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Sphingosine 1-phosphate induces vesicular endothelial growth factor expression in endothelial cells

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Angiogenesis is essential for tumor growth and vascular endothelial cell growth factor (VEGF) plays a key role in this process. Conversely, sphingosine 1-phosphate (S1P) is a biologically active sphingolipid known to play a key role in cancer progression by regulating endothelial cell proliferation and migration. In this study, the authors found that S1P increases the level of VEGF mRNA in human umbilical vein endothelial cells (HUVECs) and immortalized HUVECs (iHUVECs). Additionally, S1P was found to increase VEGF promoter activity in MS-1 mouse pancreatic islet endothelial cells. Furthermore, a pharmacological inhibitory study revealed that $G_{\alpha i/o}$-mediated phospholipase C, Akt, Erk, and p38 MAPK signaling are involved in this S1P-induced expression of VEGF. A component of AP1 transcription factor is important for S1P-induced VEGF expression. Taken together, these findings suggest that S1P enhances endothelial cell proliferation and migration by upregulating the expression of VEGF mRNA. [BMB reports 2009; 42(10): 685-690]

INTRODUCTION

Sphingosine 1-phosphate (S1P) is a bioactive lipid mediator that exerts a variety of effects, such as proliferation, survival, migration, NO synthesis, and angiogenesis in many types of cells, including vascular endothelial cells (1). Platelets are the primary source of S1P in plasma and serum because they contain sphingosine kinase, but not sphingosine lyase (2). Sphingosine kinase generates S1P from sphingosine, which is an abundant lipid found in most cell membranes, whereas sphingosine lyase breaks down S1P in most cells. The lack of sphingosine lyase expression in platelets is unique (3, 4). In other cells, intracellular S1P generated by sphingosine kinase may act in a paracrine or autocrine manner. Once secreted into the plasma, S1P binds to and is metabolically stabilized by albumin (2). S1P is involved in many physiological responses and serves as a ligand for a subset of G protein coupled cell surface receptors. To date, five mammalian S1P receptors ($S1P_{1\text{-}5}$) have been identified on different cells, and notably, $S1P_1$ and $S1P_3$ have been found to be expressed on endothelial cells and participate in proliferation, survival, and migration (5-7). The downstream targets of S1P signaling include adenylate cyclase, Ras, mitogen-activated protein kinase (MAPK), phospholipase C (PLC), c-Src tyrosine kinase, the small GTPases Rac and Rho, phospholipase D, p125FAK, and calcium homeostasis. Furthermore, neutralization of S1P with monoclonal antibodies is known to reduce the formation of blood vessels and the tumor-associated angiogenesis induced by VEGF and basic fibroblast growth factor (bFGF) (8) In addition, silencing of $S1P_1$ expression in endothelial cells or murine tumors dramatically suppresses angiogenesis and tumor growth (9). These findings clearly imply that S1P may contribute to cancer progression by regulating tumor proliferation, invasiveness and angiogenesis.

Angiogenesis is defined as the process whereby new blood vessels are formed from existing vessels, and as such, is a natural physiological process. Furthermore, the progression of angiogenesis depends on a balance between positive and negative regulators. VEGF is probably the most important angiogenic factor due to its potency, selectivity for endothelial cells, ability to regulate most steps in the angiogenic cascade, and because it is expressed in abundance by a wide variety of mammalian tumors (10). VEGF gene expression, which occurs at the transcriptional level, is induced by many stimuli, including hypoxic conditions, growth factors, hormones, angiogenic cytokines, and cellular stress (11). Furthermore, VEGF promoter contains binding sites for numerous transcription factors, such as HIF-1, Egr-1, AP-1, AP-2, p53, TCF, and Sp-1 (12), and hypoxia induces the stabilization of VEGF mRNA by binding proteins (13).

In this study, we found that S1P induced VEGF expression in human endothelial cells, and that this induction of VEGF was dependent on $G_{\alpha i}$, PLC, PI3 kinase, and MAPK. We also found that AP1 transcriptional factor is important for S1P-induced VEGF expression. These results suggest that S1P stimulates angiogenesis via VEGF expression in endothelial cells.

