Sphingosine 1-Phosphate Stimulates Cell Migration through a G_{i}-coupled Cell Surface Receptor

POTENTIAL INVOLVEMENT IN ANGIOGENESIS*

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Sphingosine 1-phosphate (SPP) has been shown to inhibit chemotaxis of a variety of cells, in some cases through intracellular actions, while in others through receptor-mediated effects. Surprisingly, we found that low concentrations of SPP (10–100 nM) increased chemotaxis of HEK293 cells overexpressing the G protein-coupled SPP receptor EDG-1. In agreement with previous findings in human breast cancer cells (Wang, F., Nohara, K., Olivero, O., Thompson, E. W., and Spiegel, S. (1999) Exp. Cell Res. 247, 17–28), SPP, at micromolar concentrations, inhibited chemotaxis of both vector- and EDG-1-overexpressing HEK293 cells. Nanomolar concentrations of SPP also induced a marked increase in chemotaxis of human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC), which express the SPP receptors EDG-1 and EDG-3, while higher concentrations of SPP were less effective. Treatment with pertussis toxin, which ADP-ribosylates and inactivates G_{i}-coupled receptors, blocked SPP-induced chemotaxis. Checkerboard analysis indicated that SPP stimulates both chemotaxis and chemokinosis. Taken together, these data suggest that SPP stimulates cell migration by binding to EDG-1. Similar to SPP, sphinganine 1-phosphate (dihydro-SPP), which also binds to this family of SPP receptors, enhanced chemotaxis; whereas, another structurally related lysophospholipid, lysophosphatidic acid, did not compete with SPP for binding nor did it have significant effects on chemotaxis of endothelial cells. Furthermore, SPP increased proliferation of HUVEC and BAEC in a pertussis toxin-sensitive manner. SPP and dihydro-SPP also stimulated tube formation of BAEC grown on collagen gels (in vitro angiogenesis), and potentiated tube formation induced by basic fibroblast growth factor. Pertussis toxin treatment blocked SPP- but not bFGF-stimulated in vitro angiogenesis. Our results suggest that SPP may play a role in angiogenesis through binding to endothelial cell G_{i}-coupled SPP receptors.

The sphingolipid metabolite sphingosine 1-phosphate (SPP) is a bioactive lipid that regulates diverse biological effects and signaling pathways (reviewed in Ref. 1). SPP increases cell proliferation (2, 3) and opposes ceramide-mediated apoptosis (4–7) through an intracellular action (8, 9), yet some of its biological effects when added exogenously are due to binding to cell surface receptors. Pertussis toxin-sensitive G proteins are involved in some of the signaling pathways activated by SPP (10–15), suggesting that it activates a receptor coupled to a G/G_{i} protein. In agreement, low concentrations of SPP activate G_{i}-protein-gated inward rectifying K\^{+} channels only when applied at the extracellular face of atrial myocytes (16).

Several reports have demonstrated that SPP inhibits cell motility. SPP inhibited chemotactic motility of mouse melanoma B16, mouse fibroblast BALB/3T3 clone A31, and several tumor cell lines at nanomolar concentrations (17–19). Moreover, SPP immobilized on glass beads markedly inhibited melanoma cell motility. However, pertussis toxin treatment did not block the effect of SPP, suggesting that in these cells SPP acts through a cell surface receptor, independently of pertussis toxin-sensitive G-proteins (20). In contrast, SPP inhibits chemotaxis of human breast cancer cells only at high (micromolar) concentrations, acting independently of EDG-1 (21).

We have recently identified SPP as a ligand for the G-protein-coupled receptor, endothelial differentiation gene-1 (EDG-1) (22). EDG-1 binds SPP with remarkable specificity and high affinity (K_{D} = 8 nM) (9, 22). Binding of SPP to EDG-1 resulted in inhibition of adenylate cyclase and activation of mitogen-activated protein kinase (both G_{i}-mediated), but did not mobilize calcium from internal stores (9, 23). In contrast, Okamoto et al. (12) found that in HEL cells overexpressing EDG-1, binding of SPP induced calcium mobilization (12).

