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Lysophosphatidic acid upregulates vascular endothelial growth factor-C and tube formation in human endothelial cells through $LPA_{1/3}$, COX-2, and NF-κB activation- and EGFR transactivation-dependent mechanisms

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ABSTRACT

Lysophosphatidic acid (LPA) is a lipid bioactive mediator which binds to G-protein-coupled receptors and activates a variety of cellular functions. LPA modulates multiple behaviors in endothelial cells, including cell proliferation and migration, capillary-like tube formation *in vitro*, activation of proteases, interactions with leukocytes, and expressions of inflammation-related genes, thereby regulating vessel formation. LPA has been reported to modulate the angiogenesis process. However, the role of LPA in the lymphangiogenesis process has not been studied. In this study, we showed that LPA upregulated vascular endothelial growth factor-C (VEGF-C) mRNA expression in human umbilical vein endothelial cells (HUVECs) and subsequent endothelial cell tube formation *in vitro* and *in vivo*. These enhancement effects were LPA_1- and LPA_3-dependent and required cyclooxygenase-2 (COX-2), endothelial growth factor receptor (EGFR) transactivation and activation of nuclear factor kappaB (NF-κB). Moreover, LPA induced the protein expressions of the lymphatic markers, Prox-1, LYVE-1, and podoplanin, in HUVECs, and these enhancement effects were dependent on LPA_1 and LPA_3 activation and EGFR transactivation. Our results demonstrated that LPA might regulate VEGF-C and lymphatic marker expression in endothelial cells, which contributes to endothelial cell tube formation *in vitro* and *in vivo*, thus facilitating endothelial cell participation in the lymphangiogenesis process. This study clarifies the signaling mechanism of LPA-regulated VEGF-C expression and lymphatic marker expressions in endothelial cells, which suggest that LPA may be a suitable target for generating therapeutics against lymphangiogenesis and tumor metastasis.

1. Introduction

Lysophosphatidic acid (LPA) is a lipid bioactive mediator that plays important roles in a variety of cellular effects including cell proliferation, migration, apoptosis, and differentiation [1–3]. LPA is abundantly stored in platelets and is present as a normal constituent of human plasma and serum at the micromolar level [4]. Most of the biological effects of LPA are mediated through cell surface receptors of the endothelial differentiation gene (EDG) family of G-protein-coupled receptors (GPCRs) [5]. To date, five types of LPA receptors, LPA_{1-5}, have been identified [5–7]. Accumulating evidence reveals that LPA modulates multiple endothelial cell functions, including cell proliferation [1,8], migration [1,9], capillary-like tube formation *in vitro* [10], activation of proteases [11], interactions with leukocytes [12,13], and expressions of inflammation-related genes [14]. Through modulating these endothelial cell functions, LPA may act as an essential regulator for blood vessel formation, therefore modulating inflammatory and angiogenesis processes.

Blood and lymphatic vessels are two of the most important constituents in microenvironments which are essential for cancer metastasis [15,16]. Angiogenesis is dependent on blood vessel formation and also is a key process in cancer metastasis [17–19]. LPA has been reported to stimulate the angiogenesis process [20,21], which contributes to cancer metastasis in several cancer types [22,23]. Lymphangiogenesis is also a key process in cancer metastasis [15], which proceeds by lymphatic capillary vessel formation driven by

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endothelial cell liberation of endothelial cells from monolayers and morphogenesis into capillary-like structures [24]. However, the role of LPA in the lymphangiogenesis process remains unclear. The progression of lymphangiogenesis is regulated by multiple signaling pathways. Involvement of vascular endothelial growth factor-C (VEGF-C) via VEGF receptor (VEGFR)-3 is one of the most critical mechanisms mediating lymphangiogenesis [25,26]. By binding with VEGFR-3, VEGF-C exhibits mitogenic activity in lymphatic endothelial cells and promotes lymphatic capillary network formation, thus facilitating the lymphangiogenesis process [27,28].