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RESULTS AND DISCUSSION

S1P increased VEGF induction in endothelial cells

In tumors, angiogenesis depends on the release of angiogenic factors by tumor cells and the tumor microenvironment, and VEGF is the primary and most potent of these angiogenic factors (14, 15). However, S1P is also a potent activator of proliferation, migration, and angiogenesis in endothelial cells (1).

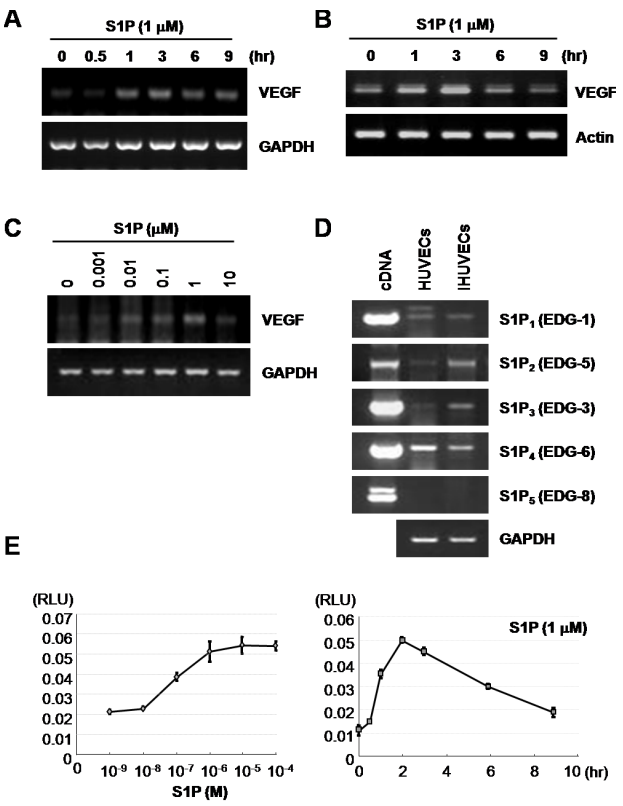

Fig. 1. S1P induces VEGF expression in endothelial cells. (A) iHUVECs and (B) HUVECs were treated with 1 μM S1P for the indicated times. Total RNA was isolated from the cells and the levels of human VEGF and GAPDH were then determined by RT-PCR. (C) iHUVECs were treated with the indicated concentrations of S1P for 3 hr (left panel), after which the levels of human VEGF and GAPDH were determined by RT-PCR. (D) Total RNA was isolated from HUVECs and iHUVECs and the levels of human $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ were then determined by RT-PCR. (E) MS-1 cells were stably transfected with the luciferase reporter gene under the control of VEGF. Transfected cells (MS-1/VEGF-luc) were incubated with 0.1% BSA in DMEM for 18 h and then treated with S1P at the indicated concentrations for 3 hr (left panel) or with 1μM S1P for the indicated times (right panel). The luciferase activities in the cell lysates (1 μg) were assayed as described in the Materials and Methods. Data represent the means ± SEs of three separate experiments conducted in triplicate. RLU: relative light unit.

