Role of the Sphingosine 1-Phosphate Receptor EDG-1 in Vascular Smooth Muscle Cell Proliferation and Migration

Michael J. Kluk, Timothy Hla

Abstract—Sphingosine 1-phosphate (S1P), a platelet-derived ligand for the EDG-1 family of G protein–coupled receptors (GPCRs), has recently emerged as a regulator of vascular development. Although S1P has potent effects on endothelial cells and vascular smooth muscle cells (VSMCs), the functions of the specific S1P receptors in the latter cell type are not known. Here we show that pup-intimal VSMCs express higher levels of EDG-1 mRNA than adult-medial VSMCs. Stable transfection of EDG-1 into adult-medial VSMCs enhanced their proliferative response to S1P, concomitant with induction of p70 S6 kinase activity and expression of cyclin D1. Pertussis toxin treatment inhibited S1P-induced p70 S6 kinase activation, cyclin D1 expression and proliferation, suggesting that EDG-1–coupling to the G, pathway is critical. Furthermore, blocking p70 S6 kinase phosphorylation with rapamycin inhibited cyclin D1 expression and proliferation, suggesting that activation of p70 S6 kinase is critical in EDG-1/G,–mediated cell proliferation. EDG-1 expression also profoundly enhanced the migratory response of adult-medial VSMCs to S1P. S1P-induced migration of adult-medial VSMCs expressing exogenous EDG-1 required G, activation but not p70 S6 kinase. These results suggest that enhanced expression of EDG-1 in VSMCs dramatically stimulates both the proliferative and migratory responses to S1P. Since EDG-1 is expressed in the pup-intimal phenotype of VSMCs, S1P signaling via EDG-1 may play a role in vascular diseases in which the proliferation and migration of VSMCs are dysregulated. (Circ Res. 2001;89:496-502.)

Key Words: vascular smooth muscle cells • sphingosine 1-phosphate • proliferation • migration

Vascular smooth muscle cell (VSMC) proliferation and migration are important in physiological processes such as blood vessel development1-2 and in pathological conditions such as atherosclerosis, hypertension, and restenosis following angioplasty.3-5 Many growth factors and cytokines regulate VSMC proliferation and migration; examples include ligands for tyrosine kinase growth factor receptors (platelet-derived growth factor [PDGF], basic fibroblast growth factor [FGF-2], and insulin-like growth factor I [IGF-I]) and ligands for G protein–coupled receptors (angiostatin II and thrombin).6,7 Identification of novel molecules that regulate VSMC proliferation and migration may improve our understanding of these complex processes and could lead to novel therapeutically approaches.

Sphingosine 1-phosphate (S1P), a bioactive sphingolipid synthesized and secreted by platelets,8 is the ligand for specific isoforms of the EDG (endothelial differentiation gene) family of G protein–coupled receptors. EDG-1, the first member of this receptor family to be cloned, was identified as an inducible transcript expressed during endothelial cell differentiation in vitro.9 Subsequent work revealed S1P to be a high-affinity ligand for EDG-1 and demonstrated that through EDG-1, S1P could promote endothelial cell survival, migration, proliferation, and adherens junction assembly, thereby regulating endothelial cell morphogenesis and angiogenesis in vivo.10-13 The other EDG isoforms that bind S1P as a high affinity ligand are EDG-3, -5, -6, and -8.14-20

Recently, S1P has emerged as a regulator of VSMC functions. First, rat VSMC have been shown to express high levels of EDG-3 and -5 mRNA and low levels of EDG-1 mRNA21; however, the functions of these different receptors in VSMCs have not been addressed. Second, S1P has been shown to increase DNA synthesis, but not cell number, in VSMCs.21-23 Third, S1P at μmol/L concentrations was found to inhibit PDGF-induced migration of VSMCs.21,22 Lastly, knockout of the EDG-1 gene in mice resulted in impaired recruitment of pericytes/VSMCs to the developing aorta.24 Taken together, these studies suggest that S1P acting via its different receptors can influence VSMC function; however, the role of specific EDG isoforms in seemingly disparate VSMC responses to S1P needs to be better defined. Herein, we report the role of EDG-1 in VSMC responses to S1P in vitro.

Materials and Methods

Materials

S1P (BioMol, Incorporated) was resuspended in phosphate-buffered saline containing 0.4% (wt/vol) fatty acid free BSA (PBS/BSA).
Rapamycin and pertussis toxin (PTX) were purchased from Calbiochem.

