Angiopoietin-2 Exocytosis Is Stimulated by Sphingosine-1-Phosphate in Human Blood and Lymphatic Endothelial Cells

Cholsoo Jang, Young Jun Koh, Nam Kyu Lim, Hyun Jung Kang, Duk Hoon Kim, Sung Kwang Park, Gyun Min Lee, Choon Ju Jeon, Gou Young Koh

Objective—Although diverse functions of angiopoietin-2 (Ang2) have been revealed, little is known about upstream signaling molecules regulating Ang2 exocytosis. We therefore investigated the mechanism of Ang2 exocytosis in human blood and lymphatic endothelial cells (BECs and LECs) by stimulation with sphingosine-1-phosphate (S1P).

Methods and Results—By immunostaining and ELISA analyses using our newly developed human Ang2-specific antibodies, Ang2 exocytosis from human endothelial cells was examined. Both exogenous and endogenous S1P trigger rapid Ang2 exocytosis in time- and dose-dependent manners. Intriguingly, S1P-induced Ang2 exocytosis is higher in LECs than BECs. These effects of S1P are mainly mediated by the endothelial differentiation gene receptor 1, which subsequently activates its downstream phospholipase C and intracellular calcium mobilization to trigger Ang2 exocytosis. Consistently, S1P also dramatically stimulates Ang2 exocytosis from the ECs of ex vivo–incubated blood vessels.

Conclusion—These results imply that the rapid secretion of Ang2 by exocytosis from endothelial cells is another possible mechanism underlying S1P-induced angiogenesis and inflammation. (Arterioscler Thromb Vasc Biol. 2009;29:401-407.)

Key Words: angiogenesis ■ angiopoietin ■ endothelial differentiation gene receptor ■ lymphangiogenesis ■ Weibel-Palade bodies

Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) were identified as key regulators of pathophysiologically angiogenesis along with vascular endothelial growth factors (VEGFs). Both Ang1 and Ang2 are secretory proteins and have an amino-terminal coiled-coil domain for their oligomerization and a carboxy-terminal fibrinogen-like domain (FLD) for binding to their receptor, Tie2. In contrast to the consistent role of Ang1 in stabilizing newly-formed vessels, more versatile functions have been reported for Ang2 such as angiogenesis, lymphangiogenesis, vascular permeability, and inflammation. Intriguingly, Ang2 is synthesized in advance and stored in Weibel-Palade bodies (WPBs) of endothelial cells (ECs), thereby being rapidly secreted to the external microenvironment by exocytosis when stimulated by extracellular signals. This implies that Ang2 activity is temporally regulated to exert controlled actions onto Tie2-expressing cells via autocrine and paracrine rather than endocrine manner. However, despite the importance of Ang2 exocytosis control, signaling pathways regulating Ang2 exocytosis are poorly understood.

Lymphatic endothelial cells (LECs) have their own characteristic morphologies, functions, and specific gene expressions compared to blood endothelial cells (BECs). Lymphatic vessels function as a key route for immune cell trafficking and interstitial fluid drainage, and directly participate in inflammation and tissue homeostasis. Interestingly, Ang2 knockout mice exhibit defective lymphatic vessel development, implicating essential role of Ang2 in development and function of LECs. Although the expression of Ang2 in LECs has been documented, presence of Ang2 granules and regulation of Ang2 secretion are still unknown.

Sphingosine-1-phosphate (S1P) is a phospholipid signaling molecule generated from the plasma-membrane sphingosine by sphingosine kinases 1 and 2. S1P is mainly secreted from platelets, monocytes, and mast cells, thereby governing various cellular processes including cell proliferation, survival, and migration. Moreover, S1P has a dual mode of action as an extracellular ligand for cell-surface receptors and an intracellular second messenger. In fact, diverse functions of S1P are mostly contributed by its G-protein coupled–receptors (GPCR), which belong to the endothelial differentiation gene (EDG) family. Among several EDG members, only EDG1 and EDG3 are expressed to mediate diverse effects of S1P on BEC physiologies like adherens junction...
assembly and cell motility. However, little is known about the role of S1P and its downstream signaling in LECs during pathophysiological conditions.