To investigate the relationship between VEGF and S1P in endothelial cells, we observed the effects of S1P on the induction of VEGF mRNA. As shown in Fig. 1A and 1B, S1P (1 μM) induced VEGF mRNA in iHUVECs and HUVECs at 1 hr after administration, and this peaked at 3 hrs. Furthermore, S1P increased the VEGF mRNA levels in iHUVECs in a dose-dependent fashion (Fig. 1C). Specifically, treatment with 0.01 μM S1P led to a marked increase in VEGF mRNA levels, which increased in response to treatment with 1 μM SIP and then decreased in response to treatment with 10 μM. Five closely related GPCRs that exhibit high affinity for S1P, $S1P_{1\text{-}5}$, have been identified. Of these, $S1P_{1\text{-}3}$ are widely expressed in various tissues, whereas $S1P_{4\text{-}5}$ are primarily expressed in the immune and nervous systems, respectively (16). In the present study, we found that iHUVECs and HUVECs expressed four of these five S1P receptors ($S1P_{1\text{-}4}$) (Fig. 1D). Furthermore, because lysophosphatidic acid (LPA) is known to induce VEGF in human ovarian cancer cells by stimulating VEGF promoter activity (17), we speculated that S1P might also increase VEGF transcription by stimulating VEGF promoter activity. To evaluate the effects of S1P on VEGF promoter activity, we generated a stable MS-1 pancreatic islet endothelial cell-line containing luciferase reporter gene under the control of the VEGF promoter. These cells were incubated with S1P, after which their luciferase activities were measured. As shown in Fig. 1E, S1P increased VEGF promoter activity in a dose and time-dependent manner. VEGF promoter activity was enhanced by treatment with 10 nM S1P and saturated in response to treatment with 1 μM S1P, which is a physiological concentration. Stimulation of VEGF promoter activity by S1P (1μM) occurred 1 hour after treatment and reached its maximum level at 2 hours. These data provide direct evidence that S1P rapidly and transiently increases VEGF mRNA levels by activating VEGF promoter in endothelial cells.

$G_{\alpha i/o}$, PLC, and intracellular calcium are involved in S1P-induced VEGF expression

$S1P_1$ is coupled exclusively to Ras-MAP kinase, the phosphoinositide (PI) 3-kinase-Akt pathway, and to the phospholipase C pathway via G_i, whereas $S1P_2$ and $S1P_3$ are coupled to the multiple G proteins, $G_{\alpha q}$, $G_{\alpha 12/13}$ and $G_{\alpha i}$, to activate the phospholipase C and Rho pathways as well as the aforementioned $G_{\alpha i}$-dependent pathways (16). To investigate the signaling pathway initiated by the S1P-mediated induction of VEGF, we examined molecules downstream of S1P. Initially, we evaluated S1P to determine if it activates PLC-mediated $[Ca^{2+}]_i$ increases in iHUVECs. S1P was found to lead to a rapid increase in $[Ca^{2+}]_i$ in a dose dependent manner in iHUVECs (Fig. 2A). Additionally, pre-incubating these cells with U73122 (a PLC inhibitor) or $BAPTA^{AM}$ (an intracellular calcium chelator) significantly inhibited the S1P-induced $[Ca^{2+}]_i$ increases. However, U73433 (an inactive analogue of U73122) and pertussis toxin (a $G_{\alpha i/o}$ inhibitor) had no effect (Fig. 2B). To evaluate the role that pharmacological inhibitors played on the induction of

