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Rho-Mediated Phosphorylation of Focal Adhesion Kinase and Myosin Light Chain in Human Endothelial Cells Stimulated with Sphingosine 1-Phosphate, a Bioactive Lysophospholipid Released from Activated Platelets[1]

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Since sphingosine 1-phosphate (Sph-1-P) is stored in abundant amounts in blood platelets and released extracellularly upon stimulation, it is important to clarify the effects of this bioactive lysophospholipid on vascular endothelial cells from the viewpoint of platelet-endothelial cell interactions. In this study, we investigated the effects of Sph-1-P on the cytoskeletal remodeling of human umbilical vein endothelial cells (HUVECs). Of a focal adhesion kinase (FAK) family of non-receptor protein-tyrosine kinases, HUVECs were found to express FAK, but scarcely proline-rich tyrosine kinase 2. Sph-1-P induced FAK tyrosine phosphorylation, myosin light chain phosphorylation, and the formation of stress fibers in HUVECs. The specific Rho inactivator C3 transferase from *Clostridium botulinum* abolished all of these cytoskeletal responses induced by Sph-1-P, while pertussis toxin only partly inhibited FAK tyrosine phosphorylation, and hardly affected myosin light chain phosphorylation and stress fiber formation. In contrast, Sph-1-P–induced intracellular Ca^{2+} mobilization was suppressed by pertussis toxin, but not at all by C3 exoenzyme. Our results suggest that Sph-1-P, a bioactive lipid released from activated platelets, induces endothelial cell cytoskeletal reorganization, mainly through Rho-mediated signaling pathways.

Key words: focal adhesion kinase, myosin light chain, Rho, sphingosine 1-phosphate, vascular endothelial cells.

It is now established that sphingolipids, including ceramide, sphingosine (Sph), and sphingosine 1-phosphate (Sph-1-P), play important roles as signal-transducing mediators (*1–3*). To clarify the involvement of sphingolipids in vascular biology, we have studied the metabolism and functional effects of Sph derivatives in platelets (*3–6*). We found that, in human platelets, Sph-1-P is rapidly formed from Sph by Sph kinase, stored intracellularly in abundant amounts, and released into the extracellular environment upon stimulation (*4, 5*). A variety of vascular events, including hemostasis, thrombosis, and atherosclerosis, are considered to comprise an integrated group of multicellular events (*7*). In this context, it is important to study the effects of Sph-1-P on endothelial cells from the viewpoint of platelet-endothelial cell interaction; platelet Sph-1-P is released extracellularly upon stimulation. We previously found that Sph-1-P acts as a human umbilical vein endothelial cell (HUVEC) survival factor; this bioactive lipid was shown to protect HUVECs from apoptosis induced by the withdrawal of growth factors and to stimulate HUVEC DNA synthesis (*6*). Very recently, the induction of HUVEC adherens junction assembly and morphogenesis by Sph-1-P has been reported (*8*). In this study, we examined the signaling pathways elicited by Sph-1-P and leading to HUVEC cytoskeletal reorganization.

MATERIALS AND METHODS

Materials—Recombinant *Clostridium botulinum* C3 exoenzyme was prepared as described previously (*9*), and kindly donated by Dr. S. Narumiya (Department of Pharmacology, Kyoto University Faculty of Medicine). In some experiments, C3 exoenzyme obtained from Upstate Biotechnology (Lake Placid, NY) was used, with similar results. Rabbit anti–myosin light chain (MLC) polyclonal antibody and mouse anti–phospho-MLC monoclonal antibody (MoAb) were kindly donated by Dr. Y. Sasaki (Asahi Chemical Industry, Fuji, Shizuoka). The following materials were obtained from the indicated suppliers: Sph-1-P (Biomol, Plymouth Meeting, PA); Sph and tetramethyl rhodamine isothiocyanate (TRITC)–conjugated phalloidin (Sigma Chemical, Louis, MO); fetal calf serum (FCS) (ICN Biomedicals, Aurora, OH); recombinant human basic fibroblast growth factor (Becton Dickinson Labware, Lincoln Park, NJ); protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden); peroxidase-conjugated goat anti–mouse