Two other related G protein-coupled receptors, EDG-3 and EDG-5, have recently been shown to bind SPP with similar high affinity (14, 24), to confer responsiveness to SPP of a serum response element-driven reporter gene when expressed in Jurkat cells, and to allow SPP-stimulated \textsuperscript{45}Ca\^{2+} efflux in Xenopus oocytes (25). In agreement, overexpression of EDG-3 in Chinese hamster ovary cells led to phospholipase C activation and calcium mobilization induced by SPP, which was significantly inhibited by pertussis toxin (26). However, low con-

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\^{1} The abbreviations used are: SPP, sphingosine 1-phosphate; BAEC, bovine aortic endothelial cells; BSA, bovine serum albumin; HUVEC, human umbilical vein endothelial cells; GPCR, G protein-coupled receptors; dihydro-SPP, sphinganine 1-phosphate; LPA, lysophosphatidic acid; IMEM, Richter's improved minimal essential medium; bFGF, basic fibroblast growth factor; EDG-1, endothelial differentiation gene-1; SPC, sphingosylphosphorylcholine; RT-PCR, reverse transcriptase-polymerase chain reaction.
centrations of SPP mobilize calcium from internal sources in BAEC in a pertussis toxin-sensitive manner without activation of phospholipase C (27), suggesting the involvement of novel, unidentified signaling pathways in SPP-induced release of intracellular calcium.

Although the biological functions of the EDG family of GPCR are not completely understood, the EDG-1 transcript was originally cloned as an immediate-early gene induced during differentiation of HUVEC, cells of the vessel wall accessible to platelet-derived ligands, into capillary-like tubules (28). Moreover, SPP signaling in HEK293 cells overexpressing EDG-1 leads, by a Rho-dependent mechanism, to formation of a network of cell-cell aggregates resembling the network formation of differentiated endothelial cells and P-cadherin expression (22). Because SPP is stored and released from activated platelets and serum concentrations of SPP are estimated to be approximately 0.5 μM (29), about 60 times greater than the KD for binding to EDG-1, we suggested that SPP might play an important role in angiogenesis acting through EDG-1 (22).

Angiogenesis, the process of new vessel formation from pre-existing ones, or neovascularization, is a critical event for a variety of physiological processes, such as wound healing, embryonic development, corpus luteum formation, and menstruation. However, angiogenesis can be activated in response to tissue damage and is important in certain pathological conditions such as tumor growth and metastasis, rheumatoid arthritis, diabetic retinopathy, psoriasis, and cardiovascular diseases (30). Conversely, in states of inadequate tissue perfusion, such as myocardial or limb ischemia, enhanced angiogenesis is essential and beneficial (31). Endothelial cell migration and formation of new capillary tubes are required events in the angiogenic response. In this study, we investigated the potential role of SPP in angiogenesis by examining regulation of endothelial cell motility, proliferation, and tube formation through SPP receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—IMEM, penicillin/streptomycin, l-glutamine, amphotericin B, fetal calf serum, and fetal bovine serum were from Biosera (Rockville, MD). Medium 199 was from Life Technologies (Gaithersburg, MD). Calf serum was from Colorado Serum Co. (Denver, CO), and Matrigel, bFGF, endothelial cell growth supplement, and rat tail type I collagen were from Collaborative Biomedical Products (Bedford, MA). SPP, dihydro-SPP, and sphingosylphosphorylcholine (SPC) were obtained from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). 1-Oleoyl-2-hydroxy-sn-glycerol-3-phosphocholine (LPA) was from Avanti Polar Lipids, Inc. (Alabaster, AB). The Diff-Quik kits were from Sigma. [methyl-3H]Thymidine (55 Ci/mmol) was purchased from Amersham Pharmacia Biotech.

**Cell Culture**—Human embryonic kidney cells (HEK293, ATCC CRL-1573) and HEK293-EDG-1 cells, kindly provided by Drs. Menq-Jer Lee and Timothy Hla, were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, with 1 mg/ml G418 sulfate for HEK293-EDG-1, as described previously (9, 22). BAEC were kindly provided by Dr. Luyuan Li and maintained in IMEM containing 10% fetal bovine serum supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), l-glutamine (2 mM), and 1 ng/ml bFGF. HUVECs were isolated as previously reported (32), and grown in medium 199 supplemented with gentamycin, 2 mM glutamine, 500 units/dl sodium heparin, 2.5 mg/dl amphotericin B, and 2 mg/dl endothelial cell growth supplement.