In this study, we provide the first direct evidence of Ang2 exocytosis in primary cultured human LECs and in the endothelial cells of ex vivo-incubated blood vessels. We also define downstream signaling pathways of S1P-induced Ang2 exocytosis both in BECs and LECs.

Methods

Cell Culture and Reagent Treatment

HUVECs were prepared from human umbilical cords by collagenase digestion as described previously. HLEC were purchased from Clonetics (Walkersville, Md). Cells (4 to 6 passages) grown in EBM-2 with supplements were starved with serum-free EBM-2 for 12 hours before treatment. The following reagents were treated: 10 μmol/L DMS for 1 hour; 200 ng/mL PTX for 12 hours; 10 μmol/L BAPTA or BAPTA-AM in DMEM, or calcium-free DMEM for 10 minutes. Appropriate dissolving agents were treated in parallel as controls. The Ang2 secretion after treatment was normalized against the Ang2 secretion before treatment to eliminate possible variance among samples.

Ang2 ELISA Analysis

Ninety-six-well plates were coated with 100 ng/well 4A1A3 in coating solution (7.5 mmol/L Na2CO3, 17.5 mmol/L NaHCO3, pH 9.6) for o/n at 4°C. After blocking with 1% BSA in PBS for 1 hour at RT, 200 μL of serially diluted standard Ang2 (from 1500 to 46.9 pg/mL) and samples (culture supernatant without dilution) were incubated for 1.5 hour at RT. After incubating with 100 ng/well biotin-labeled 4B33A11 for 1.5 hour at RT, streptavidine-HRP (1:2500, Pierce) was added for 30 minutes at RT. Tetramethylbenzidine (Sigma) was used to visualize the signal. The absorbance was determined at 450 nm using a SOFTmaxPRO ELISA reader (Molecular Devices).

Immunofluorescence Staining

ECs grown on 0.1% gelatin-coated coverslips or umbilical cord slices were fixed with 3.7% paraformaldehyde in PBS for 30 minutes. After washing with 0.1% PBST, the samples were permeabilized with 0.5% PBST for 10 minutes. The cords were incubated with 0.1% PBST for 10 minutes. The samples were incubated with blocking solution (5% BSA and 5% donkey serum in 0.1% PBST) for 30 minutes and incubated with appropriate primary antibodies in blocking solution for o/n at 4°C. The primary antibodies were (1) mouse anti-human Ang2 antibody, 4B33A11 (1:100); (2) rabbit anti-human von Willebrand factor (vWF; 1:200, Sigma); (3) rabbit anti-human Prox-1 (1:200, Reliatech, Germany); (4) rabbit anti-human EDG1 (1:100, Abcam); (5) goat anti-human EDG3 (1:50, Santa Cruz Biototechnology); (6) FITC-conjugated mouse anti-human smooth muscle actin (1:200, Sigma). After washing with 0.1% PBST, the samples were incubated with appropriate secondary antibodies (1:1000) and DAPI (1:10000) in blocking solution for 3 hours. The samples were mounted with fluorescence mounting medium and observed using a Zeiss LSM 510 confocal microscope (Carl Zeiss).

The methods for generation of Ang2 antibodies, measurement of S1P concentration, ex vivo incubation of umbilical cords, RT-PCR analyses, siRNA transfection, Matrigel plug assay, and statistics are described in supplemental materials (available online at atvb.ahajournals.org).