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Abbreviations: Sph, sphingosine; Sph-1-P, sphingosine 1-phosphate; HUVEC, human umbilical vein endothelial cell, MLC, myosin light chain; MoAb, monoclonal antibody; TRITC, tetramethyl rhodamine isothiocyanate; FCS, fetal calf serum; FAK, focal adhesion kinase; Pyk2, proline-rich tyrosine kinase 2; edg, endothelial differentiation gene

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IgG (Cappel, Durham, NC); peroxidase-conjugated goat anti–rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA); pertussis toxin (islet-activating protein) (Kaken Pharmaceutical, Tokyo); anti–focal adhesion kinase (FAK) MoAb (2A7), anti–proline-rich tyrosine kinase 2 (Pyk2) polyclonal antibody, and anti–phosphotyrosine MoAb (4G10) (Upstate Biotechnology, Lake Placid, NY); anti-FAK MoAb (77), anti-Pyk2 MoAb, and anti-phosphotyrosine MoAb (PY20) (Transduction Laboratories, Lexington, KY); fura2-AM (Dojin Chemicals, Kumamoto).

Cell Culture—HUVECs were isolated from human umbilical cord by trypsin treatment and plated onto 0.2% gelatin–coated dishes. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 20% FCS, 10 ng/ml of basic fibroblast growth factor, penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml), at 37°C under an atmosphere of 5% CO_2 and 95% room air. Cells were used between the third and the sixth passages.

When indicated, HUVECs were pretreated with 2.5 μg/ml of C3 exoenzyme for 48 h or 10 ng/ml of pertussis toxin for 12 h. In some experiments with pertussis toxin, cells were serum-deprived during preincubation, with similar results. In all cases, cells were serum-deprived for 1 h before exposure to Sph-1-P.

Immunoprecipitation—Serum-starved confluent HUVECs were treated as indicated, and lysed with ice-cold lysis buffer [1% Triton X-100, 0.5% Nonidet P-40, 50 mM Tris/HCl (pH 7.2), 1 mM EGTA, 1 mM Na_3VO_4, 0.5 mM PMSF, and 50 μg/ml of leupeptin in final concentrations]. All subsequent immunoprecipitation steps were carried out at 4°C. The lysed cells were harvested by scraping and centrifuged at 15,000 ×*g* for 10 min. The supernatants were precleared with protein G-Sepharose. The resultant supernatants were incubated overnight with 4 μg of anti-FAK MoAb (2A7) or anti-Pyk2 polyclonal antibody, followed by the addition of protein G-Sepharose, and further incubated for 1 h. The immune complexes were recovered by centrifugation, washed with lysis buffer and heated for 10 min at 100°C in SDS-PAGE sample buffer.

Western Blotting—The proteins were resolved on 8 or 15% SDS-PAGE and then electrophoretically transferred to PVDF membranes. Membranes were blocked using 1% BSA in PBS. After washing the membranes with PBS containing 0.1% Tween-80, the immunoblots were incubated with anti-FAK MoAb (77) (0.25 μg/ml), anti-Pyk2 MoAb (0 25 μg/ml), or anti-phosphotyrosine MoAbs (1 μg/ml of PY20 plus 1 μg/ml of 4G10). Antibody binding was detected using peroxidase-conjugated goat anti–mouse IgG and visualized with ECL chemiluminescence reaction reagents (Amersham Buckinghamshire, UK).

When indicated, tyrosine phosphorylation of FAK was quantified using a PDI420oe scanner and Quantity one 2.5a software for Macintosh.

MLC Phosphorylation—MLC phosphorylation in HUVECs was analyzed by Western blotting using anti–phospho-MLC MoAb and anti-MLC polyclonal antibody, as previously described (*10*).