SPP Binding Assay—HUVEC or BAEC were washed with binding buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 15 mM sodium fluoride, 2 mM deoxyxystrophan, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and leupeptin) and removed from dishes by scraping. Cells were centrifuged and resuspended in 4 mg/ml BSA. 105 cells were incubated with 0.2 nM [35S]SPP, synthesized enzymatically using recombinant sphingosine kinase (33) as described previously (9), in 0.2 ml of binding buffer plus 4 mg/ml BSA for 30 min at 4 °C in the absence or presence of 100-fold excess unlabeled SPP or other lipid competitors, added as 4 mg/ml fatty acid-free BSA complexes. Cells were then pelleted at 8,000 rpm in a microcentrifuge, washed twice with binding buffer containing 0.4 mg/ml fatty acid-free BSA, resuspended in binding buffer without BSA and bound [35S]SPP quantitated by scintillation counting. The phosphatase and protease inhibitors were included in the binding assays as a precaution against the possibility that cells which may have been damaged during scraping and plated in the wells may have expressed the receptors which received SPP binding. In addition, it has been proposed that cell surface lipid phosphatases which can cleave exogenous SPP exist (34). Nevertheless, identical specific binding of [35S]SPP was obtained in the absence of the protease and phosphatase inhibitors. It should be pointed out that SPP is not metabolized during the binding assay. When [35S]SPP was incubated in the absence or presence of endothelial cells under the same conditions as the binding assay, no decrease in the amount of [35S]SPP was detected by TLC nor did any additional bands appear.

**Reverse Transcriptase (RT)-PCR**—The cDNA encoding the open reading frame of EDG-1 was amplified with the Gene Amp RNA PCR kit (Perkin-Elmer) using RNA isolated with TRIzol Reagent (Life Technologies) and digested with RNase-free DNase I (RQ-1, Promega). The point of the RT-PCR was the same for the experiments, unless indicated otherwise, cells were serum starved for 48 h before the assays. In brief, polycarbonate filters (5 μm for BAEC and 8 μm for HUVEC) were coated with gelatin (0.1%) overnight. Cells were harvested by trypsinization, washed with serum-free IMEM containing 0.1% BSA, and were added to the upper wells (24-well well) or bottom wells (12-well well) or bottom wells (12-well well) of the Boyden chamber. In most of the experiments, unless indicated otherwise, cells were serum starved for 48 h prior to the assays.

**[3H]Thymidine Incorporation Assays**—Cells were seeded at an initial density of 5 × 104 cells per well in 24-well plates and allowed to attach overnight. Confluent BAEC were growth arrested in culture media without bFGF for 48 h and then treated for 24 h with SPP or bFGF. Since the sensitivity of BAEC to stimulation by bFGF and SPP decreased with passage number, all experiments were carried out with cells at less than passage 12. Confluent HUVEC were serum-starved for 24 h and then treated with different concentration of SPP in medium 199 containing 1% FCS and 1000 units/dl heparin for 24 h. [3H]Thymidine (1 μCi/ml) was added 8 h before termination of the assay, and [3H]thymidine incorporation into DNA was measured as described (38). Values are the means of triplicate determinations and standard deviations were routinely less than 10% of the mean.

**In Vivo Angiogenesis**—Three-dimensional collagen gel plates (24 well) were prepared by addition of 0.5 ml of a chilled solution of 0.7 mg/ml rat tail type I collagen in Dulbecco's modified Eagle's medium adjusted to neutral pH with NaHCO3. After formation of the collagen gel (about 1–2 mm thickness), BAEC were seeded at 50,000 cells/well. At 80% confluency, culture medium was changed to media without bFGF and incubation was continued for 48 h, by which time the cells formed a monolayer on the gel. The cells were then treated with different concentrations of bFGF and SPP as indicated in the Table. The gels were maintained at 37 °C for 48 h and then fixed with cold methanol. The gels were then soaked in phosphate-buffered saline/glycerol (1:1) and transferred onto glass slides. The extent of tube-like structures that formed in the gel was measured as total length per field using computer-assisted imaging with a Hamamatsu C2400 video camera and a Zeiss Axioscope microscope to quantitate the extent of tube formation. Three culture wells were used for each sample, and three microscopic fields.
SPP binding to HEK293-EDG

Previously, it has been demonstrated that human endothelial cells
not altered by these concentrations of SPP (Fig. 1, 1.5–1.9-fold, while chemotaxis of vector-transfected cells was
not shown), whereas, LPA and SPC were completely ineffective
"Experimental Procedures."

