioactivity of Sph-1-P by regulating its concentration (interacting with S1P receptors) (*27–29*, *36*).

Concluding Remarks—The levels and distribution of plasma Sph-1-P *in vivo* may be dynamically regulated by various factors. Not only an enhancement of Sph-1-P release, but also the regulation of Sph-1-P degradation on the cell surface leads to an increase in the plasma Sph-1-P level. Furthermore, the ratios of the plasma concentrations of albumin and lipoproteins (especially HDL) seem very important when the bioavailability of Sph-1-P is concerned. In various atherosclerotic diseases where the plasma levels of lipids and lipoproteins are altered and endothelial functions are disturbed, modulation of the effects of Sph-1-P should occur, and this may be of pathophysiological importance.

The authors thank Dr. L. Yang for her help. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by the Mitsubishi Pharma Research Foundation.

REFERENCES

1. Spiegel, S. and Milstien, S. (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat. Rev. Mol. Cell. Biol.* **4**, 397–407
2. Hla, T. (2003) Signaling and biological actions of sphingosine 1-phosphate. *Pharmacol. Res.* **47**, 401–407
3. Kluk, M.J. and Hla, T. (2002) Signaling of sphingosine-1-phosphate via the S1P/EDG-family of G-protein-coupled receptors. *Biochim. Biophys. Acta* **1582**, 72–80
4. Takuwa, Y. (2002) Subtype-specific differential regulation of Rho family G proteins and cell migration by the Edg family sphingosine-1-phosphate receptors. *Biochim. Biophys. Acta* **1582**, 112–120
5. Yatomi, Y., Ozaki, Y., Ohmori, T., and Igarashi, Y. (2001) Sphingosine 1-phosphate: synthesis and release. *Prostaglandins Other Lipid Mediat.* **64**, 107–122
6. Lynch, K.R. (2002) Lysophospholipid receptor nomenclature. *Biochim. Biophys. Acta* **1582**, 70–71
7. Yatomi, Y., Yamamura, S., Ruan, F., and Igarashi, Y. (1997) Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *J. Biol. Chem.* **272**, 5291–5297
8. Kimura, T., Sato, K., Kuwabara, A., Tomura, H., Ishiwara, M., Kobayashi, I., Ui, M., and Okajima, F. (2001) Sphingosine 1-phosphate may be a major component of plasma lipoproteins responsible for the cytoprotective actions in human umbilical vein endothelial cells. *J. Biol. Chem.* **276**, 31780–31785
9. Lee, M.J., Thangada, S., Claffey, K.P., Ancellin, N., Liu, C.H., Kluk, M., Volpi, M., Sha'afi, R.I., and Hla, T. (1999) Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell* **99**, 301–312
10. Ohmori, T., Yatomi, Y., Okamoto, H., Miura, Y., Rile, G., Satoh, K., and Ozaki, Y. (2001) Gi-mediated Cas tyrosine phosphorylation in vascular endothelial cells stimulated with sphingosine 1-phosphate: possible involvement in cell motility enhancement in cooperation with Rho-mediated pathways. *J. Biol. Chem.* **276**, 5274–5280
11. Igarashi, J., Bernier, S.G., and Michel, T. (2001) Sphingosine 1-phosphate and activation of endothelial nitric-oxide synthase. differential regulation of Akt and MAP kinase pathways by EDG and bradykinin receptors in vascular endothelial cells. *J. Biol. Chem.* **276**, 12420–12426
12. Morales-Ruiz, M., Lee, M.J., Zollner, S., Gratton, J.P., Scotland, R., Shiojima, I., Walsh, K., Hla, T., and Sessa, W.C. (2001) Sphingosine 1-phosphate activates Akt, nitric oxide production, and chemotaxis through a Gi protein/phosphoinositide 3-kinase pathway in endothelial cells. *J. Biol. Chem.* **276**, 19672–19677
13. Kluk, M.J. and Hla, T. (2001) Role of the sphingosine 1-phosphate receptor EDG-1 in vascular smooth muscle cell proliferation and migration. *Circ. Res.* **89**, 496–502
14. Ryu, Y., Takuwa, N., Sugimoto, N., Sakurada, S., Usui, S., Okamoto, H., Matsui, O., and Takuwa, Y. (2002) Sphingosine-1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. *Circ. Res.* **90**, 325–332
15. Sugimoto, N., Takuwa, N., Okamoto, H., Sakurada, S., and Takuwa, Y. (2003) Inhibitory and stimulatory regulation of Rac and cell motility by the G12/13-Rho and Gi pathways integrated downstream of a single G protein-coupled sphingosine-1-phosphate receptor isoform. *Mol. Cell. Biol.* **23**, 1534–1545
16. Sugiyama, A., Yatomi, Y., Ozaki, Y., and Hashimoto, K. (2000) Sphingosine 1-phosphate induces sinus tachycardia and coronary vasoconstriction in the canine heart. *Cardiovasc. Res.* **46**, 119–125
17. Ohmori, T., Yatomi, Y., Osada, M., Kazama, F., Takafuta, T., Ikeda, H., and Ozaki, Y. (2003) Sphingosine 1-phosphate induces contraction of coronary artery smooth muscle cells via S1P2. *Cardiovasc. Res.* **58**, 170–7
18. Ohmori, T., Yatomi, Y., Osada, M., and Ozaki, Y. (2004) Platelet-derived sphingosine 1-phosphate induces contraction of

