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Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing

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Lee, Hsinyu, Edward J. Goetzl, and Songzhu An. Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. *Am. J. Physiol. Cell Physiol.* 278: C612–C618, 2000.—Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are potent lipid growth factors with similar abilities to stimulate cytoskeleton-based cellular functions. Their effects are mediated by a subfamily of G protein-coupled receptors (GPCRs) encoded by endothelial differentiation genes (*edgs*). We hypothesize that large quantities of LPA and S1P generated by activated platelets may influence endothelial cell functions. Using an *in vitro* wound healing assay, we observed that LPA and S1P stimulated closure of wounded monolayers of human umbilical vein endothelial cells and adult bovine aortic endothelial cells, which express LPA receptor Edg2, and S1P receptors Edg1 and Edg3. The two major components of wound healing, cell migration and proliferation, were stimulated individually by both lipids. LPA and S1P also stimulated intracellular Ca^{2+} mobilization and mitogen-activated protein kinase (MAPK) phosphorylation. Pertussis toxin partially blocked the effects of both lipids on endothelial cell migration, MAPK phosphorylation, and Ca^{2+} mobilization, implicating $G_{i/o}$ -coupled Edg receptor signaling in endothelial cells. LPA and S1P did not cross-desensitize each other in Ca^{2+} responses, suggesting involvement of distinct receptors. Thus LPA and S1P affect endothelial cell functions through signaling pathways activated by distinct GPCRs and may contribute to the healing of wounded vasculatures.

G protein-coupled receptors; proliferation; migration; calcium; mitogen-activated protein kinase

LYSOPHOSPHATIDIC ACID (LPA) and sphingosine 1-phosphate (S1P) are potent growth factors with diverse biological activities (9, 19, 28). Concentrations of LPA and S1P reach micromolar levels in serum and account for much of the cellular effects of serum (7, 30, 32). The most prominent sources of LPA and S1P are activated platelets, injured cells, and cells stimulated by cytokines and growth factors, suggesting their potential roles in wound healing (9, 19, 28).

When tissues are wounded, damaged blood vessels recruit and activate platelets. The activated platelets play a critical role in wound healing through the release of soluble mediators of vascular dilation, permeability, and cellular proliferation. Endothelial cells also

participate in the wound repair process through enhancement of proliferation, blood coagulation, and angiogenesis. LPA and S1P have been shown to regulate endothelial cell biochemical pathways and functions including Ca^{2+} mobilization (18), proliferation (23), and tight junction permeability (27). However, the effects of LPA and S1P on wound healing properties have not been fully characterized in endothelial cells.

The cellular effects of LPA and S1P are mediated by G protein-coupled receptors (GPCRs). Recent studies have revealed a subfamily of GPCRs encoded by endothelial differentiation genes (*edgs*), also termed lysophospholipid (LP) receptors (4, 6, 14). Of the five Edg receptors with known ligands, human Edg1 (LP_{B1}), Edg3 (LP_{B2}), and Edg5 (LP_{B3}) bind and transduce signals for S1P. Human Edg2 (LP_{A1}) and Edg4 (LP_{A2}) bind and transduce signals for LPA. Intracellular signaling pathways activated by the cloned Edg receptors have been characterized in heterologous expression systems (4, 6, 14).

In this study, we demonstrate for the first time that LPA and S1P stimulate the closure of a wounded endothelial cell monolayer by increasing migration and proliferation of these cells. To determine whether the effects of LPA and S1P on endothelial cells are receptor mediated, we examined expression of mRNAs encoding the Edg receptors and investigated whether the effects of LPA and S1P were transduced by signaling pathways characteristic of those linked to the Edg receptors.

MATERIALS AND METHODS

Reagents. S1P and dihydrosphingosine (DHS) were obtained from Biomol (Plymouth, PA). 1-Oleoyl-lysophosphatidic acid (LPA), 1- β -D-galactosylsphingosine (psychosine, PS), lysophosphatidylglycerol (LPG), fatty acid-free (FAF) BSA, fibronectin, and wortmannin were purchased from Sigma (St. Louis, MO). Fura 2-AM was purchased from Molecular Probes (Eugene, OR). Cell culture inserts with 8- μ m pores were purchased from Becton Dickinson (Franklin Lakes, NJ). Pertussis toxin (PTx) was from Calbiochem (La Jolla, CA). [3 H]thymidine was purchased from Amersham (Arlington Heights, IL). *Taq* DNA polymerase was from GIBCO BRL (Gaithersburg, MD). Fetal bovine serum and calf serum (CS) were obtained from the University of California Medical Center, San Francisco (UCSF) Cell Culture Facilities.

