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

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Summary (241 words)

Sphingosine 1-phosphate (S1P) is a platelet-derived sphingolipid that elicits numerous biological responses in endothelial cells mediated by a family of G protein-coupled EDG receptors. Stimulation of EDG receptors by S1P has been shown to activate the endothelial isoform of nitric oxide synthase (eNOS) in heterologous expression systems (J Biol Chem 2000, 275: 32363). However, the signaling pathways that modulate eNOS regulation by S1P/EDG in vascular endothelial cells remain less well understood. We now report that S1P treatment of bovine aortic endothelial cells (BAEC) acutely increases eNOS enzyme activity; the EC50 for S1P activation of eNOS is ~10 nM. The magnitude of eNOS activation by S1P in BAEC is equivalent to that elicited by the agonist bradykinin. S1P treatment activates Akt, a protein kinase implicated in phosphorylation of eNOS. S1P treatment of BAEC leads to eNOS phosphorylation at Ser1179, a residue phosphorylated by Akt; an eNOS mutant in which this Akt phosphorylation site is inactivated shows attenuated S1P-induced eNOS activation. S1P-induced activation both of Akt and of eNOS is inhibited by pertussis toxin; by the phosphoinositide 3-kinase inhibitor wortmannin; and by the intracellular calcium chelator 1,2-bis-o-aminophenoxyethane-N,N,N’,N’-tetraacetic acid tetra(acetoxymethyl) ester. By contrast to S1P, activation of G protein-coupled bradykinin B2 receptors neither activates kinase Akt nor promotes Ser1179 eNOS phosphorylation despite robustly activating eNOS enzyme activity. Understanding the differential regulation of protein kinase pathways by S1P and bradykinin may lead to the identification of new points for eNOS regulation in vascular endothelial cells.
Introduction

Vascular endothelial cells respond to intercellular messengers that modulate processes as diverse as blood pressure homeostasis, platelet aggregation and angiogenesis (reviewed in (1,2)). Blood platelets secrete sphingosine 1-phosphate (S1P), a biologically active sphingolipid that has been broadly implicated in angiogenesis, platelet activation, and inhibition of apoptosis in vascular cells (3,4). S1P has also been characterized as a potent signal transducing molecule that may exert such diverse biological responses as cellular differentiation (5,6), hypertrophy (7), proliferation (8), and migration (9-11). S1P is a potent ligand for the G protein-coupled receptors termed EDG receptors (reviewed in (12)). The EDG receptor family is comprised of at least 8 independent subtypes (EDG-1-8, see refs. (12-15)). EDG receptors are coupled via pertussis toxin-sensitive G proteins to the activation of the mitogen-activated protein kinase (MAP kinase) pathway (16) and to the elevation of intracellular calcium concentration (10) in signal transduction pathways leading to angiogenesis (17). Additionally, S1P binding to the EDG-1 receptor has recently been shown to activate the endothelial isoform of nitric oxide synthase (eNOS) (18), a key signaling protein that plays a pivotal role in the maintenance of vascular homeostasis by promoting vascular smooth muscle relaxation and inhibiting platelet aggregation (1).

The EDG-1 receptor and eNOS are both localized to plasmalemmal caveolae, which are specialized sphingolipid-enriched domains in the plasma membrane that serve as sites for the sequestration of diverse signaling proteins (18,19). However, the specific intracellular signaling pathways that couple S1P/EDG receptor stimulation to eNOS activation remain less well understood. eNOS is a calcium-calmodulin-dependent enzyme: in vascular
endothelial cells, eNOS is activated in response to the transient increases in intracellular calcium initiated by the activation of diverse G protein-coupled receptors, including the bradykinin B2 receptor.

eNOS is also activated by phosphorylation by protein kinase Akt (20,21), and eNOS is inhibited by the MAP kinases ERK1/2 (22). eNOS phosphorylation by protein kinase Akt is promoted by vascular endothelial growth factor (VEGF) (20,23) and by fluid shear stress (21,24), but Akt activation has not been previously implicated in eNOS regulation by G protein-coupled receptors. The protein kinase Akt (known also as protein kinase B; reviewed in (25)) functions as a downstream effector of phosphoinositide 3-kinase (PI3-K)-dependent signaling pathways (reviewed in (26)), and plays pivotal roles in numerous cellular responses including angiogenesis (27). Although G protein-coupled receptors may regulate the PI3-K/Akt pathway, the activation of Akt by S1P-induced EDG receptor stimulation has not been previously described.

In the present study, we provide evidence that S1P potently and robustly activates eNOS in cultured vascular endothelial cells via EDG receptors in a pathway that involves G protein-dependent activation of kinase Akt. eNOS activation by the S1P/EDG pathway stands in contrast to the activation of eNOS by bradykinin B2 receptors, in which activation of eNOS appears to proceed independently of Akt-mediated phosphorylation.
Experimental Procedures

Materials.