Phalloidin Staining of Actin Filaments—For actin staining, cells were fixed in 3% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 5 min, and incubated with 0.1 μg/ml of TRITC-conjugated phalloidin for 40 min (*11*). Then, cells were viewed under a BH2 microscope (Olympus, Tokyo), and photographed on Fujicolor super G100 film (Fujifilm, Tokyo).

$[Ca^{2+}]_i$ Measurement—$[Ca^{2+}]_i$ was measured using Ca^{2+}-sensitive fluorophore fura2. Confluent HUVECs were harvested by trypsinization, and the cell pellet was resuspended in buffer containing 138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1.0 mM $MgCl_2$, 1 mg/ml of glucose, and 20 mM HEPES (pH 7.4). The cells were then incubated with 3 μM fura2-AM. After 1 h at 37°C, the cells were washed twice, adjusted to 2 × 10^6/ml, and supplemented with 1 mM $CaCl_2$. Fluorescence measurements were made using a FS100 (Kowa, Tokyo). The $[Ca^{2+}]_i$ values were determined from the ratio of fura2 fluorescence intensity at 340 and 380 nm excitation (*12*).

Data Presentation—The results presented are representative of those from three or four independent experiments or as the mean ± SD of three or four data points.

RESULTS

Expression of FAK, but Not Pyk2, in HUVECs—Although FAK has been proposed to play a central role in integrin-stimulated signaling events, a number of other cellular stimuli, including agonists acting on G protein–linked receptors, can enhance FAK tyrosine phosphorylation, leading to cytoskeletal remodeling (*13*). Recently, Pyk2, also known as related adhesion focal tyrosine kinase, cell adhesion kinase β, or calcium-dependent protein-tyrosine kinase, has been identified as a member of the FAK family protein-tyrosine kinases (*14*, *15*). In this study, we first examined the expression of FAK and Pyk2 in HUVECs. As reported (*16*), we confirmed FAK expression in HUVECs by specific anti-FAK MoAb (Fig. 1A). Under the same experimental conditions, however, Pyk2 was not detected (Fig. 1B). In platelets, which were used for comparison, not only FAK but also Pyk2 was detected (Fig. 1); Pyk2 is known to be highly expressed in cells of hematopoietic lineage (and the central nervous system) (*15*).

Sph-1-P–Induced HUVEC Cytoskeletal Reorganization and its Inhibition by C3 Exoenzyme—It is now established that the extracellular mediator activities of lysophospholipids are transduced by subfamilies of G protein–coupled receptors, of which the most completely characterized are those encoded by the endothelial differentiation genes (edgs) (*17*). Furthermore, HUVECs have been shown to express the Sph-1-P receptors Edgs-1 and 3 (*8*). We next

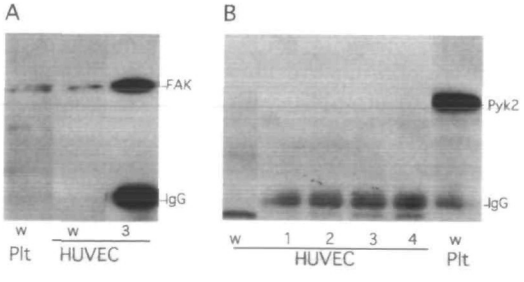

Fig 1. **Expression of FAK, but not Pyk2, in HUVECs.** HUVEC lysates were immunoprecipitated with anti-FAK MoAb (lane 3 in A) or anti-Pyk2 MoAb (lanes 1–4 in B), resolved on 8% SDS-PAGE, and then Western-blotted with anti-FAK MoAb (A) or anti-Pyk2 MoAb (B), respectively The cell numbers lysed were 1 × 10^6 (1), 2 × 10^6 (2), 3 × 10^6 (3), and 4 × 10^6 (4). Whole cell lysates (w) from HUVECs or platelets (Plt) were also analyzed.