Endothelial cell culture. Adult bovine aortic endothelial cells (ABAEC) were kindly provided by Dr. Richard Weiner at UCSF. ABAEC at passages 8–15 were used in these experiments. Cells were cultured in DMEM H16 (UCSF, Tissue Culture Facilities) supplemented with 10% of CS and passaged weekly. Human umbilical cord vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA)

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and cultured in endothelial cell growth medium (Clonetics) supplemented with 10% FCS and passaged weekly. HUVEC at passages 2–8 were used in the experiments.

In vitro endothelial cell wound healing assay. Endothelial cells were cultured in 48-well plates at 1×10^5 cells/well as confluent monolayers. The monolayers were incubated in the absence of serum for 16 h and wounded in a line across the well with a 200- μ l standard pipette tip. The wounded monolayers were then washed twice with serum-free media to remove cell debris and incubated with different concentrations of LPA, S1P, or 10% FCS. The area of cell-free wound was recorded at indicated time points using a charge-coupled device camera (C2400; NEC, Hawthorne, CA) connected to an inverted microscope (Axiovert 35; Zeiss, Thornwood, NY). The image was subsequently captured by an image-analyzing frame-grabber card (LG-3 Scientific Frame Grabber; Scion, Frederick, MD) and was analyzed by an image analysis software (NIH Image 1.55). The wound healing effect was calculated as the percentage of the remaining cell-free area compared with the area of the initial wound.

[3 H]thymidine uptake experiments. Endothelial cells were in 24-well plates at 5×10^4 cells/well for 24 h and then were incubated in the absence of serum for 16 h and with different concentrations of LPA or S1P for 16 h followed by incubation with 0.175 μ Ci/well of [3 H]thymidine for 6 h. The pulse-labeled cells were then fixed with ice-cold 5% TCA for 20 min at 4°C and followed by three quick washes with 5% TCA to removed remaining unincorporated labels. The washed cells were then lysed by 0.25 N NaOH and the radioactivity was counted by scintillation counter.

Migration assay. Migration of endothelial cells was determined by a modified Boyden chamber assay. Migration chambers with 8- μ m pores were coated with 50 μ l of fibronectin (3 μ g/ml) for 1 h. After removal of the excess coating by aspiration, 1×10^5 endothelial cells were added to the top chambers. The chambers were then transferred into wells of 24-well plates each with different treatments. After 4 h, the nonmigrated cells on the top chamber were removed with a cotton swab. The migrated cells on the bottom of the chamber were fixed with 4% glutaraldehyde and stained with 0.5% of crystal violet. The migrated cells were photographed and quantified by light microscopy at a magnification of $\times 150$ by counting the stained cells from four randomly selected fields.

RT-PCR of *edg* mRNAs. Total cellular RNA was extracted from ABAEC and HUVEC by the TRIzol reagent (GIBCO BRL), and a Superscript kit (GIBCO BRL) was used for RT synthesis of cDNAs. PCR amplification was performed with 35 cycles of 30 s at 94°C, 30 s at 62°C, and 2 min at 72°C. Oligonucleotide pairs were: 5'-dGACTCTGCTGGCAAATTC AAGCGAC and 5'-dACCCCTCCAGTGCATTGTTCA-CAG for *edg1*; 5'-dGCTCCACACACGGATGAGCAACC and 5'-dGTGGTCATTGCTGTGAAGTCCAGC for *edg2*; 5'-dCAAAATGAGGCCTTACGACGCCA and 5'-dTCCCATTCTGAAGTCTGCGTTC for human *edg3*; 5'-dTTCATTGGCAACCTG-GCTCTCTGC and 5'-dTGCGTAGAGGATCAGATGGTCACC for bovine *edg3*; 5'-dAGCTGCACAGCCGCTGCCCGT and 5'-dTGTGTGCCATGCCAGACCTTGTC for *edg4*; and 5'-dCTCTCTACGCCAAGCATTATGTGCT and 5'-dATCTAGAC-CCTCAGACCACCGTGTGGCCCTC for *edg5*. PCR products were resolved by 2% agarose gel and stained with ethidium bromide and photographed.