Various signaling pathways have been reported to upregulate VEGF-C expression. Transactivation of Her2/Neu, a member of the epidermal growth factor receptor (EGFR) family [29–31] and activation of cyclooxygenase-2 (COX-2) and nuclear factor kappaB (NF-κB) [29–32] has been shown to be essential mechanisms mediating VEGF-C upregulation in different cell lines. In addition, LPA has been shown to upregulate VEGF-A, another VEGF family member recognized as a potent angiogenic factor expressed in ovarian cancer cells [33]. Recently, PGE2, a specific GPCR agonist, was shown to upregulate VEGF-A expression in gastric cancer cells via COX-2- and subsequent EGFR transactivation-dependent mechanisms [34]. However, the role of LPA in VEGF-C expression in endothelial cells, the major cell type essential for lymphatic vessel formation, has not been investigated.

Many proinflammatory cytokines, including interleukin (IL)-1β and tumor necrosis factor (TNF)-α have been shown to upregulate VEGF-C expression in human umbilical vein endothelial cells (HUVECs) [35]. One recent study reported that high levels of IL-8 in the circulation system are closely correlated to elevated VEGF-C levels in patient with metastatic esophageal squamous cell carcinoma [36]. Since LPA has been reported to be a proinflammatory factor [37] and our previous study demonstrated that LPA significantly enhances IL-1β and IL-8 expressions in HUVECs [13], LPA might upregulate VEGF-C expression in human endothelial cells.

In the present study, we observed that LPA upregulated VEGF-C mRNA expression in HUVECs, and subsequent endothelial cell tube formation *in vitro* and *in vivo* was LPA_1- and LPA_3-dependent and required COX-2, EGFR transactivation, and activation of NF-κB. Furthermore, our results revealed that LPA enhanced the lymphatic-specific markers of Prox-1, LYVE-1 and podoplanin protein expression levels in HUVECs, which were dependent on the activation of LPA_1 and LPA_3 and subsequent EGFR transactivation. Since LPA-stimulated EGFR transactivation in HUVECs is COX-2-dependent, our findings demonstrate that LPA might regulate VEGF-C and lymphatic marker expressions in endothelial cells, which contribute to endothelial cell tube formation *in vitro* and *in vivo*, thus facilitating endothelial cell participation in the lymphangiogenesis process.

2. Materials and methods

2.1. Reagents and antibodies

Lysophosphatidic acid (LPA), pyrrolidine dithiocarbamate (PDTC), and Ki16425 were purchased from Sigma (St. Louis, MO). Sphingosine 1-phosphate (S1P) was purchased from Biomol (Plymouth, PA). Anti-human EGFR antibody, and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). AG1478 and GM 6001 were purchased from Calbiochem (La Jolla, CA). The anti-human p-EGFR (Tyr-845) antibody was purchased from Upstate Biotechnology (Lake Placid, NY). NS-398 and SC-560 were purchased from Cayman Chemicals (Ann Arbor, MI). Fetal bovine serum (FBS) and M199 were purchased from Hyclone (Logan, UT). Trypsin-EDTA was purchased from Gibco BRL (Grand Island, NY). Endothelial cell growth medium (EGM) was purchased from Cell Applications (San Diego, CA). Penicillin, streptomycin, and L-glutamine were purchased from Invitrogen (Carlsbad, CA).

2.2. Cell culture

HUVECs were isolated from fresh umbilical cords by treatment with 1% (v/v) collagenase (Sigma) in phosphate buffered saline (PBS) at 37 °C for 10 min. After elution with M199 containing 20% FBS, HUVECs were cultured on 0.04% gelatin-coated (Sigma) 10-cm plates (Greiner Bio-One, Kremsmuenster, Austria) in M199 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine (Invitrogen), 10% (v/v) FBS, and 25% (v/v) EGM, and cells underwent one passage weekly. Cells were subcultured after trypsinization (in a 0.5% (v/v) trypsin solution, supplemented with 0.2% (v/v) EDTA) and used throughout passages 2 to 4.