Fetal bovine serum (FBS) was from Hyclone (Logan, CT); all other cell culture reagents, media and LipofectAMINE were from Life Technologies (Rockville, MD). S1P and dihydro-S1P (sphinganine 1-phosphate) were from BioMol (Plymouth Meeting, PA). PD98059 and 1,2-bis-\(\alpha\)-aminoxyloxyethane-\(N,N,N',N'\)-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA) were from Calbiochem (San Diego, CA). Anti-phospho-eNOS antibody (phosphoserine 1177 in the human eNOS sequence; corresponding to Ser1179 in bovine eNOS), anti-phospho-Akt antibody (Ser473), anti-Akt antibody, Akt enzyme activity kit and ERK enzyme activity kit were from Cell Signaling Technologies (Beverly, MA). Anti-phospho-ERK1/2 antibody (Thr183/Tyr185) was from Promega (Madison, WI). Anti-eNOS monoclonal antibody was from Transduction Laboratories (Lexington, KY). Super Signal substrate for chemiluminescence detection and secondary antibodies conjugated with horseradish peroxidase were from Pierce (Rockford, IL). \(^{3}\text{H}\) L-Arginine was from Amersham (Arlington Heights, IL). Protein determinations were made with the BioRad Protein Assay Kit. All other reagents, including anti-FLAG monoclonal antibody, were from Sigma (Saint Louis, MO).

Plasmid construction.

cDNA encoding full-length human EDG-1 receptor epitope-tagged with FLAG peptide (FLAG/EDG-1, described in (16), was provided by Timothy Hla, University of Connecticut) was subcloned into pcDNA3 (Invitrogen) as previously described (18). Full-length wild type bovine eNOS cDNA subcloned into pBK-CMV was described previously (28). eNOS S1179A mutant cDNA was made with PCR-based mutagenesis and verified with standard DNA sequencing technique (22).
Cell Culture, Transfection and Drug Treatment.

BAEC were obtained from Cell Systems (Kirkland, WA), and maintained in culture in Dulbecco’s modified Eagle media (DMEM) supplemented with FBS (10%, v/v) as described (29). Cells were plated onto gelatin-coated culture dishes and studied prior to cell confluence between passages 5 - 9.

COS-7 cells were maintained in culture as described previously (30). The day before transfection, cells were split at a ratio of 1:8 in DMEM containing 10% FBS. They were then co-transfected with cDNAs encoding FLAG/EDG-1 (2 µg) and/or eNOS (0.03 µg) by using LipofectAMINE, as previously described (18). The total DNA amount was normalized using “empty” (no insert) vector plasmid DNA. For both BAEC and transfected COS-7 cells, culture media was changed to DMEM without FBS and incubation proceeded overnight prior to all experiments (10), to exclude the effects of S1P contained in FBS.

S1P and dihydro-S1P were solubilized in methanol and stored at −20 °C; the same volume of methanol was used as a vehicle-control, and the final concentration of methanol did not exceed 0.4% (v/v) in any experiment. PD98059, wortmannin and BAPTA were solubilized in dimethyl sulfoxide (DMSO) and kept at −20 °C; where indicated, DMSO (0.1% v/v) was used as a vehicle-control.

Western Blot Analyses.

Protein expression and the degree of protein phosphorylation were assayed by the western blot analysis. After drug treatments, cells in a 60 mm dish were washed with ice-cold PBS and incubated for 10 min on ice with 500 µl of lysis buffer containing: Tris (20 mM; pH 7.5), EDTA (1 mM), EGTA (1 mM), Triton-X (1%, v/v), NaCl (150 mM), Na3VO4 (1 mM), sodium pyrophosphate (2.5 mM), β-glycerophosphate (1 mM) and a cocktail of
protease inhibitors (as described in (31)). Cells were harvested by scraping and centrifuged at 4 °C for 10 min in a microfuge. The supernatant was analyzed for protein concentration, and an equal amount of cellular proteins (20 µg/lane) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then probed with the appropriate primary antibody: membrane-bound primary antibodies were visualized by using secondary antibodies conjugated with horseradish-peroxidase and Super Signal substrate, as previously described (32). Densitometric analyses of western blots were performed using a ChemiImager 4000 (Alpha-Innotech).