Western blot analysis of extracellular signal-regulated kinase. Endothelial cells plated in six-well plates were treated with the indicated concentrations of LPA or S1P for 5 min. Treated cells were then lysed with RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50

mM Tris, pH 8.0) containing protease inhibitor cocktail (Sigma) and 2 mM Na vanadate. Equal amounts of cell lysates were separated by 4–10% SDS-PAGE and electrophoretically transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham). The transferred membranes were blocked with 5% nonfat milk for 1 h, incubated with rabbit polyclonal antibody against phosphorylated extracellular signal-regulated kinase (ERK; New England BioLabs, Beverly, MA), and visualized with ECL reagents (Amersham). The blots were stripped and reprobed with antibody against p42 mitogen-activated protein kinase (MAPK; New England BioLabs) to demonstrate uniform loading of proteins.

Calcium assay. HUVEC were cultured overnight in 100-mm dishes at about 75% confluency. Cells were then trypsinized, washed, and loaded with 2.5 μ M of fura 2-AM in PBS (containing 1 mM CaCl_2) for 30 min at 37°C in the dark, washed again, and resuspended in PBS. Cuvettes containing 1×10^6 fura 2 loaded cells in 1.5 ml were mixed with a magnetic stirrer in a Perkin-Elmer LS 50B fluorometer. Fluorescence was recorded before and after the addition of phospholipids dissolved in PBS containing 0.1 mg/ml FAF human serum albumin. The fluorescence ratio obtained at 340 and 380 nm (F_{340}/F_{380}) was used as an index of intracellular calcium concentration ($[\text{Ca}^{2+}]_i$).

Statistical analysis. Data were statistically analyzed by one-way ANOVA followed by Fisher's protected least-significant differences test (StatView; Abacus Concept, Berkeley, CA). A value of $P < 0.05$ was considered statistically significant.

RESULTS

LPA and S1P stimulation of in vitro wound healing of endothelial cell monolayers. The wound healing effects of LPA and S1P on cultured endothelial cell monolayers were examined by using an in vitro wound healing assay (8). The cell-free wound gaps of ABAEC and HUVEC monolayers healed slowly in the absence of any treatment. However, in the presence of LPA and S1P, the closure of the wounded area was significantly accelerated (Fig. 1). More cells appeared in the wounded gap, which represents enhanced healing of the wounded area. The remaining cell-free area at 12–16 h as a percentage of the initial wound area was taken as an index of wound healing (Figs. 1 and 2). In control ABAEC, 68% of the wound area remained cell-free at 12 h after wounding. However, in the LPA- and S1P-treated groups, only 45% and 38% remained, respectively. Cells treated with 10% FCS showed 23% remaining as cell-free area (Fig. 2). In control HUVEC, 71% of the wound area remained cell free at 16 h after wounding, compared with 59% in LPA-treated, 17% in S1P-treated, and 35% in 10% FCS-treated groups (Fig. 2). Similar results were obtained in at least three such experiments.

The effects of LPA and S1P on the closure of the wounded endothelial cell monolayers can be attributed to two distinct processes: cell proliferation and cell migration. We next examined the effects of LPA and S1P on these two different functions of endothelial cells.

LPA and S1P stimulation of endothelial cell proliferation. Endothelial cells were starved in serum-free conditions for 16 h before treatments began. Starved cells

