Sphingosine 1-Phosphate Transactivates the Platelet-Derived Growth Factor β Receptor and Epidermal Growth Factor Receptor in Vascular Smooth Muscle Cells

Tatsuo Tanimoto, Andreea O. Lungu, Bradford C. Berk

Abstract—Sphingosine 1-phosphate (S1P) is a bioactive lipid generated during vascular injury that regulates cell growth, differentiation, survival, and motility via endothelial differentiation gene (EDG) family G protein–coupled receptors. Although several G protein–coupled receptor ligands transactivate receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR), S1P-stimulated receptor tyrosine kinase transactivation has not been well studied. We show that platelet-derived growth factor β receptor (PDGFB) and EGFR are tyrosine phosphorylated in response to S1P in rat aortic vascular smooth muscle cells (VSMCs). S1P-stimulated transactivation of PDGFB and EGFR was mediated via Gi-coupled EDG receptors. S1P-stimulated transactivation of EGFR and PDGFB was dependent on Src, reactive oxygen species, and cholesterol-rich membranes. A phosphoinositide 3-kinase–Akt pathway was activated by S1P and blocked by AG1296 and AG1478. Activation of extracellular signal–regulated kinase (ERK) 1 and ERK2 pathway by S1P was blocked only by AG1478. In Chinese hamster ovary cells that expressed exogenous EDG-1, activation of Akt and ERK1/2 in response to S1P was observed and was enhanced by coexpression of PDGFB or EGFR. S1P-mediated VSMC proliferation was shown to be secondary to transactivation, because it was suppressed by AG1296 and AG1478. These data establish S1P as an important stimulus for EGFR and PDGFB activation in VSMCs that may have important implications for the vessel response to injury. (Circ Res. 2004;94:1050-1058.)

Key Words: signal transduction ● epidermal growth factor ● sphingosine 1-phosphate

S1P, a bioactive lipid released by activated platelets, induces many biological responses, including cell proliferation, differentiation, survival, and motility.1–4 S1P is a ligand for the EDG family of G protein–coupled receptors (GPCRs). These receptors, which include EDG-1/S1P1, EDG-3/S1P3, EDG-5/S1P2, EDG-6/S1P4, and EDG-8/S1P5, all bind S1P and dihydro-S1P with high affinity but couple to different G-proteins and thus regulate diverse processes.5 Whereas EDG-1, EDG-6, and EDG-8 couple mainly to Gi, and motility.6 Conversely, EDG-5 seems to suppress VSMC motility.6 Transactivation of receptor tyrosine kinase (RTK) by binding of ligand to GPCRs has been shown to have important physiological consequences.12–16 Activation of EGFR and platelet-derived growth factor β receptor (PDGFB) by several GPCR ligands, including thrombin, angiotensin II (Ang II), lysophosphatidic acid (LPA), and endothelin-1, has been well studied.17–19 EGFR is also transactivated by PDGFB20 and insulin-like growth factor I.21 Because S1P is a relatively newly identified GPCR ligand, S1P-stimulated transactivation of RTK has not been well studied. Recently, our group and another reported that S1P transactivates the vascular endothelial growth factor receptor 2 (VEGFR2) and EGFR. S1P-mediated VSMC proliferation was shown to be secondary to transactivation, because it was suppressed by AG1296 and AG1478. These data establish S1P as an important stimulus for EGFR and PDGFB activation in VSMCs that may have important implications for the vessel response to injury.

Materials and Methods

Reagents

Sources of reagents are listed in the online data supplement, available at http://circres.ahajournals.org.
Cell Culture and Proliferation

VSMCs were isolated from the thoracic aorta of Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) and maintained in DMEM with 10% FBS, as described. To assay proliferation, VSMCs (25 000 cells) were seeded in 24-well plates in DMEM with 10% FBS. The next day, medium was changed to DMEM without FBS-containing agonists and incubated for 2 or 4 days. Then cells were trypsinized and cell number was counted. Chinese hamster ovary (CHO) cells were maintained in Ham's F12 medium supplemented with 10% FBS. CHO cells stably expressing mouse PDGFβR were a gift from Dr Harlan Ives and were maintained in 600 g/mL G418. pcDNA3.1-FLAG-EDG-1 and pcDNA3.1-EGFR were gifts from Drs Timothy Hla and Hamid Band, respectively.