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examined whether Sph-1-P stimulates FAK tyrosine phosphorylation, which was determined in HUVEC lysates by anti-FAK immunoprecipitation followed by Western blotting with anti-phosphotyrosine antibodies (Fig. 2). Sph-1-P induced a rapid and marked increase in FAK tyrosine phosphorylation within 5 min after stimulation (Fig. 2A). Sph-1-P–induced FAK tyrosine phosphorylation was con-

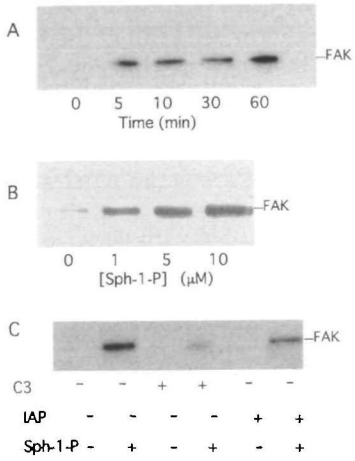

Fig 2. **Sph-1-P–induced FAK tyrosine phosphorylation in HUVECs and its inhibition by C3 exoenzyme.** Serum-starved HUVECs were stimulated with 10 μM Sph-1-P for the indicated durations (A) or with the indicated concentrations of Sph-1-P for 10 min (B) The cell lysates were immunoprecipitated with anti-FAK MoAb, and then Western-blotted with anti-phosphotyrosine MoAb. In (C), HUVECs pretreated without (–) or with C3 exoenzyme (C3) or pertussis toxin (IAP) were stimulated without (–) or with 10 μM Sph-1-P for 10 min, and then FAK tyrosine phosphorylation was analyzed.

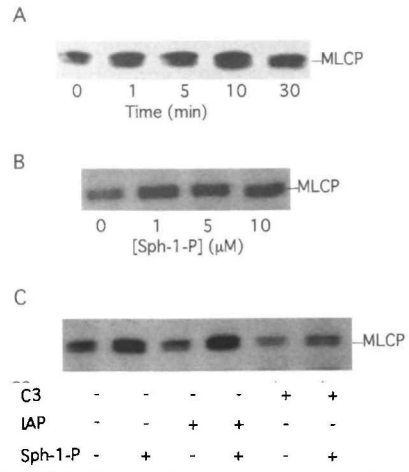

Fig. 3 **Sph-1-P–induced MLC phosphorylation in HUVECs and its inhibition by C3 exoenzyme.** Serum-starved HUVECs were stimulated with 10 μM Sph-1-P for the indicated durations (A) or with the indicated concentrations of Sph-1-P for 10 min (B) The cell lysates were resolved on 15% SDS-PAGE, and Western-blotted with anti–phospho-MLC (MLCP) MoAb. In (C), HUVECs pretreated without (–) or with C3 exoenzyme (C3) or pertussis toxin (IAP) were stimulated without (–) or with 10 μM Sph-1-P for 10 min, and then MLC phosphorylation was analyzed

