Sphingosine 1-Phosphate Activates Akt, Nitric Oxide Production, and Chemotaxis through a G_i Protein/Phosphoinositide 3-Kinase Pathway in Endothelial Cells

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Sphingosine 1-phosphate (SPP) binds to members of the endothelial differentiation gene family (EDG) of receptors and leads to diverse signaling events including cell survival, growth, migration and differentiation. However, the mechanisms of how SPP activates these proangiogenic pathways are poorly understood. Here we show that SPP signals through the EDG-1 receptor to the heterotrimeric G protein G_i, leading to activation of the serine/threonine kinase Akt and phosphorylation of the Akt substrate, endothelial nitric-oxide synthase (eNOS). Inhibition of G_i signaling, and phosphoinositide 3-kinase (PI 3-kinase) activity resulted in a decrease in SPP-induced endothelial cell chemotaxis. SPP also stimulates eNOS phosphorylation and NO release and these effects are also attenuated by inhibition of G_i signaling, PI 3-kinase, and Akt. However, inhibition of NO production did not influence SPP-induced chemotaxis but effectively blocked the chemotactic actions of vascular endothelial growth factor. Thus, SPP signals through G_i and PI 3-kinase leading to Akt activation and eNOS phosphorylation.

Sphingosine 1-phosphate (SPP) is a bioactive lipid, which can be stored and released from platelets upon their activation but can also be synthesized in response to extracellular stimuli by the sequential action of sphingomyelinase, ceramidase, and sphingosine kinase in many cell types (1). Five members of the endothelial differentiation gene family (EDG) of G protein-coupled receptors (EDG-1, EDG-3, EDG-5, EDG-6, and EDG-8) have been identified as SPP receptors in a wide variety of cell types (2–5). These receptors exhibit high affinity binding for SPP, and SPP is weakly displaced by other sphingolipids, including lysophosphatidic acid. EDG receptors differ in their association with the G protein family members. Both EDG-3 and EDG-5 potently activate G_i, G_o, G_{12}, and G_{13}, whereas EDG-1 and EDG-8 couples to G_i but not G_o. In a similar manner, EDG-6 couples to G_o whereas a role for G_{12}, G_{13} has yet to be addressed (6).

SPP participates in a wide spectrum of angiogenic activities including cell proliferation (7, 8), endothelial cell migration (9–11), morphogenesis, and survival, and is involved in the formation of mature neovessels in vivo (12, 13). All these angiogenesis signaling pathways involve the pertussis toxin (PTx)-sensitive G protein, G_i. However, the downstream effectors of G_i responsible for these effects are not well established.

It has been shown that SPP signaling via EDG-1 stimulates mitogen-activated kinase family member ERK (ERK1/ERK2), an effect inhibited by two structurally distinct phosphoinositide 3-kinase (PI 3-kinase) inhibitors (LY294002 and wortmannin). Moreover, immunoprecipitation of Grb-2 from SPP-treated cells resulted in the recovery of PI 3-kinase activity, an effect blocked by pretreatment with pertussis toxin (14). PI 3-kinase catalyzes the phosphorylation of the inositol ring of phosphatidylinositol lipids at the 3-position producing phosphatidylinositol 3,4-biphosphate and phosphatidylinositol 3,4,5-trisphosphate. One downstream effector of PI (3)-kinase is the serine/threonine kinase Akt (or protein kinase B) (15).

Upon receptor activation, Akt is recruited to the plasma membrane and binds to inositol lipids via its pleckstrin homology domain. Akt is then phosphorylated by phosphoinositide-dependent kinases, and this phosphorylation enhances its catalytic activity toward a variety of diverse substrates (16). Recently, we and others (17, 18) have shown that Akt can phosphorylate endothelial nitric-oxide synthase (eNOS) on serine 1179 (serine 1179 in bovine or serine 1177 in the human ortholog, respectively) resulting in eNOS activation and nitric oxide (NO) production (17–19). However, it is not known if SPP can stimulate Akt, eNOS phosphorylation, and NO production in intact endothelial cells.