coronary artery smooth muscle cells via S1P2. *J. Thromb. Haemost.* **2**, 203–5
19. Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C.X., Hobson, J.P., Rosenfeldt, H.M., Nava, V.E., Chae, S.S., Lee, M.J., Liu, C.H., Hla, T., Spiegel, S., and Proia, R.L. (2000) Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J. Clin. Invest.* **106**, 951–961
20. Allende, M.L., Yamashita, T., and Proia, R.L. (2003) G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation. *Blood* **102**, 3665–3667
21. Yatomi, Y., Igarashi, Y., Yang, L., Hisano, N., Qi, R., Asazuma, N., Satoh, K., Ozaki, Y., and Kume, S. (1997) Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J. Biochem.* **121**, 969–973
22. Yatomi, Y., Ruan, F., Megidish, T., Toyokuni, T., Hakomori, S., and Igarashi, Y. (1996) N, N-Dimethylsphingosine inhibition of sphingosine kinase and sphingosine 1-phosphate activity in human platelets. *Biochemistry* **35**, 626–633
23. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
24. Nakajima, K., Saito, T., Tamura, A., Suzuki, M., Nakano, T., Adachi, M., Tanaka, A., Tada, N., Nakamura, H., Campos, E. *et al.* (1993) Cholesterol in remnant-like lipoproteins in human serum using monoclonal anti apo B-100 and anti apo A-I immunoaffinity mixed gels. *Clin. Chim. Acta* **223**, 53–71
25. Yatomi, Y., Ohmori, T., Rile, G., Kazama, F., Okamoto, H., Sano, T., Satoh, K., Kume, S., Tigyi, G., Igarashi, Y., and Ozaki, Y. (2000) Sphingosine 1-phosphate as a major bioactive lysophospholipid that is released from platelets and interacts with endothelial cells. *Blood* **96**, 3431–3438
26. Murata, N., Sato, K., Kon, J., Tomura, H., Yanagita, M., Kuwabara, A., Ui, M., and Okajima, F. (2000) Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. *Biochem. J.* **352**, 809–815
27. Brindley, D.N., English, D., Pilquil, C., Buri, K., and Ling, Z.C. (2002) Lipid phosphate phosphatases regulate signal transduction through glycerolipids and sphingolipids. *Biochim. Biophys. Acta* **1582**, 33–44
28. Kai, M., Wada, I., Imai, S., Sakane, F., and Kanoh, H. (1997) Cloning and characterization of two human isozymes of Mg^{2+}-independent phosphatidic acid phosphatase. *J. Biol. Chem.* **272**, 24572–24578
29. Sciorra, V.A. and Morris, A.J. (2002) Roles for lipid phosphate phosphatases in regulation of cellular signaling. *Biochim. Biophys. Acta* **1582**, 45–51
30. Ancellin, N., Colmont, C., Su, J., Li, Q., Mittereder, N., Chae, S.S., Stefansson, S., Liau, G., and Hla, T. (2002) Extracellular export of sphingosine kinase-1 enzyme. Sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. *J. Biol. Chem.* **277**, 6667–6675
31. Okajima, F. (2002) Plasma lipoproteins behave as carriers of extracellular sphingosine 1-phosphate: is this an atherogenic mediator or an anti-atherogenic mediator? *Biochim. Biophys. Acta* **1582**, 132–137
32. Kimura, T., Sato, K., Malchinkhuu, E., Tomura, H., Tamama, K., Kuwabara, A., Murakami, M., and Okajima, F. (2003) High-density lipoprotein stimulates endothelial cell migration and survival through sphingosine 1-phosphate and its receptors. *Arterioscler. Thromb. Vasc. Biol.* **23**, 1283–1288
33. Higgins, C.F. (1994) Flip-flop: the transmembrane translocation of lipids. *Cell* **79**, 393–395
34. Ruetz, S. and Gros, P. (1994) Phosphatidylcholine translocase: a physiological role for the mdr2 gene. *Cell* **77**, 1071–1081
35. Sasaki, M., Shoji, A., Kubo, Y., Nada, S., and Yamaguchi, A. (2003) Cloning of rat ABCA7 and its preferential expression in platelets. *Biochem. Biophys. Res. Commun.* **304**, 777–782
36. Pyne, S. and Pyne, N.J. (2002) Sphingosine 1-phosphate signalling and termination at lipid phosphate receptors. *Biochim. Biophys. Acta* **1582**, 121–131