Immunoblot Analysis

Western blot analyses were performed from ≥3 experiments, as described. Densitometric analyses were performed by NIH imaging. For analysis of data from several experiments, background intensity (lane 1) was subtracted from the intensity of each lane. Next, the value of the total pixel intensity for each protein on its Western blot was set to 100%, and the relative densitometry of each lane was expressed as percent of total. This approach was used because basal phosphorylation was so low in some experiments that fold increase was not a meaningful measurement.

Immunoprecipitation

Lysates containing equal amounts (300 µg) of protein were precleared with protein A/G PLUS-agarose and immunoprecipitated as described previously. Details are available in the online data supplement.

Detergent-Free Purification of Cholesterol-Rich Membrane Fractions

Cholesterol-rich membrane (CRM) fractions were prepared as reported by Song et al. Details are available in the online data supplement.

Superoxide Measurement

The redox-sensitive fluorescent dye hydroethidine was used to evaluate in situ production of superoxide. Details are available in the online data supplement.

Statistical Analysis

Data are presented as mean±SEM for all experiments that were performed at least three times. Data were evaluated by ANOVA followed by Dunnnett’s multiple-comparison tests.

Results

S1P Stimulates Tyrosine Phosphorylation of PDGFβR and EGFR

In response to 1 µmol/L S1P, tyrosine phosphorylation of PDGFβR and EGFR increased in a time-dependent manner (Figures 1A and 1B). Both PDGFβR and EGFR tyrosine phosphorylation peaked at 5 minutes, and PDGFβR phosphorylation decreased to basal levels by 120 minutes. EGFR tyrosine phosphorylation decreased but still remained at 120 minutes. c-Src, Akt, and ERK1/2 were also phosphorylated with peaks at 2, 5, and 10 minutes, respectively. PI3K and Shc, which contribute to growth factor–stimulated activation of Akt and ERK, were also tyrosine phosphorylated in...
response to S1P. Coimmunoprecipitation of PI3K with the PDGFβR and EGFR was observed in a time-dependent manner. Interestingly, Shc was associated with EGFR but not with PDGFβR. The time course for PI3K binding to the PDGF and EGFR and phosphorylation of PI3K were similar, suggesting that after binding to the EGFR or PDGF, PI3K is activated.

The concentration response for S1P showed an approximate EC₅₀ of 1 nmol/L for phosphorylation of PDGFβR, EGFR, and Akt, and maximum phosphorylation was observed at ~100 nmol/L (not shown). In contrast, ERK1/2 phosphorylation did not saturate at 1 μmol/L S1P. Because 1 μmol/L is considered the physiological highest concentration of S1P, we did not examine higher concentrations. The EC₅₀ for ERK1/2 phosphorylation was ~30 nmol/L S1P based on the assumption that phosphorylation at 1 μmol/L was maximal. These data suggested that S1P-stimulated activation of Akt and ERK is secondary to transactivation of RTK, although there may be differences in downstream events given the concentration-response differences for Akt versus ERK1/2.

VSMCs also express LPA receptors, and S1P can cross-react with LPA receptors. In our experiments, 10 μmol/L LPA transactivated the EGFR to an extent similar to 1 μmol/L S1P, but 1 μmol/L LPA transactivated EGFR weakly and 10 μmol/L LPA transactivated the PDGFβR very weakly (data not shown). These results suggest that the effect of S1P at 1 μmol/L observed in VSMCs is mainly mediated by S1P receptors. Based on these data, cells were treated with 1 μmol/L S1P to assay the effect of several inhibitors, because phosphorylation levels of PDGFβR and EGFR were ~5-fold increased at that concentration.

SIP-Stimulated Transactivation of PDGFβR and EGFR Is Inhibited by AG1296 and AG1478

Transactivation of RTK in response to GPCR ligands depends on the tyrosine kinase activity of RTK. We examined whether S1P-induced PDGFβR and EGFR transactivation was dependent on tyrosine kinase activity using specific inhibitors. As shown in Figure 2A and the Table, AG1296, a PDGFβR tyrosine kinase inhibitor, abolished phosphorylation of PDGFβR in response to S1P (compare lanes 5 and 7, IP: PDGFβR) and partially inhibited phosphorylation of Akt (lanes 5 and 7, IB: pAkt). AG1478, a specific EGFR tyrosine kinase inhibitor, abolished EGFR phosphorylation (lanes 5 and 6, IP: EGFR) and inhibited the phosphorylation of ERK1/2 (lanes 5 and 6, IB: pERK) and Akt (lanes 5 and 6, IB: pAkt). Pretreatment of VSMCs with both AG1296 and AG1478 caused partial inhibition of the phosphorylation of Akt in response to S1P (lane 8). Specificity of the inhibitors was demonstrated by complete but selective inhibition of PDGF-BB–mediated and EGF–mediated tyrosine phosphorylation of PDGFβR and EGFR, respectively (data not shown).