Higher concentration of SPP (1–10

m

M), as previously reported
indicates low concentrations of SPP (10–100 nM) did not inhibit,
but rather enhanced chemotaxis of HEK293-EDG-1 cells by
1.5–1.9-fold, while chemotaxis of vector-transfected cells was not altered by these concentrations of SPP (Fig. 1A). The concentrations of SPP which stimulate chemotaxis of HEK293-EDG-1 cells are in the same range as the measured affinity of EDG-1 for SPP (K

i

D = 8 nM) (22) and correlate closely with binding and inhibition of forskolin-stimulated cAMP accumulation in these cells (9). These results suggest that low concentrations of SPP may increase chemotaxis by binding to EDG-1. Higher concentration of SPP (1–10 μM), as previously reported in human breast cancer cells (21), inhibited, rather than stimulated, chemotaxis of both vector and EDG-1-transfected cells.

In contrast to undetectable levels of EDG-1 mRNA in parental and vector transfected HEK293 cells (9, 39), HEK293-EDG-1 cells express very high levels of EDG-1 mRNA as detected by Northern analysis (9) and RT-PCR (Fig. 2A). Additionally, a low level of EDG-3 mRNA and EDG-5 mRNA were detected by RT-PCR in these cells (Fig. 2A). Similar to our previous results (9), both unlabeled SPP and dihydro-SPP effectively competed with 

[32P]SPP for binding to EDG-1 (data not shown), whereas, LPA and SPC were completely ineffective (Fig. 1B). Moreover, LPA, even at concentrations as high as 10 μM, and for prolonged incubations, had no significant effect on SPP binding to HEK293-EDG-1 cells.

Expression of EDG Receptors in Endothelial Cells—Previously, it has been demonstrated that human endothelial cells express high levels of EDG-1 mRNA (28). Thus, it was of interest to examine whether the other SPP receptors, EDG-3 and EDG-5 (24, 25, 40), are also expressed in HUVEC as well as BAEC which, based on indirect evidence, have been proposed to have putative G1-coupled SPP receptors linked to calcium mobilization (27). Consistent with previous studies, RT-PCR analysis clearly demonstrated an EDG-1 product of about 1300 base pairs, in agreement with the predicted size (1284 base pairs), in HUVEC and BAEC (Fig. 2, B and C). HUVEC and BAEC also apparently expressed somewhat lower levels of EDG-3 and barely detectable EDG-5 mRNA. It should be noted that the bovine SPP receptor cDNAs have not yet been cloned and sequenced; however, our PCR primers were designed to cover highly conserved regions. Restriction analysis of the HUVEC RT-PCR products yielded fragments of the expected sizes confirming their identity (data not shown). The entire open reading frame of each of the three SPP receptors is encoded within a single exon (40, 41), and RT-PCR primers which span an intron junction cannot be used to evaluate genomic DNA contamination. Therefore, controls without reverse transcriptase were performed in all cases (Fig. 2).

Effects of SPP on Chemotaxis of Endothelial Cells—Since SPP increased chemotaxis of HEK293 cells by apparently acting through EDG-1, the effect of SPP on chemotaxis of HUVEC and BAEC, which express EDG-1 and -3, was examined (Fig. 3, A and B). SPP stimulated chemotaxis of both HUVEC and BAEC, reaching a maximum effect at 1 μM (7- and 10-fold, respectively). Similar to the results with HEK293-EDG-1 cells, concentrations of SPP greater than 1 μM were less effective, although significant enhancement of chemotaxis was still evident at higher concentrations. Interestingly, bFGF (20 ng/ml), a potent angiogenic factor (42), increased chemotaxis of BAEC to the same extent as did 1 μM SPP.