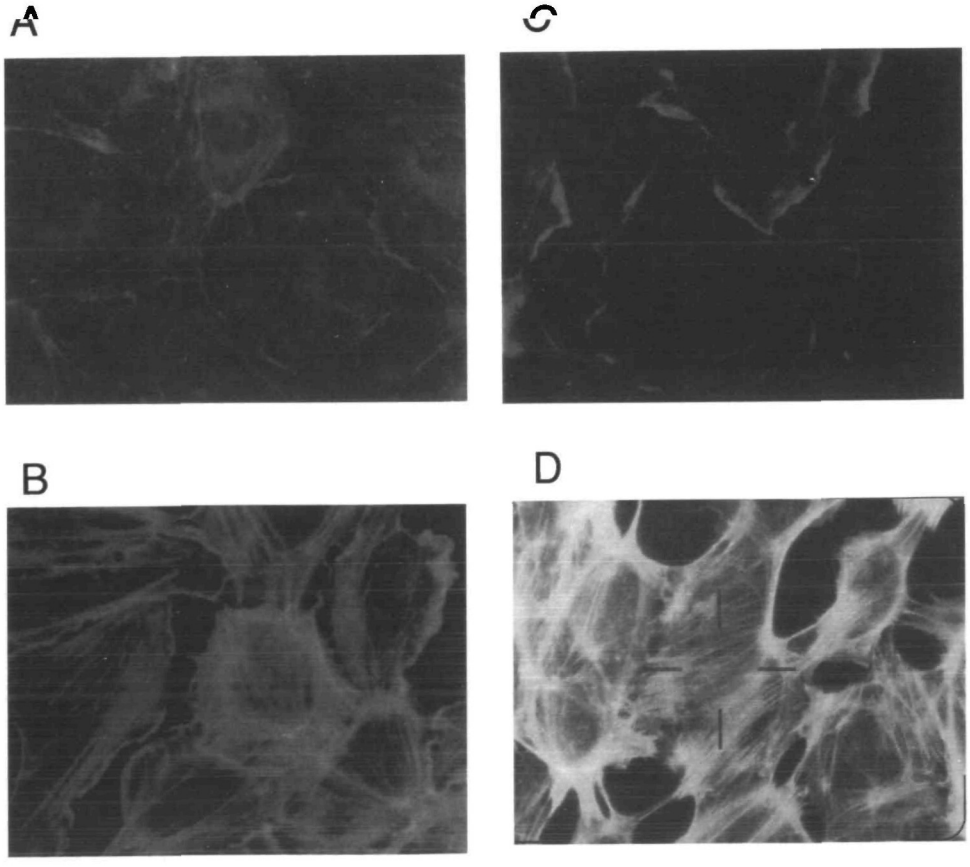

Fig 4 **Induction of HUVEC stress fiber formation by Sph-1-P and its inhibition by C3 exoenzyme.** HUVECs pretreated without (A and B) or with C3 exoenzyme (C) or pertussis toxin (IAP) (D) were stimulated without (A) or with (B-D) 10 μM Sph-1-P for 30 min. The cells were then fixed and stained with TRITC-conjugated phalloidin for actin stress fiber staining.

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centration-dependent (Fig. 2B), and not mimicked by Sph, the substrate of Sph kinase for the production of Sph-1-P (*2, 3*) (data not shown).

Rho is a member of the Ras superfamily of small GTP-binding proteins that integrate extracellular signals to actin cytoskeleton organization (*18*). To determine the involvement of Rho protein, exoenzyme C3 ADP–ribosyltransferase from *Clostridium botulinum*, which specifically ADP-ribosylates Rho on Asn-41 and inhibits Rho function (*19*), was employed. Pertussis toxin was also used to determine the involvement of Gi/o-type heterotrimeric G protein (*20*). Sph-1-P–induced FAK tyrosine phosphorylation was markedly blocked by pretreatment with C3 exoenzyme, but only partially with pertussis toxin (Fig. 2C); the levels of FAK tyrosine phosphorylation preincubated with C3 exoenzyme and pertussis toxin were 15.8 ± 2.7 and 54.0 ± 15.2% (mean ± SD, n = 4) of the control (without pretreatment), respectively.

We also examined whether Sph-1-P caused an increase in MLC phosphorylation, which is thought to underlie cytoskeletal reorganization, in a Rho-dependent manner (*21, 22*). MLC phosphorylation was examined by Western blot analysis using anti–phospho-MLC antibody, which specifically recognizes monophosphorylated (Ser-19) MLC (*23*). Sph-1-P, but not Sph (data not shown), induced a rapid and marked increase in MLC phosphorylation (Fig. 3A) in a concentration-dependent manner (Fig. 3B). As was the case with FAK tyrosine phosphorylation (Fig. 3C), MLC phosphorylation induced by Sph-1-P was markedly blocked by pretreatment with C3 exoenzyme, but hardly with pertussis toxin (Fig. 3C). Under these conditions, the amounts of MLC were confirmed to be constant by Western blotting using anti-MLC polyclonal antibody (data not shown).