**Cell Culture**

Wistar Kyoto adult rat medial and pup-intimal VSMCs were a generous gift of Dr Stephen Schwartz, University of Washington, Seattle, Wash. Cells were maintained in DMEM complete growth medium (DMEM CGM): 10% FBS, 100 U/mL penicillin, 100 \( \mu \text{g/mL} \) streptomycin, and 0.25 \( \mu \text{g/mL} \) amphotericin. Adult-medial VSMCs were transfected with full-length EDG-1 cDNA in pcDNA3.1. Stable clones were selected by G418 resistance and screened for EDG-1 expression by Northern hybridization. EDG-1–transfected and vector controls were always maintained in DMEM CGM containing 0.2 mg/mL neomycin (NEO CGM). Replication–defective EDG-1 adenovirus under a cytomegalovirus promoter was constructed using the AdEasy-1 genome plasmid and a shuttle vector.\(^9\) These reagents, in addition to the control \( \beta \)-gal adenovirus, were generously provided by Dr William Sessa, Yale University, New Haven, Conn.

**Northern Blot Analysis**

Northern blots were performed as described previously.\(^9\) Briefly, total RNA was isolated from VSMCs using RNA Stat 60 (Tel Test “B”, Incorporated). Ten micrograms of total RNA was separated on a 1% agarose gel, transferred to nylon membranes (BioRad), and hybridized overnight at 58°C in 20% formamide with mouse EDG-1, human EDG-3, rat EDG-5, and human GAPDH open-reading frame probes (Random Primed DNA Labeling Kit, Boehringer Mannheim).\(^11\) After washing (58°C), blots were visualized with a PhosphorImager (Molecular Dynamics).

**DNA Synthesis**

DNA synthesis was measured by incorporation of the thymidine analogue BrdU. Cells were seeded into 35-mm glass-bottom dishes (MatTek Corporation) in DMEM CGM (or NEO CGM for stable transfectants), allowed to recover for 24 hours before and during the 24-hour treatment period. Next, cells were treated with S1P or PBS/BSA (vehicle) for another 24 hours. When indicated, PTX (dissolved in PBS) was added to cells 2 hours before and during the 24-hour treatment period. Finally, samples were incubated with 50 \( \mu \text{mol/L} \) BrdU for 30 minutes, fixed in 70% ethanol/15 mmol/L glycine/pH 2.0 and stained for BrdU incorporation with monoclonal anti-BrdU (ICN Pharmaceuticals). Bands were visualized using ECL detection system (Amersham). Equal amounts of protein were separated on a 9% polyacrylamide gel, blotted to nitrocellulose, and probed with rabbit polyclonal antibody against phosphorylated p70 S6 kinase gel, blotted to nitrocellulose, and probed with rabbit polyclonal antibody against phosphorylated p70 S6 kinase (Cell Signaling Technology) or total p70 S6 kinase (Cell Signaling Technology), and then sheep anti-rabbit HRP-conjugated secondary antibody (ICN Pharmaceuticals).

**Cyclin D1**

Subconfluent cultures were serum-starved and stimulated as indicated in the figure legends. Cell lysates were prepared as described above and 40 \( \mu \text{g} \) of lysates was loaded per lane. Blots were probed with rabbit polyclonal antibodies against cyclin D1 (Santa Cruz) and sheep anti-rabbit HRP-conjugated secondary antibody (ICN Pharmaceuticals). In preliminary experiments, the position of cyclin D1 was determined with a monoclonal antibody to cyclin D1 (Santa Cruz). Blots were reprobed with monoclonal antibody against \( \beta \)-actin (Sigma). Bands were visualized using ECL detection system (Amersham).

**Cell Migration Assay**

VSMC migration was assayed using Transwell filters (6.5-mm diameter, 8-\( \mu \text{m} \) pore size, polycarbonate membranes) (Costar). Both surfaces were coated with fibronectin (50 \( \mu \text{g/mL} \)). VSMCs were resuspended in DMEM 0.1% BSA and used in migration assays as described previously.\(^12\) A control filter was coated, stained, and processed as all other filters and used as a blank. For adenoviral transduction experiments, 75% to 100% confluent wild-type adult-medial VSMCs were infected (MOI = 800) for 12 hours with EDG-1 adenovirus or control \( \beta \)-gal adenovirus. After infection, cells were allowed to recover in DMEM CGM before migration assay. Expression from both viruses was confirmed by \( \beta \)-gal staining, GFP fluorescence, and immunoprecipitation (EDG-1 virus) (see online Figures C through E available in the data supplement at http://www.circresaha.org).