Results

Visualization and Quantification of Human Ang2 in Endothelial Cells

Immunofluorescence staining using our newly developed anti-human Ang2 antibody (clone No. 4B33A11) revealed typical granular Ang2 localization in primary cultured HUVECs (Figure 1A, top). These Ang2 granules were colocalized with multimeric protein vWF, a major constituent of WPB. We also examined whether primary cultured HLEC contain Ang2 granules. HLEC were marked by the LEC-specific transcriptional factor Prox-1 in the nuclei (Figure 1A, middle). Intriguingly, Ang2 granules were colocalized with vWF but unevenly localized to one side of the perinuclear region of HLEC (Figure 1A, bottom). This was different from the distribution of Ang2 granules in HUVECs, in which they were widely dispersed in the cytoplasm (Figure 1A, top). Using the same antibody, we also detected Ang2 granules in the ECs of human umbilical cord blood vessels (Figure 1B). Notably, immunopositive Ang2 signals in venous ECs were greater than those in arterial ECs (Figure 1B). In contrast, no immunopositive Ang2 signals were detected in nonendothelial cells of the umbilical cord (supplemental Figure 1). Immunoblotting analyses revealed that clone 4B33A11 detected both nonreduced and reduced forms of full-length and FLD of Ang2 protein, whereas clone 4A1A3 recognized only the nonreduced form of full-length Ang2, indicating that they recognize different structural epitopes on Ang2 protein (data not shown). By combining
these antibodies, we developed a new human Ang2 ELISA with high sensitivity (limit of quantification, \( \approx 50 \) pg/mL). This ELISA method did not show any cross-reactivity with other angiopoietins, including Ang3, Ang4, and COMP-Ang1, a potent and soluble variant of Ang1\(^{25}\) (data not shown), demonstrating high specificity of the Ang2 ELISA. These sensitive and specific Ang2 antibodies allowed us to investigate upstream signaling molecules regulating Ang2 exocytosis in human ECs.

**Ang2 Exocytosis Is Stimulated by S1P in BECs and LECs**

Because both S1P and Ang2 modulate inflammation and angiogenesis,\(^5,17,18\) and BECs abundantly express S1P receptors,\(^21\) we examined whether S1P stimulates Ang2 exocytosis in vascular ECs. On various doses of S1P (0.1 to 1000 nmol/L) treatment, there were remarkable dose-dependent depletions of Ang2 granules in primary cultured HUVECs (Figure 2A, left). Consistently, S1P increased the amount of Ang2 secretion in a dose-dependent manner (Figure 2B), indicating that S1P actively stimulates Ang2 exocytosis from BECs.\(^{10}\) Time-course experiments showed that S1P-induced Ang2 exocytosis had already begun by 5 minutes after S1P treatment (1 \( \mu \)mol/L), and it was much greater than the basal level of Ang2 secretion (Figure 2C). Ang2 exocytosis occurred \( \approx 44\% \) at 5 minutes and \( \approx 77\% \) at 10 minutes during 1 hour of S1P stimulation (Figure 2C). Similarly, Ang2 exocytosis from HLECs also occurred in a S1P dose-dependent manner, but the amounts of Ang2 secretion in HLECs were greater (\( \approx 1.4\)- to 2.1-fold) than those in HUVECs (Figure 2A and 2B). Moreover, Ang2 secretion in HLECs was also rapid and the increased amounts were again higher (\( \approx 1.6\)- to 2.3-fold) than those in HUVECs (Figure 2C). On the S1P stimulation, the greater Ang2 secretion from LECs than BECs could be owing to higher intracellular Ang2 protein storage in LECs than BECs (supplemental Figure II). However, the treatment of S1P (1 \( \mu \)mol/L) did not affect Ang2 mRNA synthesis up to 12 hours in both ECs (supplemental Figure III), indicating that S1P stimulates Ang2 exocytosis without immediate synthesis of Ang2.

**TNF-\( \alpha \)-Induced Endogenous S1P Synthesis Stimulates Ang2 Exocytosis**

These observations led us to examine the effect of endogenous S1P on Ang2 exocytosis. Endogenous S1P is generated by 2 sphingosine kinases and secreted via unknown mechanisms as an extracellular signaling molecule.\(^{17,18}\) Because TNF-\( \alpha \) is one of the most powerful stimulators of sphingosine kinases,\(^7\) we treated HUVECs and HLECs with TNF-\( \alpha \) and assessed its effect on S1P secretion. Consequently, TNF-\( \alpha \) (100 ng/mL) treatment induced S1P secretion from both HUVECs and HLECs (Figure 3A). The secretion of S1P was the highest at 10 minutes after TNF-\( \alpha \) treatment, and its level was decreased over time (Figure 3A). Consistently, various doses of TNF-\( \alpha \) (0.1 to 100 ng/mL) increased Ang2 exocytosis in a dose-dependent manner, and the secreted Ang2 levels were higher (\( \approx 1.3\)- to 2.4-fold) in HLECs than HUVECs (Figure 3B). To determine whether TNF-\( \alpha \)-induced Ang2 exocytosis is mediated by newly synthesized S1P, we pretreated HUVECs and HLECs with a sphingosine kinase inhibitor, dimethylsphingosine (DMS, 10 \( \mu \)mol/L), before stimulating with S1P (1 \( \mu \)mol/L) or TNF-\( \alpha \) (100 ng/mL). As a result, DMS substantially blocked Ang2 exocytosis by TNF-\( \alpha \) but not by exogenous S1P (Figure 3C and supplemental Figure IV), suggesting that newly synthesized endogenous S1P stimulates Ang2 exocytosis in ECs.