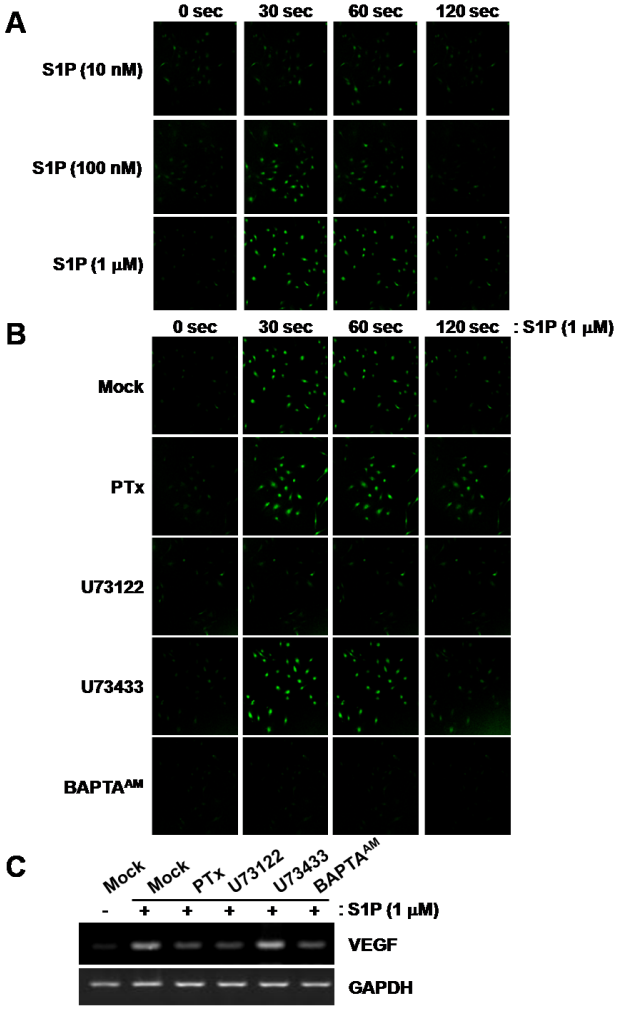

Fig. 2. VEGF induction by S1P is mediated by $G_{\alpha i/o}$, PLC, and intracellular calcium. (A) The effect of S1P on $[Ca^{2+}]_i$ in iHUVECs. Calcium responses were measured after treating the cells with S1P (10 nM, 100 nM, 1 μM). To detect the calcium signals, Fluo-3 acetoxymethyl ester (AM) was administered for 30 min. The dishes were then transferred to an inverted confocal microscope and observed using the 20X objective. (B) Samples were pretreated with PTx (100 ng/ml), U73122 (2.5 μM), U73433 (2.5 μM), or $BAPTA^{AM}$ (5 μM) for 15 min before applying 1 μM S1P and the calcium responses were then observed by confocal microscopy. (C) iHUVECs were treated with S1P (1 μM) for 3 hr in the presence of PTx (100 ng/ml), U73122 (2.5 μM), U73433 (2.5 μM), or $BAPTA^{AM}$ (5 μM) and the levels of human VEGF and GAPDH were then determined by RT-PCR.

VEGF mRNA by S1P, we pre-treated iHUVECs with PTx, U73122, or $BAPTA^{AM}$ and found that all effectively inhibited this induction, but U73433 did not inhibit S1P-induced VEGF mRNA expression (Fig. 2C). Taken together, these results indicate that S1P increases VEGF transcription via $G_{\alpha i/o}$, PLC, and intracellular calcium signaling.

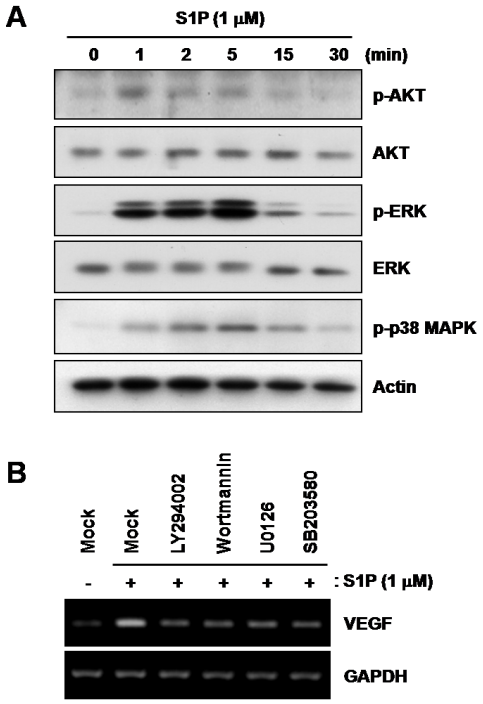

Fig. 3. VEGF induction by S1P is mediated by PI3K, p38 MAPK, and ERK1/2 MAPK. (A) iHUVECs were stimulated with 1 μM S1P for the indicated times, after which western blot analysis was conducted using the indicated antibodies. (B) iHUVECs were pretreated for 30 min with LY294002 (10 μM), wortmannin (10 μM), U0126 (10 μM), or SB203580 (20 μM) and then stimulated with 1 μM S1P for 3 hr. The levels of VEGF and GAPDH were determined by RT-PCR.