**Results**

We analyzed the expression pattern of S1P receptors in both adult-medial and pup-intimal rat VSMCs. Pup-intimal VSMC expressed EDG-1, -3, and -5 mRNA whereas adult-medial VSMCs expressed only EDG-3 and EDG-5 mRNA (Figure

---

**Figure 1.** Expression of EDG-1, -3 and -5 in rat VSMCs. A, Total RNA was prepared from VSMCs grown in DMEM CGM. Ten micrograms was loaded per lane in a 1% agarose gel. After transfer to nylon membranes, blots were probed for expression of EDG-1, -3, and -5 or GAPDH. B, Total RNA was prepared from both cell types grown until the indicated confluences in DMEM CGM. Ten micrograms was loaded per lane. Hybridization procedure for all blots is described in Materials and Methods. Data are from a representative experiment that was repeated twice.

---

Calbiochem.

At least two independent experiments performed in duplicate. *, #, **Statistically significant decreases compared with S1P PBS/BSA. #, *Statistically significant increases above vehicle control; **Statistically significant decrease compared with S1P alone (paired t test, \( P<0.05 \)). Data represent mean±SEM from at least two independent experiments performed in duplicate.

To confirm this difference in EDG-1 expression, RNA was harvested from both cell types at either 50% to 75% or 100% confluence and once again, the pup-intimal VSMCs expressed more EDG-1 than the adult-medial cells, although the latter cell type did express low levels of EDG-1 mRNA at high confluence (Figure 1B). Interestingly, EDG-1, -3, and -5 mRNA levels increased (3-fold, 2-fold, and 1.6-fold, respectively) at higher confluences in the pup-intimal VSMCs. The other S1P receptors (EDG-6 and EDG-8) were undetectable in these samples (data not shown).

Having identified a difference in EDG-1 expression levels between the two cell types, their proliferative responses to S1P were tested. Pup-intimal VSMCs had a high basal rate of proliferation because greater than 20% of cells were in S-phase. S1P increased DNA synthesis in these cells by 40% above vehicle control and this effect was inhibited by PTX (Figure 2). In contrast, the proliferation of adult-medial VSMC in basal conditions was low (<5%); however, S1P increased DNA synthesis in these cells 3-fold above vehicle control, and interestingly, this increase in DNA synthesis was not inhibited by PTX (Figure 2). Cell proliferation analysis confirmed these findings as S1P increased cell number in adult-medial VSMCs, although it was unable to significantly influence the high basal proliferation of pup-intimal VSMCs (data not shown).

Given the difference in the PTX sensitivities of S1P-induced DNA synthesis in the pup-intimal and adult-medial VSMCs, we hypothesized that the higher levels of EDG-1 in the pup-intimal cells rendered their S1P-induced DNA synthesis PTX sensitive. This is consistent with known ability of EDG-1 to couple mainly to the heterotrimeric G\(_i\) protein.26–28 This hypothesis was tested by transfecting EDG-1 into adult-medial VSMCs because these cells express little, if any, EDG-1 and because they exhibited strong proliferative responses to S1P that were not PTX sensitive.

As shown in Figure 3A, two stable adult-medial VSMC clones were derived that expressed abundant EDG-1 mRNA (EDG-1–medial cells), while the vector control clone expressed EDG-3 and EDG-5 mRNA, similar to the heterogeneous population of wild-type adult-medial cells. Next, S1P-induced DNA synthesis was assayed in all three stable clones (Figure 3B). The EDG-1–medial cells were found to respond better to S1P (7-fold increase above vehicle control [clone 1] and 9-fold increase [clone 2]) than the vector control cells (2-fold increase, consistent with the heterogeneous population of adult-medial cells). In addition, S1P-induced DNA synthesis in the EDG-1–medial VSMCs was completely PTX sensitive, whereas in vector control cells, S1P-induced DNA synthesis remained insensitive to PTX (Figure 3B). Thus, transfection of EDG-1 into adult-medial VSMCs was capable of enhancing the proliferative response to S1P and rendering it completely PTX sensitive.