**PTX-Sensitive Receptor Mediates Extracellular S1P-Induced Ang2 Exocytosis**

Because S1P functions as an extracellular ligand or an intracellular second messenger,\(^{19,20}\) we investigated which form of S1P is responsible for inducing Ang2 exocytosis. We used dehydro-S1P (dhS1P), a variant of S1P that has only extracellular but not intracellular activity.\(^{76}\) Treatment of various doses of S1P or dhS1P displayed similar efficacies in stimulating Ang2 exocytosis in ECs (Figure 3D and supple-
mental Figure IV), suggesting that extracellular S1P stimulates Ang2 exocytosis. Extracellular S1P conveys its downstream signaling via plasma membrane GPCR.17,18 To examine whether GPCR is involved in S1P-induced Ang2 exocytosis, HUVECs and HLECs were pretreated with a specific GPCR blocker, PTX (200 ng/mL),21 before S1P or TNF-α stimulation. Pretreatment of PTX markedly blocked Ang2 exocytosis by S1P or TNF-α (Figure 3E and supplemental Figure IV), implicating that GPCR is critical for mediating S1P-induced Ang2 exocytosis in ECs.

EDG1 Mainly Mediates S1P-Induced Ang2 Exocytosis

Because the EDG receptor family is the subset of GPCR that mediates S1P-dependent signaling pathway,17,18 we assessed the expression of various EDG receptors in HUVECs and HLECs. RT-PCR analyses revealed that both ECs selectively express EDG1 and EDG3 but not EDG5, EDG6, or EDG8 (Figure 4A), and quantitative RT-PCR analyses showed that the expression levels of EDG1 were much higher (13.4-fold in HUVECs and 18.7-fold in HLECs) than those of EDG3 in ECs (supplemental Figure V), which is consistent with the previous report.21 Moreover, immunostaining analyses showed that EDG1 and EDG3 dramatically translocated from plasma membrane into cytoplasm on S1P stimulation probably via receptor internalization27 (Figure 4B), suggesting that EDG receptors on EC surface are actively responsive to S1P. To further examine role of EDG1 and EDG3 in Ang2 exocytosis, we selectively reduced expression of EDG1 or EDG3 using siRNAs targeting each receptor (si1 or si3, respectively) and a scrambled random siRNA (sc) as a negative control. RT-PCR and immunostaining analyses showed that si1 or si3 but not sc selectively reduced mRNA and protein levels of EDG1 or EDG3, respectively (Figure 4C and 4D). Interestingly, S1P-induced Ang2 exocytosis was blocked by si1 but not by si3 or sc in HUVECs and HLECs (Figure 4E). Moreover, cotransfection of si1 and si3 did not further decrease the si1-induced reduction of Ang2 exocytosis (Figure 4E). These data suggest that EDG1 is a major subtype of EDG receptor mediating S1P-induced Ang2 exocytosis in both EC types.
Phospholipase C and Intracellular Calcium Are Crucial Mediators of Ang2 Exocytosis by S1P