Several reports have implicated Akt and/or NO as downstream effectors of angiogenic growth factors that can promote endothelial cell survival (20–23) and migration (24–27). These findings, in addition to reports demonstrating a role for Akt...
and NO promoting angiogenesis “in vivo” (28, 29), suggested that PI 3-kinase/Akt/eNOS pathway may function as a downstream target for the angiogenic properties of SPP. Therefore, in this study we assessed whether the PI 3-kinase/Akt/eNOS pathway is activated upon SPP stimulation in endothelial cells, thus contributing to the angiogenic properties of this bioactive lipid.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**Bovine lung microvascular endothelial cells (BLMVECs, Vec Technologies) were cultured as described previously (30, 31).

**Cell Migration Assay—**Migration assays were performed using a modified Boyden chamber (Neuroprobe, Cabin John, MD). Briefly, exponentially growing cells were harvested with trypsin (0.05%, v/v) and EDTA (0.53 mM), counted, and resuspended at a density of 0.4 × 10^6 in chemotaxis medium (Dulbecco’s modified Eagle’s medium with 1% fatty acid-free bovine serum albumin) before being placed in the upper well of a 48-well chemotaxis chamber (Neuroprobe). The lower wells of the chemotaxis chamber contained chemotaxis medium without (controls) or with VEGF (10 ng/ml) or SPP (10–500 nM). Upper and lower wells were separated by a polycrylvinylidene fluoride polycarbonate filter with 8-μm pores (Poretics Corp., Livermore, CA) coated with 100 μg/ml type I collagen (Collaborative Biomedical Products, Bedford, MA). The chamber was incubated for 4 h at 37 °C. After incubation, cells were fixed with ethanol (70%) and nonmigrating cells on the upper surface of the filter removed. Migrated cells were stained with Giemsa and counted with a microscope in triplicate. Each experiment was performed in triplicate, and migration was expressed as the number of total cells counted per well. In some experiments, BLMVECs were pre-incubated with or without L-NAME (2 mM) or LY294002 (10μM) for 30 min or 1 h, respectively, or with or without PTx (200 ng/ml) for 6 h at 37 °C. In previous experiments, this concentration of L-NAME, but not D-NAME, completely blocked VEGF- or calcium ionophore-stimulated NO production or cGMP accumulation in a reporter bioassay system as described (30, 32). In addition, the concentration of LY294002 completely abolished VEGF- or serum-stimulated Akt phosphorylation (24).

**Measurement of NO Release—**For measurement of NO, we analyzed the release of NO₂⁻, the stable breakdown product of NO, from BLMVECs treated with or without SPP (10–500 nM), LY294002 (10μM), L-N-mono-methyl-arginine (L-NMMA) (1 mM), or PTx (200 ng/ml). Cells, plated on 60-mm² dishes were equilibrated for 30 min at 37 °C in Dulbecco’s modified Eagle’s medium without fetal bovine serum. To inhibit NO release, test reagents were added for 30 min, or the supernatant was collected for analysis of NO by chemiluminescence. Samples (10 μl) containing NO₂⁻ were refluxed in glacial acetic acid containing sodium iodide. Under these conditions, NO₂⁻ was quantitatively reduced to NO, which was quantified by a chemiluminescence assay using a chemiluminescence analyzer (Analytik Jena, Jena, Germany).

**RESULTS**

**Expression of EDG Receptors in BLMVECs—**It has been shown that endothelial cells express high levels of EDG-1 transcript (2). Accordingly, we first examined whether other SPP receptors are expressed in BLMVECs. Consistent with previous studies, Northern blot analysis clearly demonstrated abundant expression of EDG-1 mRNA. BLMVECs also expressed a lower level EDG-5 transcript and barely detectable EDG-3 mRNA. However, the EDG-8 mRNA was undetectable in BLMVECs (Fig. 1). In vitro transcription for EDG-1, -3, -5, and -8 were also loaded as positive controls.

**SPP Stimulates Chemotaxis through a PTx-sensitive G Protein in BLMVECs—**Since activation of the EDG-1 receptor is important for angiogenesis, we first investigated the involvement of EDG-1 in SPP-induced chemotaxis in endothelial cells by employing a modified Boyden chamber assay. BLMVECs were subjected to a migration assay in the absence or presence of SPP (10–500 nM) as a chemotaxant. As shown in Fig. 2, SPP dose-dependently increased endothelial cell migration and the migratory activity at 500 nM was about 5–6 times that of the control. SPP induced greater cell migration than typically seen with VEGF as a chemotaxant (see Fig. 6). To investigate the possibility that a G<sub>1</sub>-coupled receptor may be involved in chemotaxis induced by SPP, BLMVECs were treated with pertussis toxin (200 ng/ml) for 6 h prior to addition of SPP. Pertussis toxin pretreatment, which ADP-ribosylates and inactivates G protein, completely abolished SPP-induced chemotaxis (Fig. 2, suggesting that SPP binding to EDG-1 increases chemotaxis in a G<sub>1</sub>-dependent manner.