The enhanced proliferation of EDG-1–medial cells in response to S1P treatment was confirmed using cell proliferation assays (Figure 4). In these experiments, while S1P maximally increased cell number by 23% in vector control cells, it more effectively increased cell number in both EDG-1–medial cells (eg, day 6: 2.3-fold in clone 1 and 1.9-fold in clone 2). Complete growth medium (10% FBS) was able to strongly induce (3- to 4-fold) the proliferation of all three cells (eg, day 4; vector control: 53 865±672 [control]; 216 678±1 697 [10% FBS]; EDG-1 clone 1: 84 378±2 562 [control]; 342 951±24 494 [10% FBS]; EDG-1 clone 2: 84 630±2352 [control]; 249 291±8 442 [10% FBS]).

Next, biochemical pathways that mediate S1P mitogenic signaling in VSMCs were investigated. First, the activation of
extracellular signal–regulated protein kinases (ERK)-1/-2 was tested in the stable transfectants because S1P has previously been shown to activate ERK-1/-2 in various cell types.10,11 S1P activated ERK-1/-2 with similar magnitude and kinetics in both vector control and EDG-1–medial cells (see online Figure A). Since phosphoinositide (P)i–3–kinase is another important mitogenic signaling molecule present in many cell types,29 its activation was also assessed in vector control and EDG-1–medial cells by measuring the phosphorylation of Akt, a critical downstream kinase.29 However, as shown in online Figure A, Akt was activated in both vector control and EDG-1–medial cells with similar magnitude and kinetics.

We reasoned that other mitogenic signaling molecules must be differentially activated to account for the enhanced mitogenicity of S1P in EDG-1–medial cells. Since p70 S6 kinase is involved in the mitogenic signaling in many cell types in response to a variety of growth factors,30,31 its activation by S1P was tested in vector control and EDG-1–medial cells. As shown in Figure 5A, p70 S6 kinase was more strongly activated in EDG-1–medial cells than in vector control cells with peak activation observed at 15 minutes. In addition, even low nmol/L (1 to 10 nmol/L) doses of S1P activated p70 S6 kinase in the EDG-1–medial cells, whereas similar doses of S1P had little, if any, effect on p70 S6 kinase in vector control cells (Figure 5B). Nevertheless, complete growth medium (10% FBS) was able to activate p70 S6 kinase in both cell types. Finally, activation of p70 S6 kinase by S1P in EDG-1–medial cells was impaired by pretreatment with PTX (Figure 5C), consistent with the PTX sensitivity of mTOR (mammalian target of rapamycin), an important kinase that is known to block p70 S6 kinase activation by inhibiting the role of p70 S6 kinase in S1P-induced proliferation was further tested. As shown in Figure 7A, treatment with rapamycin, a compound that is known to block p70 S6 kinase activation by inhibiting mTOR (mammalian target of rapamycin), an important kinase upstream of p70 S6 kinase.34,35 The latter finding suggested that p70 S6 kinase is an important component of S1P mitogenic signaling in EDG-1–medial cells. Therefore, the role of p70 S6 kinase in S1P-induced proliferation was further tested. As shown in Figure 7A, treatment with rapamycin significantly reduced S1P-stimulated proliferation in EDG-1–medial cells. The same doses of rapamycin completely blocked p70 S6 kinase activation by S1P but did not affect the activation of ERK-1/-2 (Figure 7B).

Taken together, these results suggest that expression of EDG-1 in VSMCs enhances the mitogenicity of S1P through mechanisms that involve, in part, the activation of p70 S6 kinase and increases in cyclin D1 levels. The PTX sensitivity of S1P-induced proliferation in these cells is consistent with the known ability of EDG-1 to couple mainly to the hetero-

**Figure 4.** Effect of S1P on proliferation of EDG-1–medial VSMCs. Cells were treated for up to 6 days with serum starvation medium containing PBS/BSA (vehicle control), 100 nmol/L S1P (○), or 1 μmol/L S1P (●). Medium was changed and cells were counted every 2 days. *, **Statistically significant increases above vehicle control at days 2 and 4 (vector control) and over entire time course (EDG-1 clones 1 and 2) (paired t test, P<0.05). Data represent mean±SEM of triplicate determinations from a typical experiment repeated twice.