To further define downstream pathways mediating S1P-induced Ang2 exocytosis, we examined the effect of all the known mediators of EDG receptors, namely PI3K, ERK, p38, phospholipase C (PLC), and adenylate cyclase on S1P-induced Ang2 release, using specific inhibitors of each pathway. As a result, only U73122, a PLC inhibitor, significantly blocked S1P-induced Ang2 exocytosis in HUVECs. Moreover, pretreatment of U73122 (0.1 to 10 μmol/L) suppressed S1P-induced Ang2 exocytosis in a dose-dependent manner in both ECs (Figure 5B and supplemental Figure IV), indicating that PLC plays an important role in Ang2 exocytosis by S1P. Because PLC-dependent calcium mobilization was known to promote exocytosis, we examined whether calcium mobilization mediates S1P-induced Ang2 exocytosis. We used Ca²⁺-free media and membrane-permeable or impermeable calcium chelator (BAPTA-AM or BAPTA) to deplete intracellular or extracellular calcium, respectively. Interestingly, Ang2 exocytosis was substantially suppressed by Ca²⁺-free media (≈52% and ≈60%) and BAPTA-AM pretreatment (≈32% and ≈37%) but not by BAPTA pretreatment in HUVECs and HLECs (Figure 5C and supplemental Figure IV), suggesting that intracellular calcium mediates Ang2 exocytosis in both ECs.

SIP Induces Ang2 Exocytosis From the ECs of Ex Vivo–Incubated Umbilical Cord Vessels

Our findings in primary cultured ECs led us to further investigate S1P-induced Ang2 exocytosis in ex vivo-incubated umbilical cords. Cross-sectioned slices (≈1 cm length) of the cords were treated with control buffer, S1P, or a powerful secretagogue, phorbol 12-myristate 13-acetate (PMA) as a positive control. In control buffer–treated cords, strong immunopositive Ang2 signals were specifically detected at the region of vWF-expressing ECs in the umbilical vein (Figure 6A, left). In contrast, S1P (1 μmol/L) or PMA (50 ng/mL)-treated cords displayed considerably decreased immunopositive Ang2 signals (Figure 6A, middle and data not shown). Higher magnification images confirmed that Ang2 signals were largely disappeared in the ECs of the umbilical vein treated with S1P (Figure 6B). Consistently, the amounts of Ang2 secretion were much higher in S1P- or PMA-treated cords than control (Figure 6C). To exclude any Ang2 secretion from non-ECs of cords, cross-sectioned slices of the endothelium-denuded cord were treated with S1P. Basal amount of Ang2 secretion from the endothelium-denuded cord was very low (≈14% of intact cord), and no substantial increase in S1P-induced Ang2 secretion was observed (Figure 6C), suggesting that S1P stimulates Ang2 exocytosis from the ECs of umbilical blood vessels. Furthermore, to examine roles of S1P-induced Ang2 in angiogenesis, we performed an angiogenesis assay using a Matrigel plug assay. Combination of S1P (0.5 nmol) and Ang2 (40 ng)-containing Matrigel displayed markedly higher blood vessel formation than either S1P (0.5 nmol) or Ang2 (40 ng) alone-containing Matrigel (supplementary Figure VI), suggesting that S1P-induced Ang2 release from BECs may coordinateably enhance angiogenesis.

Discussion

Using our newly developed antibodies against human Ang2, we show that a vasoactive lipid signaling molecule, S1P, potently activates endogenous Ang2 exocytosis from the WPBs of BECs, LECs, and the endothelial cells of ex vivo-incubated blood vessels. WPBs are EC-specific storage granules containing at least 10 molecules, including vWF, P-selectin, interleukin-8, CD63, and Ang2. Exocytosis of these cargo molecules from WPBs is one of the earliest responses of vascular stimulation, and plays pivotal roles in thrombosis and inflammation. Consistently, Ang2 exocytosis from WPBs also rapidly occurs within a few minutes after stimulation, indicating that Ang2 secretion is immediate and dynamic.
Our data show that basal and S1P-induced Ang2 secretions are higher in LECs than BECs. It is well known that LECs are more prone to inflammatory and angiogenic stimuli than BECs. Moreover, lymphatic capillaries have neither surrounding mural cells nor a basement membrane and exhibit occasional gaps between cells, thereby being more permeable for macromolecules than tightly associated blood vessels.