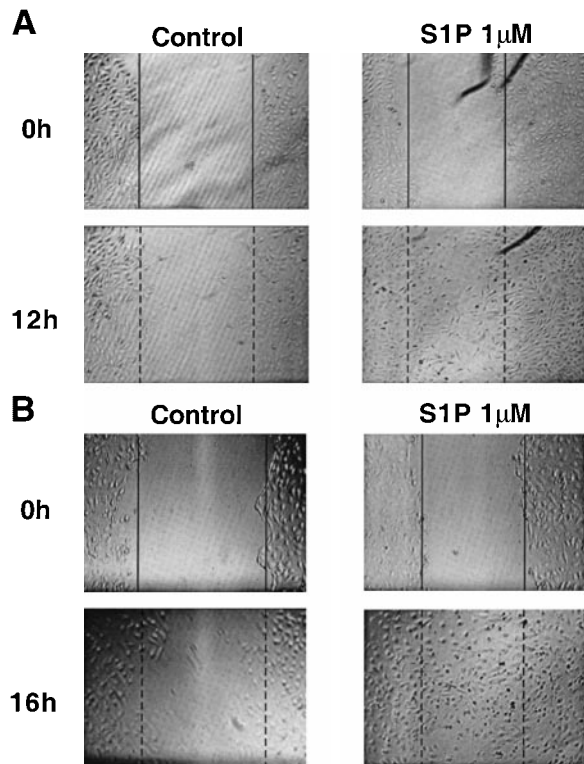


Fig. 1. Sphingosine 1-phosphate (S1P) stimulation of closure of wounded endothelial cell monolayer. Confluent endothelial cell monolayers of adult bovine aortic endothelial cells (ABAEC; *A*) or human umbilical cord vein endothelial cells (HUVEC; *B*) were wounded with a pipette tip and treated with 1 μ M S1P or vehicle (control) for indicated time. Initial wounded area was defined by lines. Cell growing into lines are considered as wound closure.

were treated with different concentrations of lipids for 16 h followed by a 6-h pulse-labeling with [3 H]thymidine. In HUVEC, LPA (0.1–10 μ M) stimulated [3 H]thymidine uptake up to twofold compared with control in a concentration-dependent manner (Fig. 3), which is consistent with a previous report (23). The same concentrations of S1P also stimulated [3 H]thymidine uptake in a similar concentration-dependent fashion. Similar results were seen in three other independent experiments.

LPA and S1P stimulation of endothelial cell migration. The second important component of wound closure is cell migration. We investigated the effects of LPA and S1P on endothelial cell migration in a modified Boyden chamber with fibronectin-coated filters (12). LPA and S1P stimulated migration of both HUVEC and ABAEC in a concentration-dependent and saturable manner (Fig. 4). S1P was more potent than LPA in stimulating endothelial cell migration in both cell types. The EC_{50} values for both LPA and S1P were estimated to be \sim 100 nM (Fig. 4*B*). Furthermore, LPG, DHS, and PS, which are lipids with structures homologous to LPA and S1P, had no effect on endothelial cell migration at concentrations up to 10 μ M (data not shown). At 10 ng/ml, basic fibroblast growth factor (bFGF), a polypeptide growth factor known to stimulate endothelial cell migration, increased ABAEC migra-

tion to a level similar to that of 100 nM S1P (data not shown).

LPA and S1P receptor expression in both human and bovine endothelial cells. To see whether the effects of LPA and S1P are mediated by Edg receptors, we first examined the expression of Edg receptors in endothelial cells. With the use of specific primers derived from human sequences, RT-PCR revealed expression of *edg1*, *edg2*, and *edg3*, but not *edg4* or *edg5* in HUVEC (Fig. 5). Similar expression patterns of Edg receptors were obtained from RT-PCR reactions for a primary culture of human capillary endothelial cells of skin origin (data not shown). Furthermore, by using primers derived from human *edg* sequences, RT-PCR revealed that at least *edg1*, *edg2*, and *edg3* were expressed in ABAEC (Fig. 5). The authenticity of the amplified products as *edg* was confirmed by DNA sequencing. These results