**Figure 5.** Activation of p70 S6 kinase in EDG-1–medial VSMCs. A, VSMCs were serum-starved and treated with 500 nmol/L S1P for the times indicated. Total cell lysates were prepared and 40 μg was loaded per lane. Blots were probed with antibody against phospho-p70 S6 kinase or total p70 S6 kinase. The experiment was repeated three times with similar results. B, After overnight serum starvation (DMEM, 0.1% BSA), cells were treated for 15 minutes with PB (PBS/BSA—vehicle control), various concentrations of S1P (1, 10, 100, 1000 nmol/L), complete growth medium containing 10% FBS (CGM), or were left untreated at time 0 (0). Total cell lysates were prepared and 40 μg was loaded per lane. C, After overnight serum starvation (DMEM 0.1% BSA), cells were stimulated with 10 nmol/L S1P for 15 minutes. In some conditions, cells were pretreated for 2 hours with PTX dissolved in PBS (400 ng/mL). For all the experiments, similar results were found for both EDG-1–medial cell clones.

**Figure 6.** Activation of cyclin D1 levels in EDG-1–medial cells. A, VSMCs were serum-starved and treated with 500 nmol/L S1P above vehicle control (vector control), or with 100 nmol/L S1P (negative control), respectively. After overnight serum starvation (DMEM 0.1% BSA), cells were stimulated with 100 nmol/L S1P for 15 minutes (P-p70 S6 Kinase). Then cells were left untreated at time 0 (0). Total cell lysates were prepared and 40 μg was loaded per lane. Blots were probed with antibody against phospho-p70 S6 kinase or total p70 S6 kinase. The experiment was repeated three times with similar results. B, After overnight serum starvation (DMEM 0.1% BSA), cells were treated with 100 nmol/L S1P for 15 minutes. In some conditions, cells were pretreated with PTX dissolved in PBS (400 ng/mL). For all the experiments, similar results were found for both EDG-1–medial cell clones.
trimeric protein, G<sub>i</sub>.<sup>26–28</sup> Also, the ability of rapamycin to impair S1P-mediated increases in cyclin D1 and in cell number suggests that activation of p70 S6 kinase is important in S1P-induced proliferation of VSMC.

In addition to proliferation, migration is a key aspect of VSMC physiology and pathophysiology.<sup>1,2</sup> Currently, little is known about S1P-mediated regulation of VSMC migration; S1P has been shown to inhibit PDGF-induced VSMC migration<sup>21,22</sup> and most recently knockout of EDG-1 was shown to impair VSMC/pericyte ensheathment of the developing aorta.<sup>24</sup> Therefore, we investigated the migratory responses of the different rat VSMCs to S1P.

As shown in Figure 8A, S1P was able to clearly induce the migration of pup-intimal VSMCs, and this effect was inhibitable by treatment with PTX, consistent with signaling via the heterotrimeric G protein, Gi. To better understand the role of EDG-1 in S1P-stimulated VSMC migration, we investigated whether exogenous EDG-1 expression could alter the migratory response of adult-medial VSMCs to S1P. In vector control cells expressing EDG-3 and EDG-5, S1P over a broad range of doses was unable to induce migration. However, in both EDG-1–medial clones, low-dose S1P (1 to 10 nmol/L) induced migration (2-fold in clone 1- and 4-fold in clone 2) (Figure 8B). Interestingly, the extent of S1P-induced migration in the two stable EDG-1–medial clones correlated with EDG-1 expression levels (Figure 3A).

Furthermore, transduction of the heterogeneous population of wild-type adult-medial VSMCs with an EDG-1 adenovirus also enabled S1P to induce migration. As shown in Figure 8C, low-dose S1P significantly increased the migration of adult-medial VSMC transduced with EDG-1 adenovirus (3-fold increase above vehicle control) but not the migration of control cells transduced with a β-gal adenovirus. As ex-
pected, EDG-1 polypeptide was expressed in VSMCs transduced with the EDG-1 adenovirus (online Figure E).

Finally, the PTX and rapamycin sensitivities of S1P-induced migration in the EDG-1–transduced cells were tested. Pretreatment with PTX completely abolished S1P-stimulated migration of the EDG-1–transduced adult-medial VSMCs whereas pretreatment with rapamycin had no effect (Figure 8D). These findings suggest that although G, activation is necessary for S1P-induced migration of VSMCs expressing EDG-1, activation of p70 S6 kinase is not required.