2.3. Reverse-transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from HUVECs using the TRIzol reagent (Gibco), and a Superscript kit (Gibco) was used for the reverse-transcription (RT) synthesis of cDNA. PCR amplification was performed using the oligonucleotide primers of human VEGF-C (5′-CTCACTTCCTGCCGATGC-3′ and 5′-GTTCGCTGCCTGACACTG-3′), LPA_1 (5′-CGGAGACTGACTGTCAGCA-3′ and 5′-GGTCCAGAACTATGCCGAGA-3′), LPA_3 (5′-TTAGCTGCTGCCGATTTCTT-3′ and 5′-ATGATGAGGAAGGCCATGAG-3′), with 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 2 min at 72 °C. The primers used to amplify GAPDH were 5′-dACCACAGTTCATGCCATCAC and 5′-dTCCACCACCCTGTTGCTGTA with 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C. PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and photographed.

2.4. Quantitative real-time PCR

Real-time PCR reactions were conducted in an iCycler iQ Real-Time detection system (Bio-Rad, Hercules, CA) using SYBR Green I (Perkin Elmer Life Sciences, Boston, MA). The thermal profile for PCR was 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. Thermocycling was carried out in a final volume of 15 μl containing 1 μl of a cDNA sample. Each sample was run in duplicate. The melting curve of each tube was examined to confirm a single peak appearance.

2.5. siRNA transfections

siRNAs targeting LPA_3, MMP-2 and EGFR were purchased from Santa Cruz Biotechnology. Sequences of 21-nucleotide siRNAs (Proligo, Boulder, CO) for targeting endogenous genes were CCGCCGCUUCCAUUUUUCCUdTdT and AGGAAAAAUGGAAGCGGCGGGdTdT (LPA_1). AUGCAGAAGUUUUACGGCUUGdTdT and CAAGCCGUAAAACUUCUGCAUdTdT (MT1-MMP), and UUCUCCGAACGUGUUCACGUdTdT, and ACGUGACACGUUCGGAGAAdTdT (scrambled). HUVECs were transfected using an optimized protocol for electroporation of HUVECs with the Nucleofector apparatus (Amaxa Biosystems, Köln, Germany). Cells at 80% confluence were trypsinized and centrifuged. Cells (1×10^6) were resuspended in 100 μl of supplemented HUVECs Nucleofector™ solution (Amaxa Biosystems) and electroporated in the presence of 2 μg of various siRNA oligonucleotides or constructs. Transfected cells were seeded onto gelatin-coated plastic dishes and used after 24 h.

2.6. Western blot analysis

Treated cells were lysed in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris; pH 8.0) containing a protease inhibitor cocktail (Sigma) and 2 mM Na vanadate. After removing the cell debris by centrifugation at 13,500 rpm for 5 min, the protein concentration was determined by the Bradford assay. Samples containing equal amounts of proteins (50 μg) were separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a PVDF membrane (Millipore, Bedford, MA). The membranes were blocked with 5% BSA in a Tris-buffered saline-Tween 20 solution. Membranes were immunoblotted with an anti-human p-EGFR antibody for 2 h, and then washed in washing buffer (PBS + 0.1% Tween 20, without 1% BSA) once for 15 min followed by two rinses for 5 min. The membranes were blocked again in new blocking buffer for 1 h at room temperature and then immunoblotted with horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h. Membranes were subsequently washed in washing buffer once for 15 min followed by two rinses for 5 min each. Proteins on each immunoblot were visualized with Renaissance® Western blot chemiluminescence reagent (NEN Life Science, Boston, MA). Blots were stripped and reprobed with an antibody against human EGFR to demonstrate uniform loading of proteins.