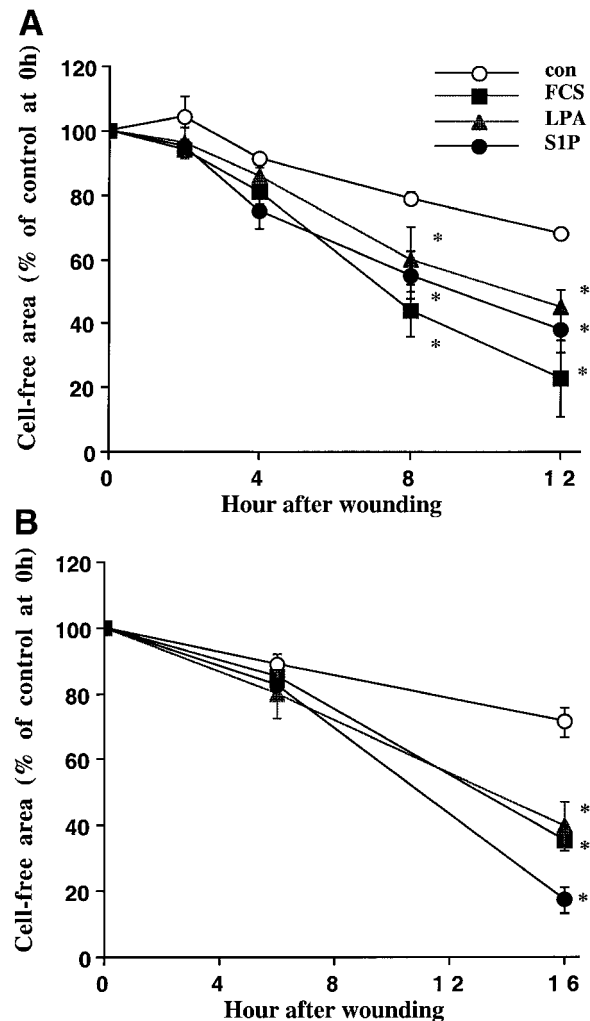


Fig. 2. Lysophosphatidic acid (LPA) and S1P stimulation of endothelial cell wound healing. Wounded ABAEC (*A*) and HUVEC (*B*) were treated with vehicle (con), 10% FCS, 10 μ M LPA, or 1 μ M S1P and recorded by a digital camera. Percentage of cell-free area at indicated time points compared with that at 0 h was determined. Each condition was performed in triplicate and data were expressed as mean \pm SD. Experiments were repeated three times, and results from a representative experiment are shown (* $P < 0.05$).

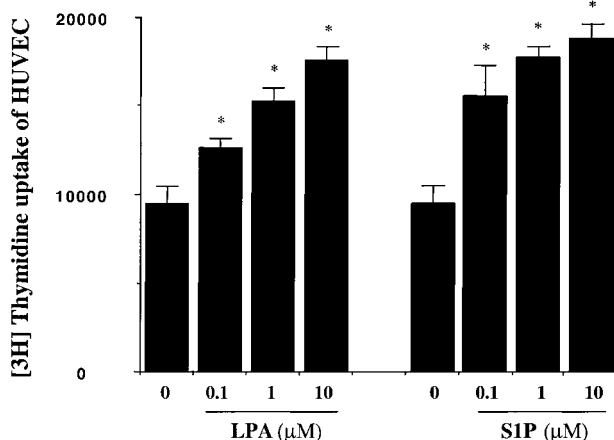


Fig. 3. LPA and S1P stimulation of endothelial cell proliferation. HUVEC plated in 24-well plates were incubated in media containing 0.1% FCS for 16 h. Cells were treated with LPA or S1P at indicated concentrations for 20 h, and then incubated for 6 h with [³H]thymidine. Incorporation of radioactivity into cells was determined by scintillation counting. Each condition was analyzed in triplicate and data were expressed as mean ± SE (**P* < 0.05).

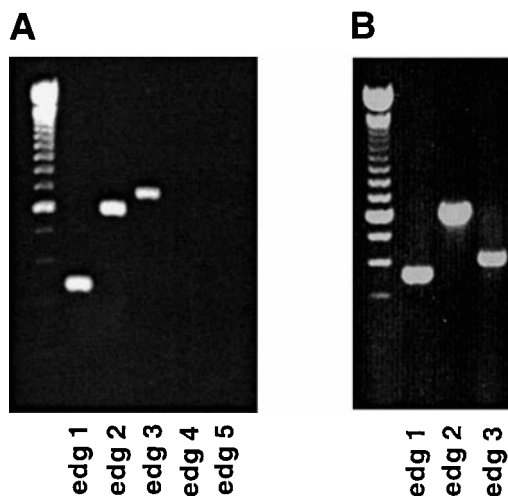


Fig. 5. RT-PCR analysis of endothelial differentiation gene (Edg) receptor expression in endothelial cells. RT-PCR products from HUVEC (A) and ABAEC (B) were separated on 2% agarose gel with 100 bp mol wt marker (with 600 bp band highlighted). A 351 bp product was amplified by primer set for both human and bovine *edg1*. A 621 bp product was amplified by primer set for human and bovine *edg2*. A 701 bp product was amplified by primer set for human *edg3*, whereas a 427 bp band was amplified by primer set for bovine *edg3*.