Discussion

VSMC proliferation and migration are important in vascular pathologies such as atherosclerosis and restenosis following angioplasty.3–5 Several growth factors have been identified which affect the proliferation and migration of VSMCs.6,7 Platelets are an important reservoir for many of these growth factors8 and after adhering to a dysfunctional endothelium or to the subendothelial matrix, platelets aggregate and subsequently release the growth factors. The bioactive lipid S1P is abundantly synthesized and secreted from platelets8 and other authors have reported that S1P influences VSMC proliferation9–11 and migration.12,13 However, the effects of S1P on VSMC migration are unclear because a recent report indicates stimulation7 while others suggest inhibition.21,22 In addition, the roles of the different EDG isoforms mediating the actions of S1P in VSMCs have not been elucidated. Therefore, in an attempt to improve our understanding of S1P receptor signaling in VSMCs, we characterized the EDG expression profile of two VSMC phenotypes and investigated whether exogenous expression of EDG-1 could alter the proliferative and migratory responses of VSMC to S1P.

Rat pup-intimal VSMCs expressed higher levels of EDG-1 mRNA than adult-medial VSMCs. In support of this finding, recent work by other authors demonstrates that EDG-1 is induced in the neointimal lesions of human in-stent restenosis.38 Interestingly, the expression of EDG-1, -3, and -5 in the pup-intimal cells increased at higher confluences (Figure 1B). The mechanisms responsible for this effect are unclear at this time, but it is possible that extracellular growth factors present in the medium and those secreted by the cells themselves33,39 may contribute to the changes observed in EDG expression. Indeed, signaling between EDG-1 and growth factor receptors such as the PDGF β-receptor has recently been reported.40

Herein, we show that exogenous expression of EDG-1 in adult-medial VSMCs enhanced S1P-induced DNA synthesis and rendered it PTX sensitive. The ability of PTX to completely block S1P-induced DNA synthesis in EDG-1–medial cells suggested that in these cells, EDG-1 couples better than EDG-3 and EDG-5 for S1P-mediated cell proliferation. Alternatively, expression of EDG-1 could affect the way EDG-3 and EDG-5 couple to G-dependent mitogenic signaling pathways.

We also report that p70 S6 kinase plays a key role in the enhanced mitogenicity of S1P in EDG-1–medial cells. p70 S6 kinase was strongly activated by S1P in EDG-1–medial cells and treatment with rapamycin, which blocked p70 S6 kinase activation but not ERK-1/2, impaired the ability of S1P to induce cyclin D1 levels and to increase cell number. This finding better defines the biochemical pathways used by S1P to induce VSMC proliferation.

Stable transfection or adenoviral infection of EDG-1 profoundly altered the migratory response of adult-medial VSMCs to S1P. In adult-medial VSMC expressing EDG-3 and EDG-5 mRNA, S1P did not significantly induce migration; however, in adult-medial VSMCs expressing exogenous EDG-1, low-dose S1P potently stimulated migration. In addition, the pup-intimal VSMCs which express endogenous levels of EDG-1 clearly migrated in response to S1P treatment. The bell-shaped migratory responses reported here have been previously observed for S1P and other GPCR ligands and may result from desensitization of the receptors by high concentrations of ligand over the 4-hour migration period.41 The S1P-stimulated VSMC migration was sensitive to PTX, consistent with coupling to the G i pathway, but insensitive to rapamycin, suggesting that p70 S6 kinase activation is not a crucial signaling aspect of S1P-induced migration.

In conclusion, these data demonstrate that expression of EDG-1 in VSMCs can significantly affect the proliferative and migratory responses to S1P and suggest that dysregulated expression and signaling of EDG-1 may be important in vascular pathophysiology where the altered migration and proliferation of VSMCs are implicated.

Acknowledgments

This work is supported by NIH grants (DK45659 and HL 67330) to T.H. T.H. is an established investigator of the American Heart Association. M.J.K. is a recipient of the Medical Scientist Training Program fellowship.

References

12. Paik HJ, Chae SS, Lee MJ, Thangada S, Hla T. Sphingosine-1-phosphate induced endothelial cell migration requires the expression of EDG-1 and...
EDG-3 receptors and rho-dependent activation of $\alpha_{i}\beta_{j}$ and $\beta_{j}$-containing integrins. *J Biol Chem.* 2001;276:11830–11837.