In this sense, highly secreted Ang2 from LECs might be more dynamically and freely diffused to Tie2-expressing cells like LECs, BECs, and monocytes/macrophages and activate them more strongly in the site of inflammation and angiogenesis. Further studies using BEC- or LEC-specific Ang2 knockout mice could provide novel insights for understanding distinct roles of Ang2 in BECs and LECs in vivo.

Using diverse signaling-specific blockers, we defined downstream pathways of Ang2 exocytosis in ECs (Figure 6D). Our results suggest that EDG1 is a main signaling pathway mediating Ang2 exocytosis because S1P-induced Ang2 secretion is highly PTX-sensitive. Moreover, much higher expressions of EDG1 than EDG3 in ECs further support a major role of EDG1, rather than EDG3, in the S1P-induced Ang2 exocytosis in ECs.

While S1P possibly promotes inflammation by activating various inflammatory molecules in WPBs, the angiogenic function of S1P might be mainly associated with Ang2 release because Ang2 is required for dormant ECs to respond to angiogenic stimuli like Ang1 and VEGF. Ang2 was known to potently destabilize quiescent ECs to exert angiogenesis and lymphangiogenesis with other growth factors. Indeed, we found that combined treatment of S1P and Ang2 potently induced angiogenesis in the Matrigel plug assay. Moreover, a recent study showed that S1P induces lymphangiogenesis via EDG1/PLC/Ca2⁺-signaling pathway, which is a main pathway for S1P-induced Ang2 exocytosis in LECs as we have shown in this study. Thus, in addition to direct roles of S1P in angiogenesis and lymphangiogenesis, S1P could have indirect roles in angiogenesis and lymphangiogenesis through Ang2 secretion. Contrary to other membrane-targeting molecules like vWF, P-selectin, or CD63, Ang2 is a soluble factor acting as a long-distance chemoattractant. Therefore, Ang2 could recruit circulating Tie2-expressing monocytes/macrophages to the site of inflammation and tumor angiogenesis. Considering different subpopulations of WPBs (eg, mutual exclusive localization of Ang2 and P-selectin even in the same ECs) and distinct mechanisms of WPB exocytosis (eg, selective secretion of vWF by a WPB lingering kiss), future studies regarding which subsets of WPBs selectively store Ang2 and what kind of other molecules are secreted with Ang2 are necessary.

To conclude, we propose that S1P derived from activated immune cells and platelets leads to rapid exocytosis of Ang2 from ECs, which may exert various physiological functions both in autocrine and paracrine manners. In these regards, our present study would provide new insights into the mechanism of cross-talk between S1P-secreting blood-borne cells and Ang2-secreting endothelial cells during dynamic vascular homeostasis and diseases.

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Disclosures

None.

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Angiopoietin-2 Exocytosis Is Stimulated by Sphingosine-1-Phosphate in Human Blood and Lymphatic Endothelial Cells

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Supplemental Materials and Methods

Materials

Flag-tagged recombinant full-length Ang2 and a deletion construct containing only fibrinogen like domain (FLD) of Ang2 were generated as previously described.\textsuperscript{1} Recombinant human VEGF-A and TNF-\(\alpha\) proteins were purchased from eBioscience (San Diego, CA). Dulbecco’s Modified Eagle Medium (DMEM) and calcium-free DMEM were purchased from GIBCO (Grand Island, NY). Endothelium basal medium-2 (EBM-2) with supplements was purchased from Clonetics (Walkersville, MD). S1P, dihydro-S1P (dhS1P), and D-erythro-N,N-dimethylsphingosine (DMS) were purchased from Biomol (Plymouth Meeting, PA). S1P was dissolved in methanol, and subsequently diluted into distilled water containing fatty acid-free bovine serum albumin (BSA) according to the manufacturer’s instructions. Pertussis toxin (PTX), U73122, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetrapotassium salt (BAPTA), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxyethyl ester (BAPTA-AM), phorbol 12-myristate 13-acetate (PMA), PD98059, SB203580, LY294002, forskolin, anti-Flag M2 antibody, anti-tubulin antibody, and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO).