**Immuno-Kinase Assay.**

The enzyme activity of Akt or ERK1/2 in BAEC was determined *in vitro* by using commercially available kinase activity kits following the supplier’s protocol. Briefly, an equal amount (70 µg) from each cell lysate described above was adjusted to 200 µl with cell lysis buffer. Akt or ERK1/2 was immunoprecipitated by using an immobilized anti-Akt or anti-ERK1/2 antibody conjugated to agarose beads, for 2 h at 4 °C. After being extensively washed, the beads were incubated with 40 µl of kinase buffer comprising Tris (25 mM; pH 7.5), β-glycerophosphate (5 mM), dithiothreitol (2 mM), Na₃VO₄ (0.1 mM), MgCl₂ (10 mM), ATP (200 µM) and 0.5 µg of recombinant glycogen synthase kinase-3 (GSK-3) protein (for Akt assay) or 1 µg of Elk-1 protein (for ERK assay), 30 min at 30 °C. Note that GSK-3 and Elk-1 are well-established substrates for Akt (33) and for ERK (34), respectively, and serve here as substrates in the BAEC-derived protein kinase reaction. The reaction was terminated by adding 13 µl of 4X SDS sample buffer and boiling. The resulting reaction mixture was then separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed for phosphorylated form of GSK-3 or Elk-1 by western blot analysis, as described above. The degree of enzyme
activation was determined as a fold increase of phosphorylation in each substrate protein relative to basal kinase activity.

**Quantitation of intracellular NO generation.**

eNOS enzyme activity was quantified as the formation of [3H] L-citrulline from [3H] L-arginine, essentially as described previously (18,35). Briefly, cells in a given well of a 6 well plate were incubated in 800 µl of buffer containing HEPES (25 mM, pH 7.3), NaCl (109 mM), KCl (5.4 mM), CaCl2 (0.9 mM), MgSO4 (1 mM) and glucose (25 mM) for 1 h at 37 °C. eNOS activity was assayed by adding a mixture of unlabelled L-arginine (10 µM), [3H] L-arginine (10 µCi/ml) and various concentrations of S1P or vehicle to the culture (each treatment was performed in triplicate cultures, and then each was assayed in duplicate). Following incubation at 37 °C for 10 min, cells were washed with ice-cold PBS, scraped into 2 ml of solution containing 20 mM sodium acetate, 1 mM L-citrulline, 2 mM EDTA and 2 mM EGTA, pH 5.5, followed by sonication. An aliquot was withdrawn for determination of the total protein content and total cellular [3H] incorporation, and the remaining sample was applied to Dowex 50WX8-400 column to separate [3H] L-citrulline. The flow-through fraction was analyzed by liquid scintillation counting; [3H] L-citrulline formation in the cells was expressed as fmol [3H] L-citrulline produced/mg cellular protein/minute.

**Other methods.**

All experiments were performed at least 3 times. Mean values for individual experiments are expressed as mean ± S.E.M. Statistical differences were analyzed by ANOVA followed by Scheffe’s F test using STAT VIEW II (Abacus Concepts). A p value less than 0.05 was considered statistically significant.
Results

We first studied the responses of BAEC to S1P by analyzing the effects of S1P on activation of protein kinases Akt and ERK1/2 (Figure 1). We exploited two independent experimental approaches to assess Akt or ERK activation in BAEC: we analyzed immunoblots probed with antibodies that specifically detect the phosphorylated (activated) forms of these kinases, and also performed in vitro kinase activity assays using protein substrates specific for kinases Akt and ERK. Note that the activation of these kinases is accompanied by the phosphorylation of specific amino acid residues in these proteins (25,36). Following the addition of S1P (100 nM) to BAEC, we detected increases in Akt phosphorylation as well as Akt enzyme activity within 1 min, reaching a maximum ~4 fold increase in activity by 2-5 min, with a gradual return to basal levels seen at 120 min following drug addition (Figure 1). Immunoblot analyses probed with an anti-Akt antibody were used to verify that these cell lysates contain equivalent amounts of Akt protein. The MAP kinases ERK1/2 also undergo robust and rapid phosphorylation and kinase activation following addition of S1P to BAEC; ERK1/2 activity and phosphorylation levels returned to the basal within 20 min following S1P addition (Figure 1). In these same cell lysates from S1P-treated BAEC, we analyzed immunoblots probed with an antibody raised against a phosphopeptide comprising the eNOS amino acid sequence around Ser1179 (1179S), the site at which eNOS undergoes phosphorylation by kinase Akt (20,21). As shown in Figure 1, S1P treatment of BAEC markedly increased eNOS phosphorylation at Ser1179, with a time course similar to that seen for Akt activation.