It has recently been shown that directional migration toward appropriate agonist ligands can be triggered via receptors coupled to G, but not by agonists for receptors coupled to two other G proteins, G and G (43). Because biochemical evidence and the yeast two-hybrid system indicate that EDG-1 is capable of interaction with G and G (44), we investigated the possibility that G proteins may be involved in the chemotactic response induced by SPP. To this end, HUVEC were treated with pertussis toxin, which ADP-riboseylates and inactivates G and G, proteins, prior to addition of SPP. Pertussis toxin pretreatment completely abolished SPP-induced chemotaxis (Fig. 3C).

SPP Stimulates Directional Migration—We next determined whether the effect of SPP was mediated by enhanced directed migration in response to the gradient of chemoattractant (chemotaxis) or by increased random motility due to the presence of the chemoattractant itself (chemokinesis). Checkerboard as-
...tions on HUVEC. As shown in Fig. 4A, LPA nor SPC had a significant effect on SPP binding (Fig. 1) in a dose-dependent manner similar to unlabeled SPP (9, 24), while neither binding to HEK293 cells overexpressing EDG-1 in a dose-dependent manner was found to be optimal for binding of SPP to its receptor (9, 24), whereas these might not be optimal conditions for binding of LPA to EDG-2 and EDG-4. For example, LPA prepared as a 1% BSA complex promoted survival of Schwann cells, while SPP as a 0.01% complex was ineffective (48). We also examined the binding affinity of SPP for its putative receptor on endothelial cells by displacing binding of [32P]SPP with increasing concentrations of unlabeled SPP. 50% of the bound [32P]SPP was competed at 10 nM unlabeled SPP in BAEC. Thus, binding of SPP to endothelial cells is also of high affinity and in agreement with the K_d of EDG-1 (8.6 nM). These results indicate that there is an excellent correlation between the K_d and the concentration-dependent effect of SPP on cell migration.

**SPP Stimulates Proliferation of HUVEC and BAEC**—Many angiogenic factors, in addition to enhancing chemotaxis, stimulate in vitro proliferation of endothelial cells (49–51). Since SPP increased chemotaxis of endothelial cells, and has previously been shown to be a potent mitogen for diverse cell types (1, 2, 52), it was of interest to examine the effects of SPP on proliferation of endothelial cells. SPP treatment of HUVEC induced a dose-dependent increase of DNA synthesis as measured by [3H]thymidine incorporation with a maximum effect at 0.1–1 μM (Fig. 5A). Similar to SPP, 1 μM dihydro-SPP also stimulated DNA synthesis in HUVEC by 1.83 ± 0.1-fold, whereas LPA at a concentration as high as 10 μM had no...
significant effect. In agreement with our previous reports in various cell types (53–55), 10 μM SPC stimulated DNA synthesis by 1.95 ± 0.1-fold.

SPP was also mitogenic for BAEC; however, a maximal effect in these cells required higher concentrations (1–10 μM). bFGF has been reported to be a potent endothelial cell mitogen (56). Surprisingly, although bFGF stimulated proliferation of BAEC, at optimal concentrations it was only as effective as 1 μM SPP and less effective than 10 μM SPP (Fig. 5B). It should be noted that sensitivity of BAEC to SPP decreased with increasing passage number, similar to a previous report on the effect of passage number on bFGF responses (57). In addition to SPP, dihydro-SPP stimulated DNA synthesis, whereas we found that LPA at a concentration up to 10 μM was not mitogenic, in fair agreement with previous studies where LPA only stimulated DNA synthesis in BAEC at concentrations around 30 μM (57).

To investigate the possibility that a Gq-coupled receptor may be involved in the proliferative response induced by SPP, endothelial cells were treated with pertussis toxin prior to addition of SPP. Both HUVEC and BAEC are more sensitive to pertussis toxin than Swiss 3T3 fibroblasts. In contrast with our previous studies with quiescent Swiss 3T3 fibroblasts (10, 11), where half of the stimulated DNA synthesis was still evident even at the highest effective concentration of pertussis toxin, pertussis toxin pretreatment of HUVEC and BAEC completely inhibited the SPP-induced mitogenic response, while it had no significant effect on DNA synthesis induced by bFGF (Fig. 5C).