PI3K and ERK1/2 MAPK are involved in S1P-induced VEGF expression

S1P activates PI3K-Akt, p44/42 MAPK, and p38 MAPK in various cells, and these activations are blocked by PTx (16). Therefore, we evaluated these signaling molecules to determine if they were involved in the induction of VEGF. As shown in Fig. 3A, S1P transiently increased the phosphorylation of Akt, Erk, and p38 MAPK in iHUVECs. In addition, we found that pharmacological inhibitors of PI3 kinase (LY294002 and wortmannin), MEK (U0126), and p38 MAPK (SB203580) all inhibited S1P-induced VEGF expression (Fig. 3B). Because S1P is also known to be a trans-activator of the EGF receptor that functions via a PKC-dependent pathway (18, 19), and pretreatment with EGF receptor inhibitor (AG1478) did not affect the VEGF induction (data not shown), we concluded that transactivation of the EGF receptor by the S1P receptor is not involved in the induction of VEGF. These findings suggest that VEGF induction by S1P is mediated by PI3K, Akt, Erk, and p38 MAPK.

AP-1 is important for S1P-mediated VEGF induction

HIFs are known to be major regulators of VEGF in response to hypoxia. Cytokines and growth factors induce VEGF induction via activation of HIFs under non-hypoxic conditions (20). Furthermore, AP-2 and Sp1 activate the VEGF promoter in response to several cytokines. In addition to binding sites for Sp1, AP-2, and HIFs, VEGF promoter contains binding sites for the transcription factors NF-κB, NF-1, TCF, Egr-1, and AP-1 (21). In the present study, immunoblotting revealed that S1P enhances the expression of c-Jun and c-Fos and activates c-Jun. However, HIF-1α and NF-κB were not induced by exogenous S1P (Fig. 4A). Taken together, these findings suggested that AP-1 is involved in S1P-mediated VEGF promoter activation. To confirm this, we over-expressed dominant negative c-Jun in MS-1 cells and then measured the VEGF mRNA levels and promoter activities. As shown in Fig. 4B, overexpression of dominant negative c-Jun led to significant inhibition of S1P-induced VEGF expression. Moreover, S1P-induced VEGF promoter activity also decreased in response to the overexpression of dominant negative c-Jun (Fig. 4C). These findings provide direct evidence that an AP1 transcription factor is involved in S1P-mediated VEGF induction.