We next analyzed the dose response to S1P for Akt and ERK1/2 activation as well as 1179S-phospho-eNOS formation in BAEC treated for 5
min with increasing concentrations of S1P. Figure 2A shows immunoblots of cell lysates probed with antibodies directed against phospho-Akt (top panel), phospho-ERK1/2 (middle panel) or \(1179S\)-phospho-eNOS (lower panel). S1P-induced activation of both Akt and ERK1/2, as well as \(1179S\)-phospho-eNOS formation, all showed similar EC\(_{50}\) values of \(~10\) nM (Figure 2B).

Treatment of BAEC with the S1P analog dihydro-S1P (which acts solely via EDG receptors (15)) led to Akt phosphorylation as well as \(1179S\)-phospho-eNOS formation (data not shown). Together, these data indicate that S1P induces the reversible receptor-mediated activation of Akt and ERK1/2, and also promotes the synthesis of \(1179S\)-phospho-eNOS, with EC\(_{50}\) values in a physiologic range.

To further characterize S1P-induced Akt and ERK activation as well as \(1179S\)-phospho-eNOS formation, BAEC were treated with various inhibitors prior to the addition of S1P. As shown in Figure 3, pretreatment with pertussis toxin completely abolished S1P-induced Akt and ERK1/2 activation as well as \(1179S\)-phospho-eNOS synthesis, indicating that the effects of S1P are dependent upon the activation of pertussis toxin-sensitive G protein pathways. Interestingly, the intracellular calcium chelator BAPTA completely inhibited Akt activation and \(1179S\)-phospho-eNOS synthesis, suggesting that activation of the PI3-K/Akt pathway by S1P is dependent upon the elevation of intracellular calcium concentration (Figure 3). In contrast, BAPTA did not block S1P-induced ERK1/2 activation; rather, calcium chelation with BAPTA led to a marked increase in the phosphorylation of these MAP kinase pathway proteins. Wortmannin, an inhibitor of PI3-K (an upstream activator of Akt), blocked S1P-mediated Akt activation and \(1179S\)-phospho-eNOS formation, but did not inhibit S1P-mediated activation of ERK1/2 (Figure 3). In contrast to the effects of
wortmannin, the kinase inhibitor PD98059 (which inhibits the MAP kinase kinase MEK, which in turn phosphorylates ERK1/2), abolished ERK1/2 activation by S1P, but blocked neither S1P-mediated activation of Akt nor 1179S-phospho-eNOS synthesis (Figure 3). These experiments demonstrate that the S1P-mediated activation of Akt and ERK1/2 and the formation of 1179S-phospho-eNOS are mediated by pertussis toxin-sensitive G proteins, and that Akt activation and 1179S-phospho-eNOS formation are calcium-dependent whereas ERK1/2 activation is not.

We next examined features of S1P-mediated eNOS activation in BAEC. Figure 4A shows the results of a S1P dose-response experiment in which cellular eNOS activity was quantitated by measuring the formation of [3H]L-citrulline in BAEC loaded with [3H]L-arginine, applying a well-characterized assay for cellular NO synthesis (35). The NOS inhibitor L-ωN-nitro-arginine completely abolishes S1P-induced NO synthesis in BAEC (Figure 4B). S1P-mediated eNOS activation is also blocked by the calcium chelator BAPTA and by pertussis toxin treatment, as shown in Figure 4. The PI3-K inhibitor wortmannin, which completely abolishes eNOS phosphorylation (Figure 3), attenuates but does not completely abrogate S1P-stimulated eNOS activation (Figure 4B): S1P mediated eNOS activation is reduced by 70% but residual S1P-augmented enzyme activity remains above basal (p < 0.05). PD98059 is without effect, suggesting that S1P-induced ERK1/2 activation (Figure 3) does not modulate eNOS in these experimental settings (Figure 4B).

We further explored the relationships between S1P-induced kinase activation and S1P-modulated eNOS activation by performing transient transfection experiments using cDNA constructs encoding eNOS and EDG-1 in COS-7 cells, following previously described co-transfection protocol (18).
It is of note that COS-7 cells do not express endogenous EDG-1 receptors (37). We studied wild-type eNOS cDNA as well as an eNOS mutant in which the putative kinase Akt phosphorylation site 1179Ser is changed to Ala (S1179A) (20-22) in cotransfection experiments with a cDNA construct encoding the EDG-1 receptor epitope-tagged with FLAG peptide (FLAG/EDG-1). Note that this epitope-tagged receptor construct retains essential features of wild type EDG-1 receptor (5,16,18,38). COS-7 cells transiently expressing FLAG/EDG-1 and/or eNOS cDNAs were treated with S1P (100 nM, 5 min); cell lysates were probed by western blot analysis with various antibodies, as shown in Figure 5. The overall expression of endogenous kinase Akt protein was unaffected by cDNA transfections; expression levels of the variously transfected cDNAs were concordant with the specific cDNA constructs used in these experiments (Figure 5A). S1P treatment induces the phosphorylation (activation) of Akt only in cells transfected with the EDG-1 cDNA. Similarly, 1179S-phospho-eNOS formation is observed only when S1P is added to cells co-transfected with EDG-1 and wild-type eNOS cDNAs. Importantly, when cells are co-transfected with EDG-1 and the S1179A mutant eNOS, S1P-induced 1179S-phospho-eNOS formation was not detected despite robust phosphorylation of Akt (Figure 5A). We next examined the effects of S1P on eNOS activation in these cells. COS-7 cells transiently expressing FLAG/EDG-1 and eNOS cDNAs (wild type or S1179A) were incubated with [3H] L-arginine and treated with S1P or vehicle; cells were harvested and lysates were analyzed for [3H] L-citrulline formation as described above. As we have observed previously (18), S1P activates eNOS in cells co-transfected with FLAG/EDG-1 and wild type eNOS, with an EC50 of ~20 nM (Figure 5B).
markedly attenuated response to S1P compared with wild type eNOS (Figure 5B), despite the fact that these cells express eNOS protein at equivalent levels (Figure 5A).