AG Compounds Suppress VSMC Proliferation in Response to S1P

To show the physiological significance of S1P-mediated RTK transactivation, we studied VSMC growth. In response to 1 μmol/L S1P, VSMC proliferation increased significantly compared with control at day 4 (Figure 2B). This was ~50% of the response observed with PDGF-BB or EGF treatment. AG1478 and AG1296 alone inhibited S1P-mediated proliferation by 30% and 31%, respectively, and together inhibited proliferation by 49% (Figure 2C), indicating an important role for RTK transactivation in S1P-stimulated growth. PDGF-BB–stimulated cell proliferation was blocked by AG1296 but not by AG1478, and EGF-stimulated growth was blocked only by AG1478.

Gi-Dependent Transactivation of PDGFβR and EGFR

Because EDG receptor signaling requires G proteins, we examined the effect of pertussis toxin (PTx), a Gi inhibitor. PTx significantly inhibited both PDGFβR and EGFR transactivation in response to S1P (Table and online Figure S1). Phosphorylation of Akt and ERK1/2 was also blocked significantly. In contrast, PDGF-BB–stimulated phosphorylation of PDGFβR and EGF-stimulated phosphorylation of EGFR were not inhibited by PTx (online Figure S1). Because Gi is common to all three S1P receptors (EDG-1, -3, and -5) reported to be expressed in VSMCs, it is possible that any one of these receptors can mediate transactivation. However, according to Kluk and Hla, EDG-1 is most important for VSMC proliferation and migration.

Transactivation of PDGFβR and EGFR in CHO Cells Overexpressing EDG-1, PDGFβR, and EGFR

To strengthen the evidence that the S1P receptor (EDG-1) mediates S1P transactivation, we transfected EDG-1 alone or with the PDGFβR and EGFR in CHO cells. Expression of EDG receptors in CHO cells has been reported previously to be very low, and expression of EDG-1, -3, or -5 could not be detected by Western blotting. Endogenous expression of Akt and ERK1/2 was observed (Figure 3C), but PDGFβR and EGFR were not detected by immunoblotting (data not shown). In CHO cells cotransfected with EDG-1 and PDGFβR, PDGFβR was transactivated in response to S1P (Figure 3A, lane 2) to a level ~50% of that observed with PDGF itself (Figure 3A, lane 3). The same results were observed in CHO cells cotransfected with EDG-1 and EGFR (Figure 3B, lanes 2 and 3). When all three constructs were cotransfected (Figures 3A and 3B, right side), both PDGFβR and EGFR transactivation in response to S1P was observed, but there was no synergism. Of interest, PDGF transactivation of the EGFR in Figure 3B, lane 6) was significantly greater than EGF transactivation of PDGFβR (Figure 3A, lane 7).

We next determined the effect of EDG-1 and RTK transactivation of activation of Akt and ERK1/2 (Figure 3C). In the absence of transfection, no activation of Akt or ERK1/2 in response to S1P was observed (Figure 3C, lane 2). Normal cell function was confirmed by Akt and ERK1/2 phosphorylation in CHO cells that overexpressed EDG-1 when stimulated with S1P (Figure 3C, lane 4). This low level of ERK1/2 and Akt activation by S1P in
EDG-1–transfected cells (lane 4) likely represents an RTK-independent signaling pathway for S1P-EDG-1 signaling. In CHO cells cotransfected with EDG-1 and PDGFβR, Akt and ERK1/2 phosphorylation in response to S1P was significantly enhanced compared with CHO cells transfected only with EDG-1 (lane 6 versus lane 4). The same results were observed in CHO cells cotransfected with EDG-1 and EGFR (lane 9 versus lane 4). When all three receptors were cotransfected, there was no additional enhancement of Akt and ERK1/2 activation in response to any agonist (Figure 3C, lanes 11 through 14), consistent with the RTK activation (Figures 3A and 3B). These data suggest that there are direct signaling pathways from EDG-1 to Akt and ERK1/2 as well as indirect pathways via PDGFβR and EGFR transactivation.