2.7. COX-2 activity assay

Determination of COX-2 activity was measured using ELISA-based detection kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's protocol. Treated cells were scraped from culture dish and suspended in 500 μl of cold buffer containing 0.1 mol/L Tris–HCl (pH 7.8) and 1 mmol/L EDTA at 4 °C. 1×10^8 of collected cells were homogenized at 4 °C and the activity was measured in a 96-well plate. Original samples served for the estimation of the total COX activity and in the other wells 24 μg SC-560 was applied for inhibiting COX-2 activity. Standard or samples (10 μL) were incubated in the presence of arachidonic acid and colorimetric substrate containing *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) in a total reaction volume of 210 μL. The COX-2 peroxidase activity was measured colorimetrically by monitoring appearance of oxidized TMPD at 590 nm by using ELISA reader. For each experiment, triplicate samples were measured for statistical significance.

2.8. Determination of NF-κB activation

The NF-κB activity was determined by using the colorimetric NF-κB/p65 ActivELISA kit (Imgenex, San Diego, CA). The cytosolic as well as nuclear extracts was prepared

according to the manufacturer's protocol. ELISA plates were precoated with anti-p65 capture antibody, and the presence of p65 was detected by the addition of a second anti-p65 detection antibody followed by alkaline phosphatase-conjugated secondary antibody and colorimetric analysis at 405 nm by using ELISA reader. For each experiment, triplicate samples were measured for statistical significance.

2.9. Determination of EGFR phosphorylation by ELISA

The concentration of phosphorylated EGFR was measured by using the human phospho-EGFR ELISA kit (R&D systems, Minneapolis, MN) following the manufacturer's instructions. 1×10^7 cells/ml of treated cells were lysed in lysis buffer (1% NP-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin). Lysates were pipetted up and down to resuspend and rock the lysates gently at 2–8 °C for 30 min. Lysates were centrifuged at 14,000 g for 5 min, and the supernatants were transfered into clean Eppendorfs. Protein concentration of each sample was determined by the Bradford assay as described above. ELISA plates were precoated with anti-phospho-EGFR capture antibody, and the presence of phospho-EGFR levels were detected by the addition of a second anti-phospho-tyrosine-HPR detection antibody and colorimetric analysis at 540 nm by using ELISA reader. For each experiment, triplicate samples were measured for statistical significance.

2.10. In vitro Matrigel tube formation assay

Matrigel (BD Pharmingen, San Diego, CA) at 0.4 ml/well was plated evenly in a 24-well plate, and incubated at 37 °C for 30 min before seeding the HUVECs (0.5×10^5 cells/well). Tube formation was studied over 6 h and photographed. The original magnification used was ×100. The Matrigel was permeablized with −20 °C methanol, blocked, and stained with a mouse anti-human antibody (Clone: WM59) against PECAM-1 (BD Pharmingen) and a goat anti-human Prox-1 antibody (Clone: AF2727, R&D systems) followed by incubation with a FITC-conjugated goat anti-mouse secondary antibody (DAKO, Carpinteria, CA) or an AlexaFluor-555-conjugated donkey anti-goat secondary antibody (Molecular Probes, Eugene, OR). After a series of further washes with PBS, samples were mounted on glass slides and viewed using a Zeiss fluorescence microscopy (Oberkochen, Germany). The original magnification used was ×100.

2.11. In vivo Matrigel plug assay

Eight-week-old Balb/c mice were given a subcutaneous injection at the abdominal midline with 0.4 ml Matrigel supplemented with medium, LPA (5 μM), or S1P (5 μM). After 7 days, the mice were killed, and vessels penetrating the Matrigel were subjected to an immunohistochemical assay and visualized by Zeiss fluorescent microscopy. The original magnification used was ×40. The area of the vascular lumen within the plugs was measured by counting the number of plugs formed by endothelial cells.