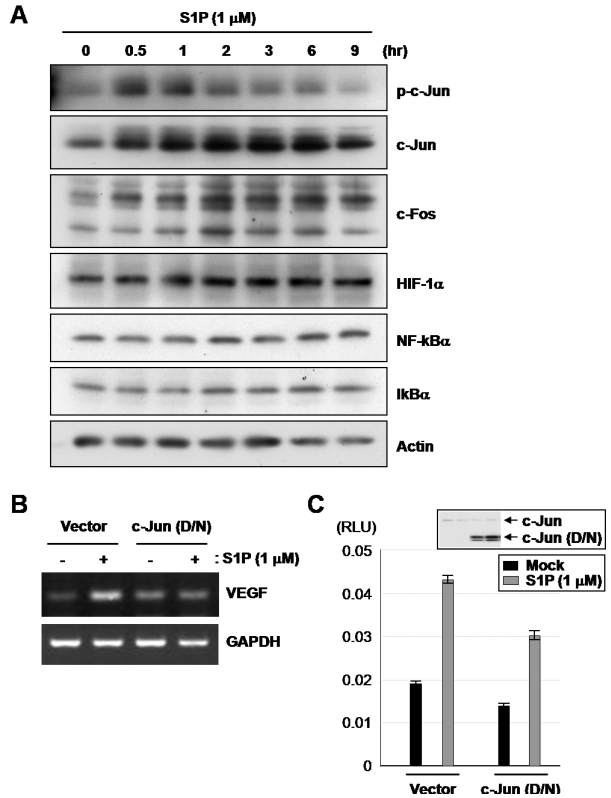

Fig. 4. AP-1 is important for S1P-mediated VEGF induction. (A) After treating iHUVECs with 1 μM S1P for the indicated times, western blots were probed with the indicated antibodies. (B) iHUVECs were transiently transfected with control vector or dominant negative (D/N) c-Jun. Transfected cells were treated with 1 μM S1P for 3 hr, after which the levels of VEGF and GAPDH were determined by RT-PCR. (C) MS-1/VEGF-luc cells were transiently transfected with control vector or dominant negative c-Jun. Transfected cells were treated with 1 μM S1P for 3 hr and then subjected to an immunoblot assay using c-Jun antibody (upper panel) or assayed for luciferase activity (lower panel).

VEGF is also known to regulate S1P signaling, and the expression of $S1P_1$ receptor in the vasculature and cross-talk between VEGFR and S1P receptors play important roles in vascular maturation. Thus, it is likely that this cross-talk, mediated by the inside-out signaling of S1P, is important for vasculogenesis and angiogenesis.

In endothelial cells, the up-regulation of VEGF promotes endothelial tube formation and angiogenesis. In addition, many cytokines, such as interleukin-1β and tumor necrosis factor, are known to regulate VEGF expression in HUVECs (22). Furthermore, a recent study reported that LPA up-regulates VEGF-C and lymphatic marker expressions in human endothelial cells (23). In the present study, we demonstrated for the first time that S1P enhances VEGF mRNA expression in human endothelial cells, and that the transcription factors G_i, PLC, PI3K, ERK, p38 MAPK, and AP1 are involved in S1P-induced VEGF mRNA expression. Taken together, the results of this study suggest that S1P up-regulates the expression of VEGF, which activates VEGF receptor in either an autocrine or paracrine fashion. Therefore, VEGF might potentiate S1P-mediated migration and mitogenesis in endothelial cells.

MATERIALS AND METHODS

Chemicals

S1P was purchased from Biomol (USA). Lipofectamine transfection reagent was purchased from Invitrogen (USA). Luciferase activity assay reagent was obtained from Promega (USA). Pertussis toxin (PTx), U73122, U73433, [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (LY294002), wortmannin, U0126, and SB203580 were purchased from Calbiochem (USA). Fluo-3 AM and 1,2-bis(2-aminophenoxy) ethane-N, N,N′,N′-tetraacetoxymethylester (BAPTA/AM) were purchased from Molecular Probes (USA).

Antibodies

Anti-phospho-AKT (Ser473) antibody was purchased from Sigma (USA). Anti-AKT, phospho-p44/42 MAP kinase (Thr202/Thr204), p44/42 MAP kinase, phospho-p38 MAPK, and phospho-c-jun (Ser63) antibody were obtained from Cell Signaling Technology Inc. (USA). Anti-c-Fos, c-Jun, NF-κB, and IκB-α antibody were purchased from Santa Cruz Biotechnology (USA). Anti-HIF-1α antibody was purchased from R&D system (USA). Anti-Actin antibody was obtained from ICN Biochemicals (USA). Horse radish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgA, IgM, IgG were obtained from

Kirkegaard & Perry Laboratories (USA).

RT-PCR

cDNA (1μg) was reverse-transcribed from total cellular RNA prepared using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions, using oligo (dT) primers and murine leukemia virus reverse transcriptase (Promega, USA). The cDNA was then amplified for 30 cycles (VEGF and $S1P_{1-5}$) or 25 cycles (GAPDH) using the primers described below. The cycling parameters used were as follows: 0.5 min at 94°C (denaturation), 0.5 min at 60°C (annealing), and 1 min at 72°C (polymerization). The products were then electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