S1P Transactivation of PDGFβR and EGFR Requires Cholesterol-Rich Membranes

It has been previously suggested that CRMs, including lipid rafts and caveolae, are important sites for GPCR-mediated transactivation of RTKs and signal transduction.33–35 To evaluate the role of CRM in S1P transactivation, we disrupted CRM by depleting cholesterol with β-cyclodextrin or filipin (Figure 4 and the Table). These agents strongly inhibited S1P-mediated transactivation of both PDGFβR (Figure 4A, lanes 4 through 6, IP: PDGFβR) and EGFR (Figure 4B, lanes 4 through 6, IP: EGFR). In contrast, PDGF-BB–stimulated and EGF-stimulated phosphorylation of their receptors was not blocked by cholesterol depletion (Figures 4A and 4B, lanes 7 through 9). These findings suggest an important role for CRM in S1P-mediated RTK transactivation.

Figure 2. Effect of PDGFβR tyrosine kinase inhibitor AG1296 and EGFR tyrosine kinase inhibitor AG1478 on signal transduction and cell proliferation. A, VSMCs were pretreated with vehicle (DMSO), 10 μmol/L AG1296, or 300 nmol/L AG1478 for 30 minutes and treated with 1 μmol/L S1P for 3 minutes. Samples immunoprecipitated with PDGF or EGFR or nonimmunoprecipitated lysate were analyzed by Western blotting. The extent of phosphorylation was quantified as in Figure 1. B, VSMCs were seeded in 24-well plates in DMEM supplemented with 10% FBS. The next day, medium was changed to DMEM without FBS containing 1 μmol/L S1P, 10 ng/mL EGF, or 20 ng/mL PDGF-BB for up to 4 days. Medium was changed and cells were counted every 2 days. Data are mean±SEM. *P<0.05. C, Using the protocol in Figure 2C, VSMC proliferation was measured with or without AG1296 or AG1478.
To evaluate the role of CRM additionally, we examined changes in subcellular location of PDGFβR, EGFR, caveolin-1, PI3K, and Shc induced by S1P using sucrose gradient fractionation (Figures 5A and 5B). Caveolin-1, PDGFβR, and EGFR were primarily localized to CRM (fractions 4 through 6) and did not change when cells were stimulated with S1P, PDGF, or EGF (Figures 5A and 5B). In contrast, PI3K and Shc were primarily located in non-CRM heavy fractions (fractions 9 through 12) basally and translocated to CRM in response to S1P in a time-dependent manner (Figures 5A and 5B). Both PI3K and Shc showed clear translocation at 5 minutes of S1P stimulation (P13K, 7% to 14%; Shc, 3% to 12%; Figure 5B), with a peak at 20 minutes for Shc. PI3K translocation was blocked by both AG1478 and AG1296, but Shc translocation was inhibited only by AG1478. These data are consistent with findings that Shc associated only with the EGFR (Figure 1) and the inhibitor studies in Figure 2.

Characterization of S1P Transactivation Pathway

To elucidate the mechanism by which S1P transactivates the EGFR and PDGFR, we used multiple inhibitors (Table and online Figures S2 through S6). Because S1P has been shown to increase calcium in VSMCs,9 we chelated calcium with BAPTA/AM (online Figure S2). This completely inhibited transactivation of PDGFβR and EGFR in response to S1P and also inhibited downstream Akt and ERK1/2 phosphorylation (online Figure S2 and the Table). Wortmannin and PD98059, specific inhibitors of PI3K and MEK-1, respectively, did not inhibit S1P-mediated RTK phosphorylation but inhibited Akt and ERK1/2, respectively, as expected (online Figure S2 and the Table). These results suggest no feedback by PI3K and MEK-1 on RTK transactivation.

Because transactivation of RTK in VSMC by Ang II is reactive oxygen species (ROS) dependent,36 we measured ROS in response to S1P. S1P stimulated superoxide generation to an extent comparable to Ang II (Figure 6). Next we examined the effect of antioxidants on the transactivation of RTK by S1P. N-acetyl cysteine, Tiron, and ebselen inhibited S1P transactivation of PDGFβR and EGFR (Table and online Figure S3). Thus, in VSMCs, S1P-stimulated transactivation of RTK is ROS dependent.