We next explored the differential regulation of S1P-mediated responses by directly comparing the signaling pathways elicited by S1P with those activated by bradykinin, a well known activator of eNOS in BAEC (39). As shown in Figure 6A, bradykinin (1 µM) induces the activation (phosphorylation) of ERK1/2 in BAEC, as we have reported previously (Figure 6A, (22)). However, bradykinin does not induce the activation of Akt or formation of 1179S-phospho-eNOS, although the same cell preparations respond to S1P (Figure 6A). We compared bradykinin and S1P-mediated eNOS activation in BAEC, as shown in Figure 6B. Although bradykinin and S1P both activate eNOS to a similar extent, bradykinin-induced eNOS activation is not inhibited by pertussis toxin nor by wortmannin, in contrast to the inhibitory effects of these agents on S1P-mediated eNOS activation. PD98059 has no substantive effect on bradykinin-dependent eNOS activation in this experimental system. It is notable that bradykinin and S1P activate eNOS to a similar magnitude (Figure 6B). When cells are treated with both bradykinin and S1P in combination, there is no additive increase in eNOS activity compared to cells treated individually with bradykinin or S1P.
Discussion

These studies have provided several lines of evidence demonstrating
that S1P induces eNOS activation in cultured endothelial cells, at least in part
mediated by eNOS Ser1179 phosphorylation and involving the PI3-K/Akt
pathway. As shown in Figure 1, the addition of S1P rapidly activates Akt
kinase and ERK1/2, concomitant with eNOS phosphorylation on serine 1179.
S1P-mediated Akt activation and eNOS 1179S-phosphorylation persists for
up to two hours, whereas ERK1/2 phosphorylation returns to basal levels
within 20 minutes, suggesting that activation of the MAP kinase pathway may
be involved in shorter-term regulation of eNOS than the PI3-K/Akt pathway
(Figure 1). Dose-response experiments (Figure 2) show that the effects of
S1P on activation of Akt, ERK1/2, and eNOS 1179S-phosphorylation all
show a similar EC50 of ~10 nM. Our observation of an EC50 for S1P-
mediated eNOS activation in the low nanomolar range is consistent with
values previously observed for other S1P-mediated responses (5).
Concentrations of S1P in human blood have been measured in the range of
several hundred nanomolar (40). However, the extent to which extracellular
S1P may be protein-bound (or otherwise complexed) has not been rigorously
determined, such that the concentration of free “biologically active” S1P
available for interaction with EDG receptors may be considerably lower.
Moreover, local blood concentrations of S1P may acutely increase when the
compound is released from activated platelets (4,40), suggesting that levels of
S1P may be subject to physiological regulation. Thus, the potency of S1P in
modulating eNOS signaling pathways in endothelial cells indicates that this
compound may dynamically regulate NO-dependent signal transduction
pathways in the vascular wall in physiological or pathophysiological states.

S1P-mediated activation of Akt, ERK1/2 and eNOS 1179S-
phosphorylation are alike completely blocked by pertussis toxin (Figure 3), indicating that these signaling responses are all mediated by pertussis toxin-sensitive G protein pathways in endothelial cells. These results demonstrate that the responses to S1P are mediated by G protein-coupled cell surface (EDG) receptors, rather than via intracellular mechanisms. Many of EDG receptor-mediated signaling events involve the activation of pertussis toxin-sensitive G proteins, but it has not previously been reported that S1P activates the PI3-K/Akt pathway. Other G protein-coupled receptors have been observed to involve the PI3-K/Akt pathway (41-43) and recent studies have provided insight into the mechanisms whereby G protein subunits modulate PI3-K activity. For example, the PI3-K catalytic subunit P110γ, together with its regulatory subunit P101, can be activated by G protein βγ subunits, leading to PI3-K activation (44). Other investigators have more recently shown that PI3-K catalytic subunit P110β can directly respond to G protein βγ subunit (45). Interestingly, lysophosphatidic acid, another ligand for EDG receptors, induces PI3-K/Akt activation in COS-7 cells (42). Taken together with our observations, these studies suggest that endothelial cells have signal transduction machinery that connects EDG receptor stimulation with the activation of PI3-K/Akt pathway.