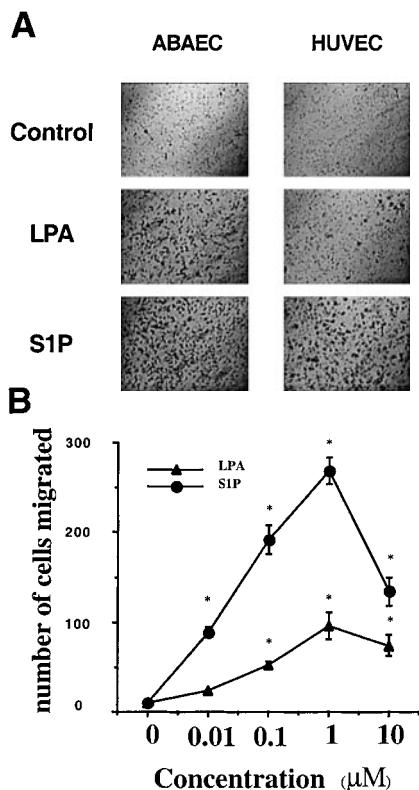


Fig. 4. LPA and S1P stimulation of endothelial cell migration. A: ABAEC (left) or HUVEC (right) were plated on top wells of migration chambers (8-μm pores) that were coated with 3 μg/ml of fibronectin. 10 μM LPA, 1 μM S1P, or vehicle (control) was added to bottom chambers. After 4 h, unmigrated cells on top chambers were removed, and migrated cells were fixed, stained with 0.5% crystal violet, and photographed. B: number of migrated HUVEC after treatment with indicated concentrations of LPA or S1P. Migrated cells were counted in four random ×150 amplified fields. Each condition was done in triplicate and data were represented as mean ± SE (**P* < 0.05).

indicated expression of *edg1*, *edg2*, and *edg3* in endothelial cells of different origins.

LPA and S1P effects on Ca²⁺ mobilization in endothelial cells. To further substantiate the receptor-dependent effects of LPA and S1P, we tested whether LPA and S1P elicited cellular effects through biochemical pathways known to be utilized by the Edg receptors. It has been shown that LPA and S1P mobilize [Ca²⁺]_i through both G_i- and G_q-coupled Edg receptors (3, 21, 26). Here, we observed that LPA and S1P mobilized [Ca²⁺]_i in endothelial cells in a PTx-sensitive manner (Fig. 6). In HUVEC, S1P stimulated increases in [Ca²⁺]_i in a concentration-dependent manner (10 nM to 10 μM) as measured by fura 2 fluorometry (data not shown). This effect was inhibited by pretreatment with PTx (Fig. 6A), suggesting the involvement of G_{i/o} proteins. LPA also induced significant increases in [Ca²⁺]_i in these cells, albeit the magnitude of the responses was smaller than that evoked by S1P (Fig. 6B). Pretreatment of endothelial cells with 10 nM S1P abolished increases in [Ca²⁺]_i elicited by subsequent treatment with S1P (Fig. 6B, second arrow). However, pretreatment with 1 μM LPA had no effect on S1P-stimulated Ca²⁺ mobilization (data not shown), nor did the pretreatment with S1P have any effect on the response to LPA (Fig. 6B, third arrow). This lack of heterologous cross-desensitization strongly suggests that S1P and LPA utilize different receptors in mobilizing [Ca²⁺]_i in HUVEC, consistent with the results using other cell types, including bovine endothelial cells (18).

PTx inhibition of LPA and S1P effects on endothelial cell migration and ERK activation. We further investigated whether the effects of LPA and S1P on other endothelial cell functions can be blocked by PTx, which would further support the involvement of G_{i/o}-coupled Edg receptors. The stimulatory effects of LPA and S1P