SPP Induces Capillary-like Tube Formation in Vitro—Later stages of angiogenesis require morphological alterations of endothelial cells, which result in lumen formation (31). Critical steps in angiogenesis, such as migration and differentiation, have been studied using an in vitro model of angiogenesis in which cultured endothelial cells are induced to invade a three-dimensional collagen gel where they form a network of capillary-like structures or tubes when stimulated by angiogenic factors (58, 59). This phenomenon is thought to mimic the formation of new blood vessels in vivo. In agreement with previous studies (59), confluent monolayers of BAEC treated with bFGF (50 ng/ml) formed networks of capillary-like tubular structures within the gel (Fig. 6A). In contrast, little invasion or network cord formation and only a few short capillary sprouts originating from untreated BAEC embedded in collagen gels were detected. SPP evoked a dose-dependent increase in the formation of capillary-like tubes of BAEC invading the collagen gel. Apparently thinner tubes were formed in response to lower doses of SPP than those formed in response to bFGF. Quantitative evaluation of tube formation revealed that SPP, similar to bFGF, markedly increased the length of the endothelial tubular structures (Fig. 6B). There was an additive effect when SPP was applied together with bFGF, suggesting that SPP can potentiate the effect of bFGF on in vitro angiogenesis. Similar to SPP, dihydro-SPP, also markedly enhanced capillary-like tube formation of BAEC, whereas LPA was completely inactive and SPC had a small but significant effect (Fig. 6C). Quiescent HUVEC and BAEC were incubated in the absence (filled bars) or presence of 20 ng/ml pertussis toxin (open or hatched bars). After 2 h, cells were washed and exposed to the indicated concentrations of SPP or bFGF for 24 h and [3H]thymidine incorporation was measured. Data are expressed as fold increases compared with non-stimulated cells.

![Image](https://example.com/image.png)

**DISCUSSION**

Previously, many studies have shown that SPP inhibits chemotaxis of diverse cell types (17, 19, 20, 60, 61). Although in human breast cancer cells, SPP inhibited chemotaxis independently of EDG-1 (21), a wealth of evidence suggests that in many other cell lines, SPP inhibits chemotaxis through unidentified cell surface receptors (17, 20, 62). Unexpectedly, we found...
increased chemotaxis of EDG-1-transfected but not vector-
with its affinity for EDG-1, SPP at nanomolar concentrations
receptor EDG-1 markedly increased cell motility. In accord
relative to the maximum response elicited by bFGF.

A

bFGF

-          +

SPP (μM)  0  0.1  1  15

B

Tube Formation (mm²/mm²)

0  5  10  15  20  25  30  35  40

SPP (μM)  0  0.1  1  15

C

Tube Formation (% maximum)

0  20  40  60  80  100

SPP (μM)  0  0.1  1  15

FIG. 6. Induction of in vitro angiogenesis in collagen gels by SPP is attenuated by pertussis toxin. BAEC were treated with the indicated concentration of SPP in the absence or presence of bFGF (50 ng/ml) and capillary tube formation on collagen gels was examined as described under “Experimental Procedures.” A, representative phase-
contrast micrographs of BAEC after 4 days of incubation in normal
medium (upper panels) or in the presence of 50 ng/ml bFGF (lower
panels) and the indicated concentrations of SPP. B, quantitative anal-
ysis of capillary tube formation. Data are expressed as length of tubes per square millimeter (n = 4 pairs of duplicates). Asterisks indicate
statistical significance determined by Student’s t test (p < 0.01). C,
BAEC were treated in the absence or presence of pertussis toxin (PT; 20
ng/ml), without or with the indicated lysophospholipids (1 μM) or bFGF
(50 ng/ml) and capillary tube formation on collagen gels was determined
as described under “Experimental Procedures.” Data are expressed
relative to the maximum response elicited by bFGF.

in this study that binding of SPP to its Gᵢ protein-coupled
receptor EDG-1 markedly increased cell motility. In accord
with its affinity for EDG-1, SPP at nanomolar concentrations
increased chemotaxis of EDG-1-transfected but not vector-
transfected HEK293 cells, as well as HUVEC and BAEC which
constitutively express EDG-1.