**SPP-induced Akt Activation and Endothelial Cell Migration Is Blocked by Pertussis Toxin and Inhibitors of PI 3-Kinase—**It has been shown that the SPP binding to EDG-1 triggers activation of ERK1/ERK2, an effect inhibited by PI 3-kinase inhibitors (14). Recent work from us and others has...
shown that inhibitors of PI 3-kinase, as well as dominant-negative suppression of Akt activity, attenuate growth factor-induced migration (24, 33). To directly test the role of PI 3-kinase in SPP-induced cell migration, we first examined if SPP can activate Akt phosphorylation. As seen in Fig. 3A, SPP stimulated Akt phosphorylation in a time-dependent manner with maximal activation occurring within 5 min and sustained phosphorylation lasting for up to 15 min. SPP-stimulated phosphorylation of Akt on Ser^{773} was antagonized by preincubation of BLMVECs with LY294002 and PTx. Next, we examined the effects of the LY294002 on basal and SPP-stimulated cell migration in endothelial cells. As shown previously, SPP stimulated cell migration (lane 4) and this effect was completely inhibited by PTx pretreatment (lane 6) whereas PTx did not influence basal migration (lane 3). Furthermore, SPP-stimulated cell migration was blocked by ~50% by LY294002 (from 90.9 ± 4.9 to 43.7 ± 7.0 migrated cells/field for cells treated with SPP and cells treated with SPP and LY294002, respectively) (lane 5), although basal migration (without stimulation) was not affected by this drug (lane 2). Collectively, these data indicate that SPP signals via G_{i} to PI 3-kinase and Akt and the PI 3-kinase pathway is in part responsible for endothelial cell migration initiated by this bioactive lipid.

**SPP Stimulates NO Production through Akt Activation in a PTx-sensitive, PI 3-Kinase, Akt-dependent Manner**—Recently, we have shown that Akt can phosphorylate bovine eNOS on serine 1179, resulting in eNOS activation and NO production (17). To examine whether SPP-induced Akt activation is coupled to NO production, we examined the effects of SPP on eNOS phosphorylation and NO release. SPP increased NO production in a dose-dependent manner in BLMVECs (Fig. 4A), an effect blocked by the NOS inhibitor, l-NAME (data not shown). Maximal SPP-induced NO release was abrogated by PTx (lane 5), whereas PTx did not influence basal NO synthesis (lane 6). As previously reported for VEGF-stimulated NO production, LY294002 attenuated SPP-induced NO release (from 1.0 ± 0.1 to 0.4 ± 0.1 pmol/μg of protein for cells treated with SPP and cells treated with SPP and LY294002, respectively).

Next, we documented the phosphorylation state of eNOS by immunoblot analysis (Fig. 4B). As predicted, SPP rapidly stimulated eNOS phosphorylation on serine 1179 (upper panel) without changing total eNOS levels (bottom panel). Maximal phosphorylation was seen at 5 min, which decreased over 20 min to levels slightly above basal values. SPP-stimulated phosphorylation of eNOS was antagonized by preincubation of BLMVECs with PTx and LY294002. These findings correlate well with the results in Figs. 3A and 4A, respectively.

Finally, to more firmly establish a link between SPP signaling to Akt, we infected endothelial cells with adenoviruses expressing either a dominant negative form of Akt (AAA-Akt) or GFP and assessed NO release and eNOS phosphorylation. As seen in Fig. 5A, SPP-induced NO release was abrogated by expression of AAA-Akt. In addition, SPP-induced phosphorylation of eNOS was markedly suppressed (Fig. 5B). Collectively, our results indicate that SPP binding to EDG-1 signals through G_{i}, leading to PI 3-kinase-dependent Akt stimulation, eNOS phosphorylation, and NO production.