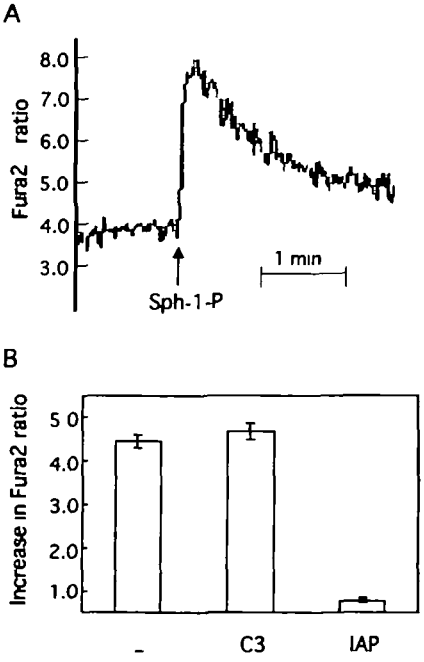

Fig. 5. **Inhibition of Sph-1-P–induced HUVEC intracellular Ca^{2+} mobilization by pertussis toxin, but not by C3 exoenzyme.** (A) Fura2-loaded HUVECs were stimulated with 1 μM Sph-1-P, and the $[Ca^{2+}]_i$ changes were monitored by the ratio of fura2 fluorescence. (B) HUVECs were pretreated without (–) or with C3 exoenzyme (C3) or pertussis toxin (IAP). The cells were then stimulated with 1 μM Sph-1-P, and the increases in $[Ca^{2+}]_i$ (the ratio of fura2 fluorescence) were measured. The results are presented as the mean ± SD (n = 3).

In agreement with the Sph-1-P induction of HUVEC FAK and MLC phosphorylation, Sph-1-P induced cytoskeletal remodeling. Confluent monolayers of HUVECs displayed a faint ring of polymerized actin at their periphery when stained with TRITC-conjugated phalloidin and examined by fluorescence microscopy (Fig. 4A). Upon stimulation with Sph-1-P, a marked expression of actin stress fibers was observed (Fig. 4B), as reported recently (*8*). As expected, the Sph-1-P effect was not mimicked by Sph (data not shown) and was inhibited by pretreatment with C3 exoenzyme (Fig. 4C), but not with pertussis toxin (Fig. 4D). These results indicate that Sph-1-P induces HUVEC cytoskeletal reorganization (involving FAK and MLC phosphorylation) through Rho activation.

Failure of C3 Exoenzyme to Affect Sph-1-P–Induced HUVEC Intracellular Ca^{2+} Mobilization—Since it is well known that Sph-1-P induces intracellular Ca^{2+} mobilization (*2, 4*), the activation of Ca^{2+}/calmodulin-dependent MLC kinase (*24*) may be the mechanism by which Sph-1-P induces MLC phosphorylation and the related-cytoskeletal reorganization. To test this possibility, we loaded control or C3 exoenzyme- or pertussis toxin–treated HUVECs with the Ca^{2+} indicator fura2 and determined intracellular Ca^{2+} mobilization. As reported recently (*8*), Sph-1-P induced a rapid increase in $[Ca^{2+}]_i$ in HUVECs (Fig. 5A), and pertussis toxin almost completely abolished this Sph-1-P–induced response (Fig. 5B). This may be related to the fact that phospholipase C activation and the resultant Ca^{2+} mobilization mediated by Edg-1 (which is abundantly expressed in HUVECs (*8*)) are sensitive to pertussis toxin (*25*). In contrast, C3 exoenzyme failed to affect intracellular Ca^{2+} mobilization induced by Sph-1-P (Fig. 5B). Accordingly, Rho is not involved in Sph-1-P–induced Ca^{2+} mobilization and hence the activation of Ca^{2+}/calmodulin-dependent MLC kinase.