Although a recent study demonstrated that LPA can bind to
EDG-1 leading to receptor phosphorylation, ERK activation, as
well as Rho-dependent morphogenesis and P-cadherin expres-
sion (39), in our study, LPA, even at a concentration as high as
10 μM, did not compete for binding of radiolabeled SPP to
HEK293-EDG-1 cells. In agreement, LPA did not displace bound
[^32P]SPP from endothelial cells nor did it stimulate
chemotaxis of HUVEC. In contrast, dihydro-SPP, which binds
to EDG-1 (9), EDG-3, and EDG-5 (24), was as potent as SPP in
induction of chemotaxis. However, LPA, similarly to SPP, can
mimic serum in inducing invasion of carcinoma and hepatoma
cells into monolayers of mesothelial cells (63). Although the
underlying mechanism of this effect is not clear, it may involve
increased cell adhesion rather than enhanced cell motility.
Moreover, in most other cell types, LPA stimulates chemokine-
sis and chemotaxis (62, 64–66), which might be due to binding
to its specific receptors, EDG-2 and EDG-4 (45–48).

We have previously shown that the SPP-induced cAMP de-
crease (9) and ERK2 activation in EDG-1-transfected cells (22)
were completely blocked by pretreatment with pertussis toxin,
which uncouples Gᵢ from GPCR. Similarly, preincubation with
pertussis toxin abolished the effect of SPP on migration of
endothelial cells. Collectively, these findings suggest that bind-
ing of SPP to the serpentine receptor EDG-1 on the endothelial
cell surface activates a pertussis toxin-sensitive Gᵢ protein cru-
cial for chemotaxis. In agreement, it has recently been demon-
strated that activation of Gᵢ coupled receptors and the subse-
quent release of Gᵢ₃ dimers is required to initiate signal
transduction leading to directed cell migration (43, 67). It
should be noted that the receptors for all known leukocyte
chemoattractants, including the chemokines, are members of a
seven-transmembrane domain superfamily and coupled to a
variety of Gᵢ subunits (68, 69). Moreover, pertussis toxin in-
hibits chemotaxis by preventing chemoattractant receptors
from activating trimeric G proteins of the Gᵢ subfamily (67).

Endothelial cells are accessible to platelet-derived ligands in
the serum, and might be the target of serum-borne SPP which
regulates their proliferation, migration, and differentiation
into capillary-like tubules, important aspects of angiogenesis
(31). SPP is well established as a potent mitogen for diverse cell
types (reviewed in Ref. 1). Indeed, we found that SPP also
stimulated DNA synthesis in endothelial cells and this was
completely blocked by pertussis toxin, suggesting a role for Gᵢ
in this process. In agreement, SPP was recently reported to
stimulate HUVEC proliferation (70), albeit at somewhat higher
concentrations which resembled the dose-response curve that
we found for BAEC. It is possible that differences in sensitivity
to SPP might arise from differences in passage numbers as has
been shown for responses to bFGF (57). Interestingly, SPP
stimulated DNA synthesis in Swiss 3T3 fibroblasts only at high
concentrations and in contrast to endothelial cells, was only
partially inhibited by pertussis toxin (10). Moreover, in these
cells, DNA synthesis was significantly and specifically in-
creased by microinjection of SPP (9). Pertussis toxin reduced
the level of DNA synthesis caused by exogenous SPP to approx-
imately the same level as that induced by microinjected SPP
(9). Thus it is likely that in fibroblasts, both intracellular as
well as receptor-mediated responses to SPP are involved in its
mitogenic effect. It is possible that there is a complex interplay
between cell surface receptor signaling and intracellular tar-
gets for SPP, which can contribute to its mitogenic response in
certain cell types. Thus, SPP, may act in a similar manner to
other bioactive lipids, such as leukotriene B4 (LTB₄), which act
through cell surface receptors and also have intracellular tar-
gets. LTB₄ is a potent chemoattractant that is primarily in-
Sphingosine 1-Phosphate Stimulates Endothelial Cell Migration

Some of the downstream effects of SPP signaling through EDG-1, such as decreased cAMP (9, 23) and activation of ERK2 (22), are pertussis toxin-sensitive, while others, such as morphogenetic differentiation, are pertussis toxin-insensitive but inhibited by the C3 exoenzyme (22) which blocks signaling through the small GTPase Rho (77). Thus it appears that EDG-1 can couple to G13 proteins as previously reported (44), as well as to G12/13 proteins which are thought to regulate Rho (78, 79), and thus might also be important for SPP-induced chemotaxis and angiogenesis. Recently, it has been demonstrated that disruption of the gene encoding G13 impaired the ability of endothelial cells to organize into a vascular system and greatly impaired migratory responses (80), suggesting that in addition to Gt, other proteins including GR, might be required for regulation of cell movement. In agreement, T-lymphoma cell invasion is dependent on SPP receptor-mediated RhoA and phospholipase C signaling pathways which lead to pseudopodia formation (74).