Generation of Ang2 antibodies

Generation of mouse monoclonal antibodies was performed as described previously.\textsuperscript{2} Briefly, purified recombinant human Ang2 protein was used as an antigen to immunize BALB/c mice. After four subcutaneous and one intravenous injection of Ang2 every 2 weeks, splenocytes were collected and immortalized by fusion with mouse myeloma cells (F0; ATCC CRL-1645). The cells were grown in 96-well plates in hypoxanthine
aminopterin thymidine medium, and the culture supernatant was screened by ELISA using purified Ang2 protein as a coating antigen. Two mouse monoclonal antibodies against human Ang2 (clone No. 4A1A3 and 4B33A11) were purified from the culture supernatant of hybridomas grown in serum-free medium by affinity chromatography on a protein G-Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ).

Measurement of S1P concentration

HUVEC and HLEC were grown to a full confluence in 60-mm dishes (~1 x 10^6 cells/dish) in EBM-2 containing serum, and then were subjected to serum starvation in serum-free EBM-2 for 12 hr before being stimulated with TNF-α (100 ng/mL) in 0.6 mL of serum-free EBM-2 containing only 0.1% fatty acid-free BSA (as a carrier for secreted S1P). Then the media was collected and subjected to S1P measurement.

To measure S1P in the media, we used the S1P competitive ELISA assay kit (Echelon Biosciences, San Diego, CA) according to the manufacturer’s protocol. According to our experience, the lowest detection limit of the kit is ~20 nmol/L in 300 µL of sample. According to the data sheet, specifically, the antibody in the kit has no cross-reactivity with 13 related-lipids, including ceramide, ceramide-1-phosphate, lysophosphatidic acid, and dihydrosphingosine.

Ex vivo-incubation of human umbilical cords

After perfusion with PBS, the middle portions of umbilical cords were cut at 1 cm with a sterile surgical knife. In some cases, venous and arterial endothelial cells were wiped off with a sterile cotton swab. The cord slices were transferred to 6-well plates and Ang2 secretion was measured by ELISA before and after treatment. The cord slices
were cryosectioned for immunofluorescence staining.

**RT-PCR analyses**

RNA was extracted using the Total RNA Isolation System (Promega, Madison, WI), and each cDNA was made using the Reverse Transcription System (Promega). Quantitative real-time PCR was performed with the SYBR Premix Ex Taq\textsuperscript{TM} (Takara, Japan) using the iCycler iQ5 Real-time PCR system (Bio-Rad, Hercules, CA). PCR reactions were performed with appropriate primers (Supplemental Table I) and cycles.

**SiRNA transfection**

SiRNAs targeted to human EDG1 or EDG3 were constructed as previously described.\textsuperscript{3} The siRNA duplexes and a negative control random scrambled siRNA were synthesized by Bioneer Inc. (Daejeon, Korea). The transfection of siRNA duplex into HUVEC and HLEC was performed using an X-tremeGENE siRNA transfection agent according to the manufacturer's instructions (Roche, Herts, UK). Briefly, after incubation of 1 \( \mu \)g siRNA duplex with 5 \( \mu \)L transfection agent in 100 \( \mu \)L serum-free EBM-2 media for 20 min, the mixture was directly added to the cells grown on 6-well plates with 50% confluence (final volume \( \sim 2.2 \) mL of serum-free EBM-2 media). The media was changed after 4 hr-incubation and the cells were further incubated for 8 hr, and RNA was purified to perform RT-PCR analyses. The measurements of Ang2 secretion were performed at 72 hr after transfection with siRNA duplex.

**Matrigel plug assay**

The growth factor-reduced Matrigel (100 \( \mu \)l; BD Biosciences, San Jose, CA) containing
S1P (0.5 nmol), Ang2 (40 ng), or both S1P and Ang2 was subcutaneously implanted into adult male C57BL/6 mice, and then the extents of angiogenesis were compared at 14 days after the implantation. Blood vessels were immunostained with anti-PECAM-1 antibody (diluted 1:1000; Reliatech, Germany). The measurements of blood vessel densities in the Matrigel were made from immunostained tissue sections by photographic analysis using ImageJ software (http://rsb.info.nih.gov/ij) after converting the images into 8-bit gray scale. The measurements were made at a screen magnification of 100X, each 0.81 mm$^2$ in area at 0.9 mm inside the boundaries of the Matrigel, and 3 mice were used per group. To exclude background fluorescence, only pixels over a certain level (>50 intensity value) were taken.