Src-dependent transactivation of EGFR in response to GPCR ligands has been well studied.13-15 To evaluate the role of Src, we treated VSMCs with the Src inhibitor PP2 and adenoviral dominant-negative c-Src. Both treatments blocked PDGFβR and EGFR transactivation in response to S1P (Table and online Figures S4 and S5). Interestingly, PP2 partially inhibited PDGF-BB–induced and EGF–induced phosphorylation of PDGFβR and EGFR, respectively. As reported previously, some phosphorylation sites in RTK are Src dependent.37,38 EGFR transactivation by several GPCR ligands depends on matrix metalloprotease (MMP) activation, release of HB-EGF, and binding of HB-EGF to EGFR.12,13,31 Therefore, we examined whether S1P-mediated EGFR and PDGFβR transactivation was MMP dependent. EGFR transactivation by S1P was blocked by the MMP inhibitors GM 6001 and o-phenanthroline (Table and online Figure S6A). In contrast, S1P-stimulated transactivation of PDGFβR was not attenuated by MMP inhibitors or by PDGF-BB–neutralizing anti-
body (Table and online Figure S6B). These data suggest that PDGFβR transactivation by S1P does not require MMP activation and that the pathway differs from EGFR transactivation.

Discussion

The major finding of this study is that S1P transactivates PDGFβR and EGFR in VSMCs via an EDG receptor, probably EDG-1. Both EGFR and PDGFβR transactivation required Gi activation, receptor tyrosine kinase activity, Src, ROS, and intact cholesterol-rich membrane domains, as shown in Figure 7. Based on the time course in Figure 1, a sequential pathway of Src-RTK-Akt/ERK is plausible, although given the limitations imposed by subcellular location and signal amplification, the time course is merely suggestive. Novel findings for S1P transactivation were the requirement for ROS generation and translocation of p85PI3K and Shc to cholesterol-rich membranes (caveolae in Figure 7). Transactivation is critical for S1P signaling, because >70% inhibition of Akt and ERK1/2 activation occurred in the presence of AG1478 and AG1296 (Figure 2 and the Table). S1P transactivation requires EDG receptors (as opposed to intracellular metabolism), because sphingosine did not stimulate EGFR and PDGFβR phosphorylation (data not shown). Importantly, because both EGFR and PDGFβR contributed to VSMC growth in response to S1P, we propose that these pathways are important for S1P-mediated VSMC effects, such as proliferation and migration. Because many of the conclusions are based on the use of inhibitors, additional investigation using different approaches will be needed to confirm our findings.

Transactivation of RTK in response to activation of many GPCRs has been reported. EGFR transactivation by ligands such as thrombin, Ang II, lysophosphatidic acid, and endothelin-1 has been well studied. Because S1P is a relatively newly identified GPCR ligand, S1P-stimulated transactivation of RTK has not been well studied. One report suggested involvement of EGFR transactivation in response...
to S1P because ERK1/2 activation was inhibited by AG1478. Boguslawski et al. reported tyrosine phosphorylation of a 175- to 185-kDa protein in response to S1P stimulation, consistent with the PDGFR. In this study we report that transactivation of EGFR by S1P likely depends on MMP, similar to other GPCR ligands. However, we have not observed the activation of specific MMP or production of HB-EGF. Additional investigations are needed to confirm the involvement of MMP in EGFR transactivation. In contrast, PDGF/βR transactivation was not MMP dependent, similar to results we have previously reported for S1P-mediated transactivation of VEGFR2/Flk-1/KDR in endothelial cells.

The present study is consistent with previous data for transactivation of PDGFβR and EGFR by Ang II in VSMCs.

**Figure 5.** Translocation of PI3K and Shc to CRM fractions in response to S1P stimulation. A, VSMCs were pretreated with vehicle (DMSO), 10 μmol/L AG1296, or 300 nmol/L AG1478 for 30 minutes and exposed to 1 μmol/L S1P for 2, 5, or 10 minutes, 20 ng/mL PDGF-BB for 5 minutes, or 10 ng/mL EGF for 2 minutes. Sucrose gradient fractionation was performed and fractions separated by SDS-PAGE and analyzed by Western blotting. B, Distribution in 12 fractions of each protein were quantified and expressed as percent of total. Results are mean±SEM of 3 determinations. To normalize the results for different experiments and blots, we made multiple exposures and used exposures that provided a similar absolute density for caveolin-1 in the CRM fraction. This exposure was then used to determine the total pixel intensity for each protein on its Western blot, and the value was set to 100%.

![Figure 5](image)

**Figure 6.** S1P stimulates ROS production in VSMCs. Superoxide generation by S1P (100 nmol/L for 2 minutes) or Ang II (100 nmol/L for 1 minute) was determined by fluorescence microscopy. Methanol (MeOH) and PBS were used as vehicle controls for S1P and Ang II, respectively.