In summary, in this study we have demonstrated that SPP has appropriate properties to be considered as a bona fide angiogenic factor, i.e. it stimulates chemokinetic and chemotactic motility, proliferation of vascular endothelial cells, and stimulates angiogenesis in vitro, similarly to the known angiogenic factor bFGF. Because bFGF and SPP have an additive effect on formation of capillary-like tubes by endothelial cells invading collagen gels, SPP may be a specific type of angiogenic factor. It is possible that SPP plays a role in normal blood vessel formation or in injury, when local production of SPP could be increased by activated platelets, and extravasation of intravascular fluid could also present SPP into tissues at concentrations sufficient to promote angiogenesis and wound healing. Elucidation of the molecular mechanisms by which SPP stimulates cell migration and angiogenesis might provide clues for development of new therapeutic agents to either promote or block these processes by targeting the EDG family of GPCR.

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REFERENCES

1. Spiegel, S., Cuvilier, O., Edsall, L. C., Kohama, T., Menzeleev, R., Olah, Z., Olivera, A., Piriyanov, G., Thomas, D. M., Tu, Z., Van Brocklyn, J. R., and Wang, P. (1998) Ann. N. Y. Acad. Sci. 845, 11–18

2. Zhang, H., Desai, N. N., Sollner, A., Suki, T., Brooker, G., and Spiegel, S. (1991) J. Cell Biol. 114, 155–167

3. Olivera, A., and Spiegel, S. (1993) Nature 365, 557–560

4. Cuvilier, O., Piriyanov, G., Kleuser, B., Uesaka, P. G., Caso, O. A., Gutfred, S., and Spiegel, S. (1996) Nature 381, 800–803

5. Hamada, K., Nakamura, H., Oda, T., Hirano, T., Shimizu, N., Uyama, H., and Takuwa, Y. (1998) Biochem. Biophys. Res. Commun. 244, 745–750

6. Kleuser, B., Cuvilier, O., and Spiegel, S. (1998) Cancer Res. 58, 1817–1824

7. Machwate, M., Rodan, S. B., Rodan, G. A., and Harada, S. I. (1998) Mol. Pharmacol. 54, 70–77

8. Peretz, G. I., Knudson, C. M., Leykin, L., Korsmeyser, S. J., and Tilly, J. L. (1997) Nat. Med. 3, 1228–1322

9. Van Brocklyn, J. R., Lee, M. J., Menzeleev, R., Olivera, A., Edsall, L., Cuvilier, O., Thomas, D. M., Coopman, P. J., Wang, D., and Spiegel, S. (1998) J. Cell Biol. 142, 229–240

10. Goodmote, K. A., Mattie, M. E., Berger, A., and Spiegel, S. (1995) J. Biol. Chem. 270, 10272–10277

11. Wu, J., Spiegel, S., and Sturgill, T. W. (1995) J. Biol. Chem. 270, 11448–11458

12. Okamoto, H., Takeshi, N., Koda, K., Okazaki, H., Chang, K., Yatomi, Y., and Hu, D. (1998) J. Biol. Chem. 273, 27104–27110

13. Sato, K., Tomura, H., Okazaki, Y., and Takada, Y. (1999) Biochem. Biophys. Res. Commun. 250, 643–649

14. Kimura, S., Sillard, R., Harii, K., and Takuwa, Y. (1999) J. Biol. Chem. 274, 126–133

15. van Koppen, C. J., Meyer zu Heringdorf, D., Lasser, K. T., Zhang, J., Jakobs, K. H., Bunnemann, M., and Pott, L. (1996) J. Biol. Chem. 271, 2082–2087

16. Sadahira, Y., Ruan, F., Hakomori, S., and Igarashi, Y. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9068–9069

J. P. Hobson and S. Spiegel, unpublished observation.