We were surprised to find that the intracellular calcium chelator BAPTA abrogated Akt activation in BAEC (Figure 3). It is unlikely that this effect of BAPTA reflects non-specific or toxic effects of the compound, since identical treatments with BAPTA did not block S1P-mediated ERK1/2 activation. To our knowledge, there has been no prior report documenting that the activation of PI3-K/Akt by G protein-coupled receptors is calcium-dependent. However, the fact that BAPTA abrogates Akt activation (and eNOS 1179S-phosphorylation) suggests that elevation of intracellular
calcium concentration may be required for S1P-mediated activation of the PI3-K/Akt pathway in endothelial cells. Interestingly, BAPTA did not abolish ERK1/2 phosphorylation, but instead reproducibly induced hyperphosphorylation of these kinases (Figure 3). The differential effects of BAPTA on ERK1/2 versus Akt pathways may indicate the divergence of signaling pathways downstream from S1P-mediated EDG receptor activation in endothelial cells. However, it must be also noted that different EDG receptor subtypes may have significantly different pharmacological properties in response to the same lipid ligands (38,46,47). Although we have shown that the EDG-1 receptor subtype is fully able to activate Akt and eNOS in transfected COS-7 cells (Figure 5), it is quite plausible that BAEC express more than one EDG receptor isoform (9,10). Thus, other EDG receptors such as EDG-3 may also be able to activate Akt and/or eNOS in endothelial cells.

The PI3-K inhibitor wortmannin inhibited S1P-mediated Akt activation and eNOS 1179S-phosphorylation, while having no substantive effect on S1P-mediated ERK1/2 activation (Figure 3). By contrast the MEK inhibitor PD98059 blocked ERK1/2 activation while having no substantive effect on S1P-mediated Akt activation, eNOS 1179S-phosphorylation (Figure 3) or eNOS enzyme activity (Figure 4). Thus in these experimental settings, S1P-induced MAP kinase activation does not seem to play a major role in eNOS regulation. Nonetheless, MAP kinase activation by S1P plays many other important roles, for example, the formation of cadherin-based cell-cell junctions (5,17) or cell migration (9,10) in vascular endothelial cells.

The present study provides the first evidence that G protein-coupled receptor stimulation can lead to Akt-dependent eNOS phosphorylation. However, not all of S1P’s effect on eNOS activation is due to eNOS 1179S-phosphorylation: as shown in Figure 5, the S1179A eNOS mutant still
undergoes S1P-dependent activation (albeit less than the wild-type enzyme), and the PI3-K inhibitor wortmannin only partially blocks S1P-induced eNOS activation (Figure 4), while this inhibitor completely blocks S1P-induced Akt activation (Figure 3). These results also point up key differences between two different G protein-coupled receptors, the EDG receptor pathway activated by S1P, and B2 receptor pathway activated by bradykinin. In contrast to S1P, bradykinin does not activate Akt kinase nor promote eNOS 1179S-phosphorylation (Figure 6). However, bradykinin still promotes robust activation of eNOS to levels comparable to S1P without appearing to involve the PI3-K pathway.

S1P and EDG receptors are implicated in signal transduction pathways leading to angiogenesis (17). Interestingly, eNOS and PI3-K/Akt are also independently implicated in angiogenesis. For example, angiogenic polypeptide growth factors such as VEGF lead to eNOS enzyme activation (48,49), and overexpression of a dominant-negative PI3-K construct markedly attenuates angiogenesis (27). Moreover, these signaling molecules share common features of their subcellular localization: plasmalemmal caveolae serve as the sites for localization of eNOS (19), EDG-1 receptor (18), as well as members of the PI3-K/Akt pathway (50). The present study establishes that S1P activates eNOS as well as the PI3-K/Akt pathway, and may implicate this signaling system in regulation of angiogenic responses in vascular endothelial cells.