**Activation of eNOS and NO Are Not Involved in SPP-stimulated Chemotaxis of BLMVECs**—As a downstream signal of PI 3-kinase/Akt, we examined the involvement of NO in SPP-induced chemotaxis in BLMVECs because VEGF-stimulated endothelial cell migration can be blocked by l-arginine-substituted analogues that inhibit NOS. Notably, the NOS inhibitor l-NAME (2 mM) had no effect on the chemotaxis produced by SPP (Fig. 6A), even though the same concentration of l-NAME blocked VEGF-induced cell migration (Fig. 6B), as shown previously (24). Therefore, SPP activates chemotaxis without requiring activation of eNOS.
of Gᵢ that participates in the chemotactic responses. Recently, three different groups have shown that neutrophils or peritoneal macrophages deficient in the PI 3-kinase/Akt/eNOS signaling pathway in chemotaxis. The precise mechanism of how activation of Akt leads to chemotaxis is not known but is blocked by PTx, suggesting an important role for Gᵢ-protein coupled SPP receptors to cell migration. Furthermore, the EDG-1 mRNA is the most prominently expressed SPP receptor in BLMVECs in contrast to the lower levels of EDG-5 mRNA and the barely detectable EDG-3 mRNA. Together, these data suggest that the activation of Gᵢ coupled to the EDG-1 receptor is necessary for SPP-induced chemotaxis in BLMVECs.

Directional cell motility is driven by chemoattractants that bind to G protein coupled receptors receptors (interleukin-8 and fMLP) or growth factors that signal through receptor tyrosine kinases (VEGF, basic fibroblast growth factor, and platelet-derived growth factor). In both cases, many studies have shown that PI 3-kinase activation in a PTx-sensitive manner. However, SPP-stimulated NO production does not influence the tractional forces leading to cell migration. Since vasodilation and increases in endothelial cell permeability also accompany an angiogenic response, it is possible that SPP activation of Gᵢ-dependent PI 3-kinase/Akt/eNOS leading to NO production may regulate local blood flow and permeability during angiogenesis.

SPP has been implicated in both inhibition and stimulation of chemotactic responses. SPP inhibits B16/F10 melanoma cell motility through a pertussis toxin-insensitive pathway (34) and inhibits chemoinvasiveness and motility of breast cancer cell lines (35). In contrast, SPP induces endothelial cell chemotaxis, and this effect is blocked by PTx (9, 11, 36). Similarly, SPP also stimulates the migration of HEK 293 and Chinese hamster ovary cells overexpressing EDG-1 or EDG-3 in a PTx-sensitive manner. However, EDG-5 transfection into Chinese hamster ovary cells does not correlate with induction of cell migration (11, 37). Our results are consistent with these latter studies showing that SPP-induced chemotaxis in endothelial cells is activated by Gᵢ-coupled SPP receptors to cell migration. Furthermore, the EDG-1 mRNA is the most prominently expressed SPP receptor in BLMVECs in contrast to the lower levels of EDG-5 mRNA and the barely detectable EDG-3 mRNA. Together, these data suggest that the activation of Gᵢ coupled to the EDG-1 receptor is necessary for SPP-induced chemotaxis in BLMVECs.

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FIG. 6. NO is not involved in SPP-stimulated chemotaxis of BLMVECs. A, BLMVECs were pretreated without or with PTx (200 ng/ml) for 6 h or l-NAME (2 mM) for 30 min, trypsinized, and resuspended in chemotaxis medium. Then, a migration experiment was performed as above. Data points represent the mean number of migrating cells/field (± S.D.) calculated in three different wells. Representative results from two separate experiments are shown. B, BLMVECs were pretreated without or with l-NAME for 30 min, trypsinized, and resuspended in chemotaxis medium and migration assays performed in response to VEGF (10 ng/ml). Data points represent the mean number of migrating cells/field (± S.D.) calculated in three different wells. Representative results from two separate experiments are shown.

likely though direct modulation of actin polymerization/depolymerization pathways.