![Figure 6](image)

**Figure 7.** Model for S1P-mediated transactivation of PDGFβR and EGFR.

![Figure 7](image)
that showed key roles for ROS\textsuperscript{40} and caveolae.\textsuperscript{36} We found that S1P increased superoxide generation to a similar extent as Ang II, and transactivation of both PDGFR\(\beta\)R and EGFR was inhibited by antioxidants. S1P transactivation of VEGFR2/Flk-1/KDR in endothelial cells was not ROS dependent,\textsuperscript{22} which is logical, because S1P does not generate ROS in endothelial cells.\textsuperscript{41} Thus, ROS generation in response to S1P is a specific feature of VSMCs. A novel finding in our study was translocation of p85PI3K and Shc to CRM and association of these molecules with PDGFR\(\beta\)R and EGFR. These results strongly support the involvement of caveolae or lipid rafts as the subcellular location where transactivation of PDGFR\(\beta\)R and EGFR occurs.

The present study suggests that there is a hierarchy in the transactivation of EGFR and PDGFR\(\beta\)R when they are expressed together, as in VSMCs. Specifically, we found for Akt activation that both EGFR and PDGFR\(\beta\)R were involved but for ERK1/2 activation only EGFR transactivation was involved (Figure 2A). We speculate that this is a consequence of Shc being recruited only to EGFR or crosstalk between EGFR and the PDGFR\(\beta\)R. This may occur if the EGFR is more easily transactivated and inhibits signaling of the PDGFR\(\beta\)R. This concept is also supported by the finding that when the PDGFR\(\beta\)R was expressed alone in CHO cells, activation of both Akt and ERK was observed. Also, in CHO cells transfected only with EGFR but without EDG-1, EGFR was phosphorylated by S1P. CHO cells do not express EDG-1,-3, or -5, but there is a possibility that another S1P receptor might be expressed in this cell, and overexpressed EGFR was transactivated slightly by S1P stimulation. In fact, highly overexpressed EGFR usually autophosphorylated without stimulation. Under our conditions, expression levels might not be sufficient for autophosphorylation without stimulation, but because expression is higher than the level in VSMCs, EGFR may be activated by a weak stimulation. We have not examined the actual sites phosphorylated in EGFR and PDGFR\(\beta\)R in response to S1P, but we speculate that the PI3K binding sites are phosphorylated in both PDGFR\(\beta\)R and EGFR whereas the Shc binding sites are tyrosine phosphorylated only in the EGFR.

An interesting model of reverse transactivation was demonstrated for the PDGFR and EDG-1.\textsuperscript{24} One study showed that PDGFR binding to the PDGFR activated sphingosine kinase, which then generated S1P. S1P was secreted and activated EDG-1 on the cell surface. This report and our present data suggest a sequential loop of transactivation between EDG receptors and PDGFR, which may potentiate signal transduction and play an important role in S1P and PDGFR signaling. It should be noted that other recent studies question the existence of reverse transactivation.\textsuperscript{32} Our data that PTx did not block PDGFR-BB–stimulated or EGFR-stimulated ERK and Akt phosphorylation also argue against reverse transactivation (online Figure S1). Additional investigations are needed to clarify the possibility of a sequential loop of transactivation.

There is increasing evidence for an important role for S1P and its receptors in vessel growth and development. Both endothelial cells and VSMCs express multiple S1P receptors, and S1P stimulates proliferation and migration of these cells. We have shown that S1P induced RTK transactivation via EDG-1 in CHO cells cotransfected with EDG-1 and RTK. Two other EDG receptors (EDG-3 and EDG-5) expressed in VSMCs may also participate in RTK transactivation. The expression levels of these receptors are high in VSMCs from several rat strains\textsuperscript{6,7,9} as well as in human VSMCs and airway SMCs.\textsuperscript{10,11,43} Based on present data, we believe that EDG-1 mainly contributes to RTK transactivation in VSMCs, but additional study is needed to confirm the role of EDG-1. S1P is released by activated platelets and is also synthesized in endothelial cells and VSMCs. We have now demonstrated that S1P transactivates three RTKs (VEGFR2/Flk-1/KDR, PDGFR\(\beta\)R, and EGFR) that are highly expressed in endothelial cells\textsuperscript{22} and VSMCs. These observations suggest that RTK transactivation in response to S1P plays an important role in vascular physiology, especially at sites of arterial injury and thrombosis.