Remarkable differences between S1P and bradykinin-mediated regulation of PI3-K/Akt, MAP kinase and eNOS may identify additional points for control of nitric oxide-dependent signal transduction pathways in vascular endothelial cells. Furthermore, it is notable that S1P, which had not been previously identified as an activator of NO synthesis in endothelial cells,
has been shown in these studies to activate eNOS to a level comparable to that elicited by bradykinin. These cellular responses to S1P occur at concentrations well within the physiological range of this sphingolipid. Taken together, our observations suggest that sphingosine 1-phosphate may have an important role in the modulation of NO-dependent signaling in the vasculature.
Footnotes

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The abbreviations used in this paper are: S1P, sphingosine 1-phosphate; MAP kinase, mitogen-activated protein kinase; ERK, extracellular-stimuli regulated kinase; eNOS, endothelial isoform of nitric oxide synthase; VEGF, vascular endothelial growth factor; PI3-K, phosphoinositide 3-kinase; FBS, fetal bovine serum; dihydro-S1P, sphinganine 1-phosphate; BAPTA, 1,2-bis-o-aminophenoxyethane-N,N,N’,N’-tetraacetic acid tetra(acetoxymethyl) ester; FLAG/EDG-1, EDG-1 receptor epitope-tagged with FLAG peptide; DMEM, Dulbecco’s modified Eagle Media; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; GSK-3, glycogen synthase kinase-3.
References
Figure Legends

Figure 1. S1P-induced activation of Akt, ERK1/2 and eNOS Serine phosphorylation.

Panel A shows the results of immunoblots and immuno-kinase assays analyzed in BAEC lysates prepared from cells treated with S1P (100 nM) for the indicated times. Cell lysates (20 µg/lane) were resolved by SDS-PAGE and probed using antibodies directed against phospho-Akt, Akt, phospho-ERK1/2, and phospho-eNOS (Ser1179) antibodies. Signals corresponding to the proteins indicated on the left of each panel were shown. The same cell lysates (70 µg/lane) were also subjected to “immuno-kinase assays” as described in detail under Experimental Procedures. Akt or ERK1/2 were immunoprecipitated from BAEC lysates and incubated with their corresponding substrate proteins in the presence of ATP. The reaction mixture was then resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the antibodies specific to phosphorylated forms of the substrate proteins. Signals corresponding to phospho-GSK-3 (Akt substrate) and phospho-Elk-1 (ERK1/2 substrate) are shown as “Akt activity” and “ERK activity”, respectively. Shown are the representative data of 4 independent experiments. Panel B shows the results of densitometric analyses from pooled data, plotting the fold increase of the degree of phosphorylation of Akt, ERK1/2 and eNOS at the time indicated, relative to the signals present at t = 0. Each data point represents the mean ± S.E.M. derived from 4 independent experiments.

Figure 2. Dose response for S1P-mediated activation of Akt, ERK1/2 and eNOS Serine phosphorylation.

Panel A shows the results of immunoblot analyses using the BAEC cell
lysates prepared from cells that had been treated with S1P (5 min) at the indicated concentrations. Cell lysates (20 µg/lane) were resolved by SDS-PAGE and probed with antibodies directed against phospho-Akt, phospho-ERK1/2, and phospho-eNOS (Ser1179), as indicated. Shown are the representative data of 4 independent experiments. **Panel B** shows the results of densitometric analyses from pooled data, plotting the fold increase of the degree of phosphorylation of Akt, ERK1/2 and eNOS at the S1P-concentration indicated, relative to the signals obtained in the absence of S1P. Each data point represents the mean ± S.E.M. derived from 4 independent experiments.

**Figure 3. Pharmacological characterization of S1P-mediated phosphorylation of Akt, ERK1/2 and eNOS.**

Shown are the results of western blot analyses for phosphorylated forms of Akt, ERK1/2 and eNOS, using the cell lysates of BAEC prepared from cells treated with S1P. Prior to S1P treatment (100 nM for 5 min), BAEC had been treated with various reagents (pertussis toxin (PTx), 50 ng/ml for overnight; BAPTA, 20 µM for 30 min; wortmannin (WORT), 500 nM for 30 min; PD98059, 10 µM for 30 min), or vehicle. Equal amount of cell lysate (20 µg/lane) were separated by SDS-PAGE and probed with antibodies directed against phospho-Akt, phospho-ERK1/2, and phospho-eNOS (Ser1179) antibodies, as indicated. Equal loading of samples was also confirmed by re-probing the immunoblots with antibodies against eNOS and Akt (not shown). Shown are data representative of 4 independent experiments.