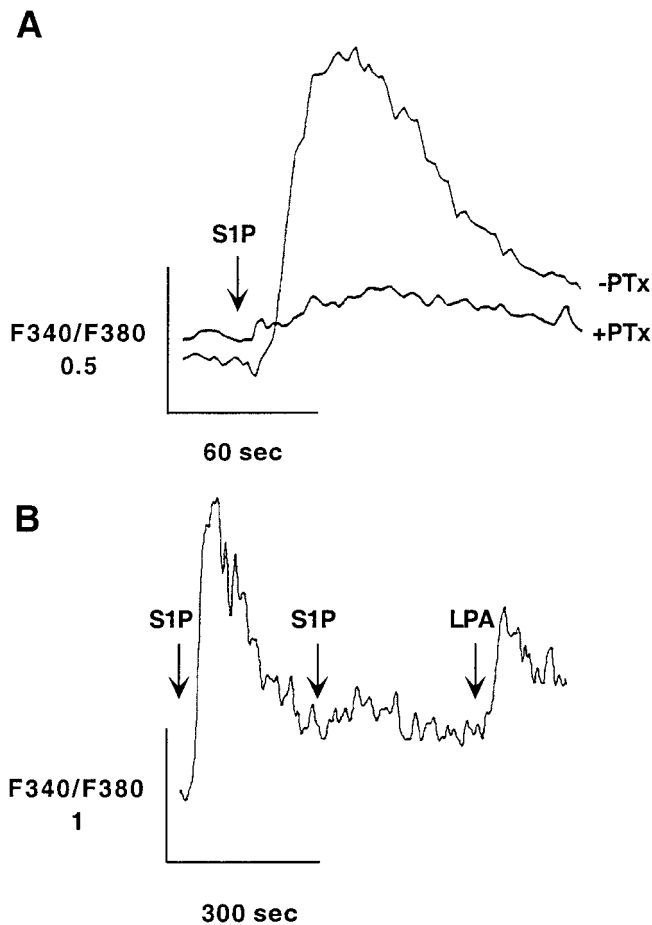


Fig. 6. Mobilization of intracellular Ca^{2+} by LPA and S1P in HUVEC. *A*: pertussis toxin (PTx)-sensitive effect of S1P. HUVEC were pretreated with 50 ng/ml of PTx (+PTx) or vehicle (-PTx) for 16 h and loaded with fura 2. Fura 2-loaded cells were then stimulated with 10 nM S1P at arrow. $[\text{Ca}^{2+}]_i$ was measured by fluorometry and expressed as a ratio between F_{340} and F_{380} . *B*: lack of cross-desensitization between LPA and S1P. Typical trace of F_{340}/F_{380} signals after sequential addition of 10 nM S1P, 10 nM S1P, and 1 μM LPA at arrows.

on ABAEC migration were suppressed by pretreatment with 15 ng/ml PTx (Fig. 7A). PTx inhibited LPA- and S1P-stimulated migration of ABAEC by $52 \pm 14\%$ and $41 \pm 10\%$ (mean \pm SD, $n = 4$), respectively. In contrast, PTx had no inhibitory effects on bFGF-stimulated migration (data not shown). Similar results were observed in HUVEC cells (data not shown).

Moreover, ERK phosphorylation stimulated by LPA and S1P was also inhibited by PTx treatment (Fig. 7B). These results suggest that the effect of LPA and S1P on endothelial cell migration and ERK activation are partially mediated through $G_{i/o}$ -coupled receptors.

DISCUSSION

Previous studies have established that multiple effects of LPA and S1P are transduced through receptors coupled to PTx-sensitive G proteins (9, 13, 19, 28, 31). Recent studies of the cloned Edg receptors revealed that Edg1, Edg2 and Edg3 all use G_i to transduce, at least some of, their signals (9, 19, 28). Here, we observed that the effects of LPA and S1P on endothelial

cell migration, MAPK phosphorylation, and Ca^{2+} mobilization were suppressed by PTx treatment. This inhibition by PTx suggested that effects of LPA and S1P on endothelial cells are partially mediated through $G_{i/o}$ -coupled Edg receptors.

It has been shown that LPA and S1P activate Rho, a small G protein involved in cell migration. C3 exoenzyme, a *C. botulinum* toxin that inhibits Rho activity, has been shown to inhibit endothelial cell migration activated by wound healing (1). It has been reported that Edg receptors mediated the activation of Rho (2, 17). These observations suggest that LPA and S1P regulate endothelial cell migration through activation of Rho. Phosphatidylinositol 3-kinase (PI3K), activated by LPA and S1P, is also involved in cell movement (16).