2.12. Cyflow analysis

Sub-confluent HUVECs were starved for 16 h and treated as indicated. Suspensions of 10^6 cells in 200 μl PBS with 0.1% fatty acid-free bovine serum albumin (BSA) received 2 μl of a rabbit anti-human Prox-1 antibody (Abcam, Cambridge, MA), goat anti-human LYVE-1 (R&D systems), or mouse anti-human podoplanin (Santa Cruz Biotechnology) and were then incubated for 1 h at 4 °C. Antibody-conjugated cells were washed with PBS three times and incubated with FITC-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR), donkey anti-goat secondary antibody (Molecular Probes), or goat anti-mouse secondary antibody (DAKO) for 2 h at 4 °C. Fluorescent signals were determined by Cyflow® SL (Partec, Münster, Germany) and analyzed by WinMDI version 2.8 software.

2.13. Statistical analysis

Significant differences between treatment groups were tested using analysis of variance (ANOVA) followed by Duncan's new multiple-range tests (StatView; Abacus Concept, Berkeley, CA). Each experiment was repeated at least three times. A value of $p<0.05$ was considered statistically significant.

3. Results

3.1. LPA-induced VEGF-C mRNA expression in HUVECs is mediated through LPA_1 and LPA_3

Many proinflammatory cytokines such as IL-1β and TNF-α upregulate VEGF-C expression in HUVECs [35]. Our previous study demonstrated that LPA upregulates IL-1β expression in HUVECs [13]. Moreover, we further reported that LPA-induced IL-1β mRNA