**Figure 4. eNOS activation by S1P.**

Shown are the results of an eNOS activaty assay performed in BAEC
treated with S1P as indicated. **Panel A: Dose-response.** BAEC were treated with increasing concentrations of S1P and subjected to eNOS activity assays (assessed as [³H] L-citrulline formation from [³H] L-arginine) as described in detail under *Experimental Procedures.* Each data point represents the mean ± S.E.M. derived from 4 independent cell preparations. **Panel B:** Perturbation of S1P-mediated eNOS activation. Prior to treatment with S1P (100 nM) or vehicle, BAEC were treated with various reagents as indicated (Nω-nitro-L-arginine (L-NNA), 100 nM for 30 min; pertussis toxin (PTx), 50 ng/ml for overnight; BAPTA, 20 µM for 30 min; wortmannin (WORT), 500 nM for 30 min; PD98059, 10 µM for 30 min). Then they were subjected to eNOS activation assay as described above. Each data point represents mean ± S.E.M. derived from 4 independent cell preparations.

**Figure 5.** Effects of S1P on Akt and eNOS signaling in COS-7 cells co-transfected with EDG-1 plus wild-type or S1179A mutant eNOS.

**Panel A: Effects of S1P on phosphorylation of Akt and eNOS.** Shown are the results of western blot analyses in COS-7 cells co-transfected with cDNA encoding FLAG/EDG-1 and/or eNOS (wild type eNOS (WT eNOS) or S1179A mutant), as indicated. The total DNA amount was normalized with “empty” vector plasmid DNA for each transfection. Cells were treated with S1P (100 nM for 5 min) or vehicle, as indicated. Cell lysates (20 µg/lane) were separated by SDS-PAGE and probed using antibodies directed against the FLAG epitope, Akt, phospho-Akt, phospho-eNOS (Ser1179), or eNOS, as indicated. Shown are the results of a representative that was independently replicated three times with equivalent results. **Panel B: Effects of S1P on eNOS activity.** Shown are the results of eNOS activity assays performed in COS-7 cells co-transfected with FLAG/EDG-1 and eNOS.
(wild type or S1179A). Cells were treated with increasing concentrations of S1P and subjected to eNOS activation assay as described in detail under Experimental Procedures. Each data point represents the mean ± S.E.M. derived from 3 independent cell preparations, each performed in duplicate. *; P < 0.05 vs. vehicle treatment. †; P < 0.05 vs. wild type-eNOS transfected cells.

Figure 6. Differential regulation of Akt, ERK1/2 and eNOS by S1P and bradykinin in BAEC.

Panel A: Effects of S1P and bradykinin on phosphorylation of Akt, ERK1/2 and eNOS. Shown are the results of immunoblots probed with antibodies directed against phosphorylated forms of Akt, ERK1/2 and eNOS. BAEC were treated with S1P (100 nM) or bradykinin (BK, 1 µM) for the times indicated on the same day of experiment. Equal amount of cell lysate (20 µg/lane) was resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies directed against phospho-Akt, phospho-ERK1/2, and phospho-eNOS, as indicated. Equal loading of samples was also confirmed by re-probing the immunoblots with antibodies against eNOS and Akt (not shown). Shown are the results from a representative data from an experiment that was independently repeated for three times with equivalent results. Note that bradykinin induces phosphorylation of ERK1/2 only, but not eNOS or Akt, whereas S1P induces phosphorylation of all these proteins. Panel B: The effects of S1P and bradykinin on eNOS activity. Shown are the results of eNOS activity assays performed in BAEC treated with S1P (100 nM) or bradykinin (1 µM). In some cultures, cells had been treated with various reagents as indicated (pertussis toxin (PTx), 50 ng/ml overnight; wortmannin (WORT), 500 nM for
30 min; PD98059, 10 µM for 30 min), prior to addition of BK. Some cells were treated with both S1P and BK in combination (indicated as BK + S1P). Each data point represents the mean ± S.E.M. derived from 3 independent cell preparations, each performed in triplicate.
A) 
phospho-Akt

phospho-ERK1/2

phospho-eNOS

[S1P], (M)

0 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}

B) 

Phosphorylation (Fold increase)

[S1P], (M)

0 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}
A) immunoblot

- total-Akt
- total-eNOS
- FLAG/EDG-1
- phospho-Akt
- phospho-eNOS

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B) [3H] Citrulline formation (fimole/mg prot/min)

- WT eNOS
- S1179A eNOS

[S1P]

0, 10^-9, 10^-8, 10^-7, 10^-6

*" and "†" indicate significance levels.
Legend for addendum: the effects of dihydro-S1P on Akt and eNOS in BAEC.

Shown are the results of western blot analysis in BAEC lysates prepared from cells that had been treated with S1P or dihydro-S1P (for 5 min) at the indicated concentrations. Cell lysates (20 μg/lane) were resolved by SDS-PAGE and probed with antibodies directed against phospho-Akt, Akt, phospho-eNOS (ser1179) and eNOS, as indicated. Note that both S1P and dihydro-S1P induce phosphorylation of Akt and eNOS to comparable extent.