DISCUSSION

In this study, we first showed that HUVECs express only FAK, but not Pyk2, as a FAK family of non–receptor protein-tyrosine kinases, and that Sph-1-P induces FAK tyrosine phosphorylation. Furthermore, MLC phosphorylation and actin stress fiber formation were shown to be induced by Sph-1-P. All of these responses induced by Sph-1-P were blocked by C3 exoenzyme, an inhibitor of the small G protein Rho (*19*), suggesting a requirement for Rho-regulated signaling pathways. In contrast, pertussis toxin only partially inhibited FAK tyrosine phosphorylation, and hardly affected myosin light chain phosphorylation and stress fiber formation. The Rho subtype involved is probably RhoA because RhoA is the predominant substrate of C3 transferase in HUVECs (*26*) and because microinjection of constitutively active V14RhoA produces cell contraction and stress fiber formation (*27*). It is likely that Sph-1-P activates Rho *via* the HUVEC surface receptors Edgs, which interact with heterotrimeric G proteins such as G_{13} and G_q (*8*); these G proteins, together with G_{12}, have been shown to mediate Rho activation (*28*).

It is well established that an increase in intracellular Ca^{2+} concentration leads to the activation of Ca^{2+}/calmodu-

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lin-dependent MLC kinase, which phosphorylates Thr-18 and Ser-19 of the MLC and induces a conformational change in MLC and the resultant actin-myosin interaction (*24*). Since Sph-1-P induces a marked intracellular Ca^{2+} mobilization, the activation of MLC kinase may be the target responsible for MLC phosphorylation and the resultant actin stress fiber formation in HUVECs treated with this phospholipid. However, pertussis toxin, which strongly inhibited Sph-1-P–induced intracellular Ca^{2+} mobilization, failed to block Sph-1-P–induced cytoskeletal changes, indicating that MLC kinase may not play a major role in MLC phosphorylation in HUVECs stimulated with this bioactive lipid. Recently, it was reported that thrombin activates the Rho/Rho kinase pathway to inactivate MLC phosphatase, leading to MLC phosphorylation/contraction in HUVECs (*27*). Sph-1-P may use similar signaling pathways in the induction of HUVEC cytoskeletal changes. MLC phosphatase, but not MLC kinase, may play a major role in the control of MLC phosphorylation/dephosphorylation in HUVECs.

As described above, pertussis toxin inhibited FAK tyrosine phosphorylation induced by Sph-1-P, although only partially and much more weakly than C3 exoenzyme. In HUVECs, protein kinase C activation has been suggested to be one of signaling events responsible for FAK tyrosine phosphorylation (*29*, *30*). Since Sph-1-P–induced phosphoinositide hydrolysis (*25*), as monitored in this study by intracellular Ca^{2+} mobilization, was abolished by pertussis toxin, inhibition of FAK phosphorylation by this toxin may be related to the inhibition of phosphoinositide hydrolysis and the resultant protein kinase C activation induced by Sph-1-P.

Sph-1-P is attracting much attention as a lysophospholipid mediator released by activated platelets (*3–5*). Accordingly, to clarify the effects of this phospholipid on endothelial cells is very important in the context of platelet–endothelial cell interactions. Sph-1-P not only elicits cytoskeletal reorganization but also induces cell adherens junction assembly and morphogenesis in HUVECs (*8*). Furthermore, Sph-1-P is a potent stimulator of the expression of adhesion molecules such as E-selectin and VCAM-1, although Sph-1-P, in this case, reportedly acts as an intracellular messenger that activates NF-κB (*31*). Sph-1-P released from activated platelets may play important role(s) in various pathological conditions in which critical platelet–endothelial interactions occur (such as thrombosis, atherosclerosis, and angiogenesis).

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