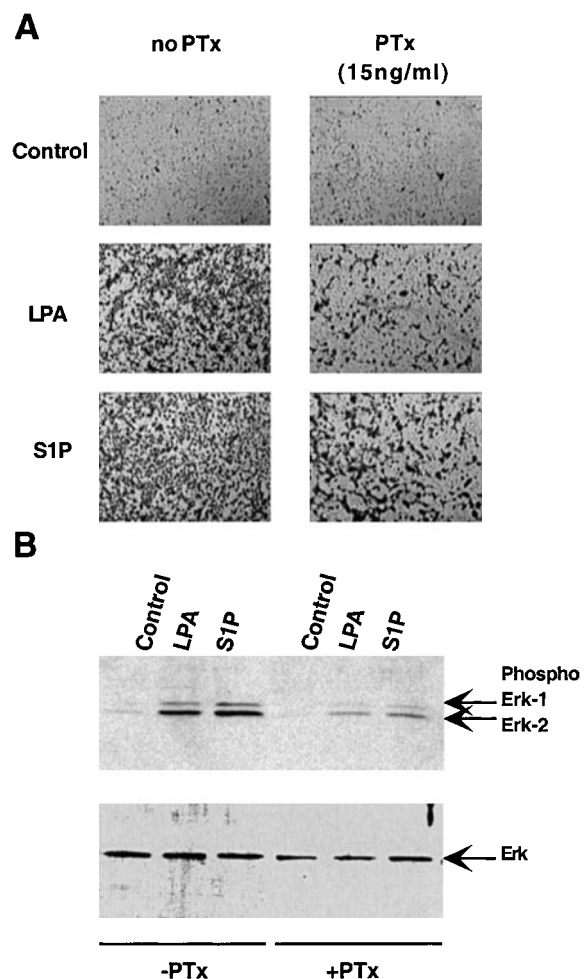


Fig. 7. PTx inhibition of LPA and S1P effects on endothelial cells. *A*: ABAEC pretreated with 15 ng/ml of PTx (right panel) or no PTx (left) for 16 h were subsequently treated with either vehicle (control), 10 μM LPA, or 1 μM S1P for 4 h to induce migration. Migrated cells were fixed, stained, and photographed. Results from a representative experiment is shown. *B*: HUVEC were pretreated with vehicle (-PTx) or 50 ng/ml of PTx (+PTx) for 16 h before being treated with 10 μM LPA, 1 μM S1P, or vehicle (control) for 5 min. Cell lysates were prepared and subjected to immunoblot with an antibody against phosphorylated extracellular signal-regulated kinase (ERK)-1 and ERK-2 (top). Blot was stripped and reprobed with anti-ERK antibody (recognized both phosphorylated and nonphosphorylated forms of ERK) to show even loading of proteins (bottom).

We observed that LPA and S1P effects on endothelial cell migration can be partially inhibited by 20 μ M wortmannin, a specific PI3K inhibitor (data not shown). Collectively, these results suggest that LPA and S1P effects on endothelial cell migration are mediated by Edg receptors and multiple G protein-activated downstream effectors.

Our results showed that the effects of LPA and S1P on endothelial cells had EC₅₀ values in physiological concentration range and were saturatable at 10 μ M concentration, supporting a receptor-mediated mechanism (Fig. 4). By RT-PCR, we further showed that both HUVEC and ABAEC express receptors for LPA (Edg2) and S1P (Edg1, Edg3) at the mRNA level (Fig. 5). The complete repertoire of Edg receptors that mediate the effects of LPA and S1P in endothelial cells has yet to be determined because additional Edg receptors may exist. The future development of LPA and S1P antagonists will address whether the effects of LPA and S1P are evoked directly by these lipid phosphates.

Mediators released from platelets play important roles in the regulation of endothelial cell functions, including wound healing. In ex vivo experiments, it was shown that perfusion of platelets facilitated endothelial cell wound healing in isolated blood vessels (15). Peptide growth factors bFGF, vascular endothelial growth factor, transforming growth factor- β (TGF- β), epidermal growth factor, and platelet-derived growth factor, many of them released from platelets, affect endothelial cell functions including wound healing (5, 20, 22, 24, 29). However, nonpeptide molecules, such as bioactive lipids, also play important roles. These lipids may exert their effects directly, or indirectly through synthesis and release of peptide growth factors. It has been shown that LPA stimulates the secretion of TGF- β in keratinocytes (25) and insulin-like growth factor II in human breast (11) and ovarian cancer cells (10). Increasing evidence suggests that LPA and S1P, released in large quantities from activated platelets, may regulate endothelial cell function. We now show that LPA and S1P stimulate wound healing of endothelial cells in vitro, which is attributable to the combined stimulatory effects of endothelial cell proliferation and migration. We also observed that LPA and S1P stimulated urokinase activity in endothelial cells (data not shown). Consequently, these effects of LPA and S1P may result in enhanced angiogenesis. Understanding the mechanisms by which LPA and S1P regulate endothelial cell function may add insight into pathological angiogenic processes.

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