**Statistics**

Values presented are means ± standard deviation (SD). Significant differences between means were determined by Student’s $t$-test or analysis of variance followed by the Student-Newman-Keuls test. Statistical significance was set at $P<0.05$. 
Supplemental References


Supplemental Figure Legends

Supplemental Figure I. Ang2 is specifically expressed in the vein endothelial cells of human umbilical cords. The perivascular regions (upper) and the surrounding muscle regions (lower) of the *ex vivo*-incubated human umbilical cord sections were co-immunostained for smooth muscle actin (SMA, green) and Ang2 (red), and counterstained with DAPI. Scale bars, 10 µm.

Supplemental Figure II. HLEC stores more intracellular Ang2 than HUVEC. (A) ECs were treated with control buffer (-) or 1 μmol/L S1P (+) for 20 min, and Ang2 secretion was measured by ELISA (n=4). *, P<0.05 vs control. #, P<0.05 vs HUVEC. (B) ECs were treated with control buffer (-) or 1 μmol/L S1P (+) for 20 min, and the amounts of intracellular Ang2 were measured by immunoblotting (IB). Tubulin is used as control. Three independent experiments show similar results.

Supplemental Figure III. S1P does not affect Ang2 mRNA synthesis in ECs. RT-PCR analyses of Ang2 in ECs after treatment with S1P (1 μmol/L) or VEGF-A (10 ng/mL) for the indicated times. GAPDH is used as control. Three independent experiments show similar results.

Supplemental Figure V. S1P induces Ang2 exocytosis via PTX-sensitive receptors, PLC and calcium signaling pathway. (A-E) Ang2 secretion was measured by ELISA (n=4). (A and C), HUVEC pretreated with 10 μmol/L DMS (A) or 200 ng/mL PTX (C) was treated with S1P (S, 1 μmol/L) or TNF-α (T, 100 ng/mL) for 20 min. *, P<0.05 vs
control. * \( P<0.05 \) vs S1P or TNF-α. (B) HUVEC was treated with S1P or dhS1P for 20 min. (D) HUVEC pretreated with U73122 was treated with S1P for 20 min. (E) HUVEC pretreated with DMEM containing BAPTA (B) or BAPTA-AM (A), or Ca\(^{2+}\)-free DMEM (C) was treated with S1P for 20 min. *, \( P<0.05 \) vs control. #, \( P<0.05 \) vs S1P.

**Supplemental Figure V. ECs express much higher levels of EDG1 than EDG3.**
Quantitative real-time RT-PCR analyses of EDG1 and EDG3 in ECs. GAPDH was used to normalize the expression levels of EDG1 and EDG3. Three independent experiments show similar results. *, \( P<0.05 \) vs EDG3.

**Supplemental Figure VI. Combined treatment of S1P and Ang2 potently induces angiogenesis in the Matrigel plug assay.** Matrigel (100 µl) containing control buffer (C), Ang2 (A, 40 ng), S1P (S, 0.5 nmol), or both S1P and Ang2 (S+A) was subcutaneously implanted into adult male C57BL/6 mice, and then the densities of PECAM-1-positive blood vessels were measured at 14 days after the implantation. The dotted lines indicate the boundaries of Matrigel. *, \( P<0.05 \) vs C. #, \( P<0.05 \) vs S1P only. Scale bars, 200 µm.
<table>
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<tr>
<td><strong>EDG1</strong></td>
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<tr>
<td>Forward: 5'-caaggccacgagcagtc-3'</td>
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<td><strong>EDG3</strong></td>
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Supplemental Figure III

**HUVEC**

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**HLEC**

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<td>Ang2</td>
<td>Ang2</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAPDH</td>
</tr>
</tbody>
</table>
Supplemental Figure VI

Blood vessel density (%)

- C
- S1P
- Ang2
- S1P+Ang2

PECAM-1

- C
- S1P
- Ang2
- S1P+Ang2

Blood vessel density (%)

- C
- S
- A
- S+A

* * *

#