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References


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Supplementary text

**Reagents:** S1P was purchased from Sigma (St. Louis, MO) or Biomol (Plymouth Meeting, PA). LPA, O-phenanthroline, N-acetylcysteine (NAC), 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron), ebselen, diphenylene iodonium (DPI), EGF and anti-FLAG monoclonal antibody were purchased from Sigma. PDGF-BB and anti-PDGF-BB neutralizing monoclonal antibody were purchased from R & D Systems (Minneapolis, MN). AG 1478, AG 1296, pertussis toxin (PTx), 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM), 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), PD98059, wortmannin and GM6001 were purchased from Calbiochem (San Diego, CA). Antibodies to phospho-ERK1/2, phospho-Akt (Ser473) and Akt were from Cell Signaling Technologies (Beverly, MA). Antibody to caveolin-1 (monoclonal) was from Transduction Laboratories (Lexington, KY). Antibodies to PDGFβR (polyclonal), c-Shc (monoclonal), p85 phosphatidyl inositol 3 kinase (PI3K) (polyclonal) and phospho-tyrosine (monoclonal, clone 4G10) were from Upstate biotechnology (Lake Placid, NY). Antibodies to EGFR (polyclonal), PDGFβR (polyclonal), ERK1 (polyclonal), ERK2 (polyclonal) and protein A/G PLUS-agarose were from Santa Cruz (Santa Cruz, CA). Antibody to phospho-c-Src (pY418, polyclonal) was from Biosource International (Camarillo, CA). Antibodies to EDG1 (polyclonal), EDG-3 (monoclonal) and EDG-5 (monoclonal) were from Exalpha Biologicals (Watertown, MA). Horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody, anti-mouse secondary antibody and chemiluminescence ECL detection kit were from Amersham Pharmacia Biotech (Piscataway, NJ).
**Detergent-free purification of cholesterol-rich membrane (CRM) fractions:** CRM fractions were prepared as reported by Song et al.\textsuperscript{29} After stimulation, dishes were washed twice with ice-cold phosphate-buffered saline, VSMC (two ~80% confluent 150-mm dishes) were scraped into 2 ml of Buffer A (500 mM sodium carbonate, pH 11.0, 25 mM MES, 0.15 M NaCl, 0.1% protease inhibitor cocktail solution (Sigma)). Homogenization was carried out sequentially in the following order using a Dounce homogenizer (20 strokes), and a sonicator (three 10s bursts; Ultrasonic Homogenizer 4710 Series, Cole-Parmer Instrument Co., Chicago, IL). The homogenate was then adjusted to 45% sucrose by the addition of 2 ml of buffer B (90% sucrose, 25 mM MES, pH 6.5, 0.15 M NaCl, 0.1% protease inhibitor cocktail solution) and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient was formed by layering 4ml of buffer C (35% sucrose, 250 mM sodium carbonate, pH 11.0, 25 mM MES, 0.15 M NaCl, 0.1% protease inhibitor cocktail solution) and 4 ml of buffer D (5% sucrose, 250 mM sodium carbonate, pH 11.0, 25 mM MES, 0.15 M NaCl, 0.1% protease inhibitor cocktail solution) and centrifuged at 28,000 rpm for 16–20 h in SORVALL DiscoveryTM 100S Ultracentrifuge equipped with SurespinTM 630 (17 ml) rotor (Kendro Laboratory Products, Newtown, CT). Then 12 x 1 ml fractions were collected from top to bottom of each tube.

**Superoxide measurement:** The redox sensitive fluorescent dye, hydroethidine (HE) was used to evaluate *in situ* production of superoxide.\textsuperscript{30} VSMC were cultured in glass bottom microwell dishes (MatTek, Ashland, MA), and at ~80% confluence, cells were serum starved for 2 days. After stimulation cells were washed twice with PBS and incubated in
HBSS containing CaCl$_2$, glucose and 3 µM HE for 15 min at 37°C in a CO$_2$ incubator. Then, cells were washed twice with PBS and fluorescence was analyzed using an Olympus microscope. Because of short-time incubation, HE stain did not concentrate to nucleus and it reflects *in situ* production of ROS.
Figure legends for supplemental information

Figure S1. Effect of PTx on transactivation of PDGF\(\beta\)R and EGFR. A: VSMC were pretreated with 50 ng/ml PTx for 20 h and treated with 1 \(\mu\)M S1P for 3 min, 20 ng/ml PDGF-BB for 5 min or 10 ng/ml EGF for 2 min. Samples immunoprecipitated with PDGFR or EGFR, or non-immunoprecipitated lysate were separated by SDS-PAGE and analyzed by western blotting. Blots are representative of three experiments. Densitometric analyses of immunoblots were performed by NIH image. The relative densitometry of each lane was expressed as % of total as described in Methods.