PCR was conducted using the following primer sets: VEGF, 5'-GAGGAGGGCAGAATCATCACG-3' and 5'-ATCGCATGAGGGGCACACAGG-3'; $S1P_1$ (EDG1), 5′-CCACAACGGGAGCAATAACT-3′ and 5′-CAGAATGACGATGGAGAGCA-3′; $S1P_2$ (EDG5), 5′-CCAAGCATTATGTGCTGTGC-3′ and 5′-CAGAAGGAGGATGCTGAAGG-3′; $S1P_3$ (EDG3), 5′-CCATCCTCTTCAAGGCTCAG-3′ and 5′-TTCGGAGAGTGGCTGCTATT-3′; $S1P_4$ (EDG6), 5′-CCAAGCGCTACATCCTCTTC-3′ and 5′-CAGAGGTTGGAGCCAAAGAC-3′; $S1P_5$ (EDG8), 5′-GGTCATCGTCCTGCATTACA-3′ and 5′-AGCAGATCCGACAACGTGA-3′; GAPDH, 5'-CGGGAAGCTTGTGATCAATGG-3' and 5'-GGCAGTGATGGCATGGACTG-3'.

Cell culture and the establishment of a stable cell line

HUVECs were isolated from human umbilical cords by collagenase digestion (Worthington Biochemical, USA), and then maintained in 20% FBS-containing M-199 medium. Immortalized HUVECs (iHUVECs) (kindly provided by Dr. Ashlee Moses, Oregon Health & Sciences University) were cultured on collagen-coated flasks in EGM-MV medium (BioWhittaker, USA) as previously described (24). MS-1 cells were maintained in DMEM supplemented with 10% FBS. All cell types were grown in a 5% CO_2 humidified atmosphere. To establish a stable reporter cell line (MS-1/VEGF-Luc), MS-1 cells were transfected by electroporation with VEGF-Luc plasmid (kindly provided by Dr. Hiroyasu Esumi, National Cancer Center Research Institute East, Japan) encoding luciferase under the control of promoter sequences of VEGF. Positive clones were selected using 1 mg/ml of G418, and their transcriptional responses were tested by performing luciferase activity assays after treatment with phorbol myristoyl acetate (PMA). c-Jun dominant negative expression vector was a gift from Dr. Tom Curran (St. Jude Children's Research Hospital, USA). To induce the transient expression of c-Jun dominant negative, MS-1/VEGF-Luc cells were plated on 12-well plates and transfected using lipofectamine. Transfected cells were then cultured for one day, starved for one day in DMEM with 0.1% BSA, and then treated with S1P in 0.1% BSA.

Luciferase reporter gene assays

MS-1/VEGF-Luc cells were lysed with luciferase assay buffer (20 mM Tris-HCl, pH 7.8, 1% Triton X-100, 150 mM NaCl, 2 mM DTT), after which the lysates (5 μl) were mixed with luciferase activity assay reagent (25 μl) and the luminescence produced after 5 s was measured using a luminometer (Labsystems, Finland). Data are presented as fold relative light units versus vehicle-treated control cultures.

Measurement of $[Ca^{2+}]_i$ by confocal microscopy

$[Ca^{2+}]_i$ was measured by confocal microscopy (Zeiss LSM 510 Meta, Germany) using the calcium-sensitive indicator, Fluo-3/AM (Molecular Probes), as previously described (25). Briefly, iHUVECs were loaded with 5 μM Fluo-3 in DMEM at room temperature for 30 min. After washing with the same medium, cells were incubated for 15 min in the absence of Fluo-3 to de-esterify the dye. The cells were evaluated by confocal microscopy using an excitation wavelength of 488 nm and an emission wavelength of 515 nm.

Western blot analysis

Whole cell lysates were prepared in lysis buffer (20 mM Tri-HCl, pH 7.4, 150 mM NaCl, 20 mM NaF, 1 mM EDTA, 1 mM PMSF). Proteins (20 μg/lane) were separated on a denaturing 10% SDS-polyacrylamide gel and then transferred to nitrocellulose. Membranes were incubated with primary antibody overnight at 4°C, after which secondary antibody linked to horseradish peroxidase and diluted 1 : 10,000 was applied. The signals were then visualized using the ECL technique.

Acknowledgements

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