It has recently been demonstrated that overexpression of EDG-1 receptor and eNOS in COS-7 cells led to SPP-dependent eNOS activation (42); however, the mechanism was not explored. We and others (17, 18, 43, 44) have shown that VEGF, fluid shear stress, estrogen, or IGF-1 can stimulate Akt and subsequent eNOS phosphorylation on serine 1179, resulting in eNOS activation and NO production. Moreover, substitution of serine 1179 with aspartate leads to constitutive activation of eNOS and NO production due to enhanced electron flux from the reductase domain to the oxygenase domain (45). In agreement with these studies, here we show that SPP induces eNOS phosphorylation on serine 1179 and NO production in endothelial cells. SPP-induced eNOS phosphorylation and NO release were inhibited by PTx, LY294002, and dominant negative Akt, suggesting that SPP binding to EDG-1 signals via G, to PI 3-kinase/Akt leading to eNOS phosphorylation and NO production. It should be noted that the PI 3-kinase inhibitor LY294002 was not able to completely block SPP-stimulated NO production at a concentration that abrogates Akt activation, suggesting other pathways of eNOS activation exist. Furthermore, similar results were obtained with dominant negative Akt. Indeed, SPP increases cytoplasmic calcium in endothelial cells via a PTx-sensitive mechanism (12), thus providing calcium to activate calmodulin and the binding of calmodulin to eNOS leading to an increase in eNOS activity (46).

The present study also shows that production of NO is not required for endothelial cell migration mediated by SPP. In contrast, previous reports have pointed to an important role of NO in VEGF-induced endothelial cell migration (24–27). These apparently contradictory observations most likely reflect the diversity of mechanisms activated in endothelial cells in response to different chemotactants. However, it is feasible that the increase in NO production mediated by SPP may be involved in other angiogenic functions promoted by this bioactive lipid, including cell survival through inactivation of caspase-3 (23) and enhancement of blood flow and permeability. Thus, our findings define two additional proangiogenic actions of SPP: activation of Akt and NO release. Further elucidation of the precise roles of Akt and NO in the context of angiogenesis will increase our understanding of the important role of SPP during vascular development, angiogenesis, and vessel homeostasis.
34. Yamamura, S., Yatomi, Y., Ruan, F., Sweeney, E. A., Hakomori, S., and Igarashi, Y. (1997) Biochemistry 36, 10751–10759
35. Wang, F., Nobara, K., Olivera, A., Thompson, E. W., and Spiegel, S. (1999) Exp. Cell Res. 247, 17–28
36. Kimura, T., Watanabe, T., Sato, K., Kon, J., Tomura, H., Tamama, K., Kuwabara, A., Kanda, T., Kobayashi, I., Ohta, H., Ui, M., and Okajima, F. (2000) Biochem. J. 348, 71–76
37. Kon, J., Sato, K., Watanabe, T., Tomura, H., Kuwabara, A., Kimura, T., Tamama, K., Ishizuka, T., Murata, N., Kanda, T., Kobayashi, I., Ohta, H., Ui, M., and Okajima, F. (1999) J. Biol. Chem. 274, 23940–23947
38. Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A. V., and Wu, D. (2000) Science 287, 1046–1049
39. Sasaki, T., Irie-Sasaki, J., Jones, R. G., Oliveira-dos-Santos, A. J., Stanford, W. L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Kozieradzki, I., Jozza, N., Mak, T. W., Ohashi, P. S., Suzuki, A., and Penninger, J. M. (2000) Science 287, 1040–1046
40. Hirsch, E., Kataev, V. L., Garlanda, C., Azzolino, L., Pirola, L., Silengo, L., Sozzani, S., Mantovani, A., Altruda, F., and Wymann, M. P. (2000) Science 287, 1049–1053
41. Zheng, D. Q., Woodard, A. S., Tallini, G., and Languino, L. R. (2000) J. Biol. Chem. 275, 24565–24574
42. Igarashi, J., and Michel, T. (2000) J. Biol. Chem. 275, 32363–32370
43. Fisslthaler, B., Dimmeler, S., Hermann, C., Busse, R., and Fleming, I. (2000) Acta Physiol. Scand. 168, 81–88
44. Haynes, M. P., Sinha, D., Russell, K. S., Collinge, M., Fulton, D., Morales-Ruiz, M., Sessa, W. C., and Bender, J. R. (2000) Circ. Res. 87, 677–682
45. McCabe, T. J., Fulton, D., Roman, L. J., and Sessa, W. C. (2000) J. Biol. Chem. 275, 6123–6128
46. Forstermann, U., Gorsky, L. D., Pellock, J. S., Ishii, K., Schmidt, H. H., Heller, M., and Murad, F. (1990) Mol. Pharmacol. 38, 7–13
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