Figure S2. Effect of BAPTA/AM, wortmannin and PD98059 on the transactivation of PDGF\(\beta\)R and EGFR. VSMC were pretreated with vehicle (DMSO) or 20 \(\mu\)M BAPTA/AM, 100 nM wortmannin or 25 \(\mu\)M PD98059 for 30 min and exposed to 1 \(\mu\)M S1P for 3 min. Total lysates or immunoprecipitated samples were separated by SDS-PAGE and analyzed by western blotting. Blots are representative of three experiments.
Figure S3. Effect of antioxidants on the transactivation of PDGFβR and EGFR. VSMC were pretreated with vehicle (DMSO) or antioxidants (10 mM NAC, 10 mM Tiron or 40 µM ebselen) for 30 min and exposed to 1 µM S1P for 3 min. Samples were immunoprecipitated, separated by SDS-PAGE and analyzed by western blotting. Blots are representative of three experiments.

Figure S4. Involvement of Src family kinase in S1P stimulated transactivation of PDGFβR and EGFR: Effect of PP2. VSMC were pretreated with 10 µM PP2 for 30 minutes and treated with 1 µM S1P for 3 min, 20 ng/ml PDGF-BB for 5 min or 10 ng/ml EGF for 2 min. Total cell lysates or immunoprecipitated samples were separated by SDS-PAGE and analyzed by western blotting. Blots are representative of three experiments.

Figure S5. Involvement of Src family kinase in S1P stimulated transactivation of PDGFβR and EGFR: Effect of DN-Src. VSMC were infected with adenovirus-LacZ or adenovirus-dominant negative c-Src (100 MOI) and treated with 1 µM S1P for 3 min, 20 ng/ml PDGF-BB for 5 min or 10 ng/ml EGF for 2 min. Samples were immunoprecipitated, separated
by SDS-PAGE and analyzed by western blotting. Blots are representative of three experiments.

**Figure S6. Effect of MMPs and secreted PDGF on S1P stimulated transactivation.** VSMC were pretreated with 1 µM GM 6001, 10 µM o-phenanthroline or 10 µg/ml PDGF-BB neutralizing antibody for 30 min and treated with 1 µM S1P for 3 min and either 10 ng/ml EGF for 2 min (A) or 20 ng/ml PDGF-BB for 5 min (B). Samples were immunoprecipitated, separated by SDS-PAGE and analyzed by western blotting. Blots are representative of three experiments.
Fig. S1

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Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8
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IB: pY (4G10)
IB: Akt
IB: pAkt
IB: pERK1/2
IB: ERK1/2

P-PDGFR (% of total)
P-EGFR (% of total)
P-Akt (% of total)
P-ERK1/2 (% of total)

Lysate
IP: EGFR
IP: PDGFβR
Fig. S3

**IP: PDGFR**

- **IB: pY (4G10)**
  - 180 kDa

- **IB: PDGFR**
  - 180 kDa
  - 160 kDa

**IP: EGFR**

- **IB: pY (4G10)**
  - 170 kDa

- **IB: EGFR**
  - 170 kDa

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Lane 1: Negative
Lane 2: Positive
Lane 3: Tiron
Lane 4: Ebselen
Lane 5: Agonist
Lane 6: NAC
Lane 7: Tiron
Lane 8: S1P
IP: PDGF-\(\beta\)R

IB: pY (4G10)

IB: PDGF-\(\beta\)R

\(\square\) 180 kDa

\(\square\) 180 kDa

\(\square\) 160 kDa

P-PDGFR (% of total)

IP: EGFR

IB: pY (4G10)

IB: EGFR

\(\square\) 170 kDa

\(\square\) 170 kDa

P-EGFR (% of total)

Lysate

IB: c-Src

\(\square\) 60 kDa

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Lane 1 2 3 4 5 6 7 8

Fig. S5
Fig. S6A

A

**IP: EGFR**

**IB: pY (4G10)**

**IB: EGFR**

**P-EGFR (% of total)**

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**Fig. S6A**
**B**

**IP: PDGF-R**

**IB: pY (4G10)**

**IB: PDGF-R**

**P-PDGFR (% of total)**

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*Fig. S6B*