Role of the Sphingosine 1-Phosphate Receptor EDG-1 in Vascular Smooth Muscle Cell Proliferation and Migration
Michael J. Kluk and Timothy Hla

_Circ Res._ 2001;89:496-502; originally published online August 30, 2001;
doi: 10.1161/hh1801.096338
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/89/6/496

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2001/08/31/hh1801.096338.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/
Supplemental Figure A

Vector Control | EDG-1-Medial VSMC
---|---

<table>
<thead>
<tr>
<th>S1P</th>
<th>0</th>
<th>1'</th>
<th>2'</th>
<th>5'</th>
<th>15'</th>
<th>30'</th>
<th>60'</th>
</tr>
</thead>
</table>

![Image](vector-control-s1p.png)

<table>
<thead>
<tr>
<th>S1P</th>
<th>0</th>
<th>1'</th>
<th>2'</th>
<th>5'</th>
<th>15'</th>
<th>30'</th>
<th>60'</th>
</tr>
</thead>
</table>

![Image](edg-1-medial-vsmc-s1p.png)

Time (min)

Phospho-ERK-1/-2

Phospho-Akt

After serum starvation, cells were treated for the indicated times with 500 nM S1P or were left untreated (0). Cells were then washed twice with PBS and lysates were prepared as described in materials and methods. 40 ug of total protein was separated by PAGE and then transferred to nitrocellulose. The blots were probed with anti-phospho-ERK-1/-2 or anti-phospho-Akt antibodies (Cell Signaling Technology).
Supplemental Figure B

Effect of S1P on p70 S6 Kinase in Pup-Intimal VSMC.

<table>
<thead>
<tr>
<th>S1P (nM)</th>
<th>0</th>
<th>PB</th>
<th>1</th>
<th>10</th>
<th>10²</th>
<th>10³</th>
<th>PDGF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Phospho-p70 S6 Kinase

β-Actin

Pup-intimal VSMC were serum starved for 24 hours in DMEM 0.1% BSA and then treated for 15 minutes with S1P (1,10,100,1000 nM), vehicle control (PB), PDGF (40ng/ml) or were left untreated(0). The cells were then harvested as described in materials and methods and 40 ug of whole cell lysates were separated per lane on a 9% polyacrylamide gel. After transfer, the blots were probed with antibodies against phospho-p70 S6 kinase and β-actin.
Supplemental Figure C

Beta Galactosidase Stain of Transduced VSMC.

EDG-1 Adenovirus  β-Gal Adenovirus

After 12 hour infection (MOI=800), the cells were washed twice with PBS and fixed in 4% paraformaldehyde/PBS for 15 minutes at room temperature. After washing again with PBS, the cells were incubated with 0.2% Bluo-Gal (Gibco BRL) in staining solution (1mM MgCl₂, 150mM NaCl, 60mM Na₂HPO₄, 40mM NaH₂PO₄, 3mM K₄Fe(CN)₆·3H₂O and 3mM K₃Fe(CN)₆). Photos were taken with a digital camera.
Supplemental Figure D

GFP Fluorescence of Transduced VSMC

β-Gal Adenovirus

EDG-1 Adenovirus

GFP is expressed separately from EDG-1 in the EDG-1 adenoviral construct. However, GFP is not included in the β-Gal adenoviral construct. Photos were taken with a 20X objective.
Supplemental Figure E

Immunoprecipitation of EDG-1 from VSMC Transduced with EDG-1 Adenovirus

Lane: 1 2 3 4 5

Heavy Chain

→ M2-EDG-1

Light Chain

Lanes:
1: anti-M2 antibody + lysis buffer
2: anti-M2 antibody + β-Gal lysate
3: β-Gal lysate alone
4: anti-M2 antibody + EDG-1 lysate
5: EDG-1 lysate alone

After 12 hour infection with EDG-1 (M2 tag) or β-Gal adenovirus (MOI =800), cells were washed twice with PBS, scraped from the dish and centrifuged at 250g for 5 minutes. The cell pellet was resuspended in buffer containing 50mM Tris pH 7.8, 5mM MgCl₂, 1mM EGTA, 20mM CHAPS, and 20% glycerol. The samples were mixed for 30 minutes at 4°C, centrifuged at 20,000g for 20 minutes and then 600μg of supernatant was incubated overnight with 20μg anti-M2 monoclonal antibody (Sigma) and 25μl of protein A beads (Pierce). The next day, the samples were washed, eluted from the beads with 2X sample buffer and loaded in a 11% polyacrylamide gel. Proteins were transferred to nitrocellulose membrane and hybridized overnight with anti-M2 antibody (1:500) in TBST/5% nonfat dry milk. After washing, the blot was probed with HRP conjugate goat anti-mouse secondary antibody (1:5000) and bands were visualized with ECL detection system (Amersham).