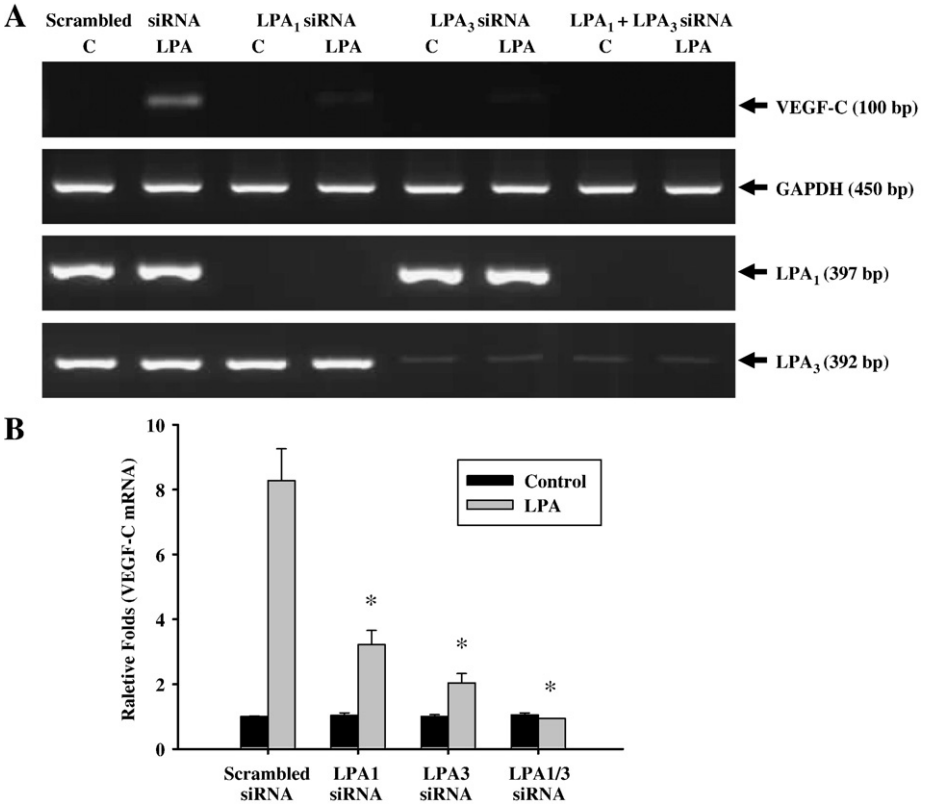

Fig. 1. Effects of LPA receptor siRNAs on VEGF-C mRNA expression in HUVECs. HUVECs were transfected with scrambled, LPA_1, LPA_3, or LPA_1+LPA_3 siRNA. Twenty-four hours after transfection, HUVECs were starved and treated with 5 μM LPA for 2 h. VEGF-C mRNA levels were assessed by RT-PCR, followed by PCR (A) or real-time PCR (B). The expression levels of LPA_1 and LPA_3 in each panel were used to evaluate the efficiency of each siRNA. The amount of GAPDH was used as a loading control in each panel. C represents the untreated control. *Statistically different as compared to the scrambled siRNA-transfected sample treated by LPA ($p<0.05$). Similar experiments were repeated three times, and a representative result is shown in the figure.

expression in HUVECs is mediated through LPA_1 and LPA_3 [14]. Therefore, we further investigated if LPA upregulates VEGF-C mRNA expression and whether LPA_1 or LPA_3 mediate LPA-enhanced VEGF-C mRNA expression in HUVECs. RNA interference was used to knock-down expression levels of certain LPA receptors. HUVECs were transfected with scrambled, LPA_1, LPA_3, or LPA_1+LPA_3 siRNA. By the RT-PCR analysis, our results demonstrated that LPA significantly upregulated VEGF-C mRNA expression in scrambled siRNA-transfected HUVECs (Fig. 1A). Moreover, HUVECs transfected with LPA_1 or LPA_3 siRNA showed significantly decreased expression of LPA_1 or LPA_3 mRNA in LPA-treated and untreated samples. The expression patterns of GAPDH, which was used as the loading control, did not significantly differ in LPA-treated or untreated samples (Fig. 1A). LPA-induced VEGF-C mRNA expression was lower in HUVECs transfected with LPA_1, LPA_3, or LPA_1+LPA_3 siRNA but not in those transfected with scrambled siRNA (Fig. 1). Co-transfection of LPA_1 and LPA_3 siRNAs also significantly suppressed LPA-enhanced VEGF-C mRNA expression in HUVECs (Fig. 1A). Real-time PCR results also showed that LPA_1, LPA_3, or LPA_1+LPA_3 siRNA exhibited suppressive effects on LPA-induced VEGF-C mRNA expression in HUVECs (Fig. 1B). These results demonstrate that LPA-enhanced VEGF-C mRNA expression in HUVECs and this enhancement effect was mediated by both LPA_1 and LPA_3.

3.2. LPA-induced endothelial cell tube formation in vitro and in vivo is mediated by LPA_1 and LPA_3

To determine whether LPA_1/LPA_3-dependent VEGF-C mRNA expression has any physiological significance, we next investigated if the LPA-induced HUVEC tube formation *in vitro* and *in vivo* is mediated via both receptors. HUVECs were transfected with scrambled, LPA_1, LPA_3, or LPA_1+LPA_3 siRNA and then seeded onto Matrigel-coated plates. We observed that LPA profoundly enhanced HUVEC tube formation *in vitro*, and tube formation was significantly suppressed in HUVECs transfected with LPA_1, LPA_3, and LPA_1+LPA_3 siRNA, respectively (Fig. 2). We further determined if LPA induces mouse endothelial cell tube formation *in vivo*. Herein, we used S1P, another lysophospholipid known to activate endothelial cell tube formation *in vitro* [10], as a positive control in the following experiments. Matrigel-containing medium, LPA (5 μM), or S1P (5 μM) was subcutaneously injected into the abdominal area of Balb/c mice. Vascularized plugs were removed and processed by H&E staining to identify if the area was covered by vessels. As shown in Fig. 3A, new vessel formation was observed in LPA-containing Matrigel plugs. In addition, S1P also showed enhancement effects on new vessel formation *in vivo* (Fig. 3A). These results confirmed that LPA can induce endothelial cell tube formation *in vivo*. Furthermore,

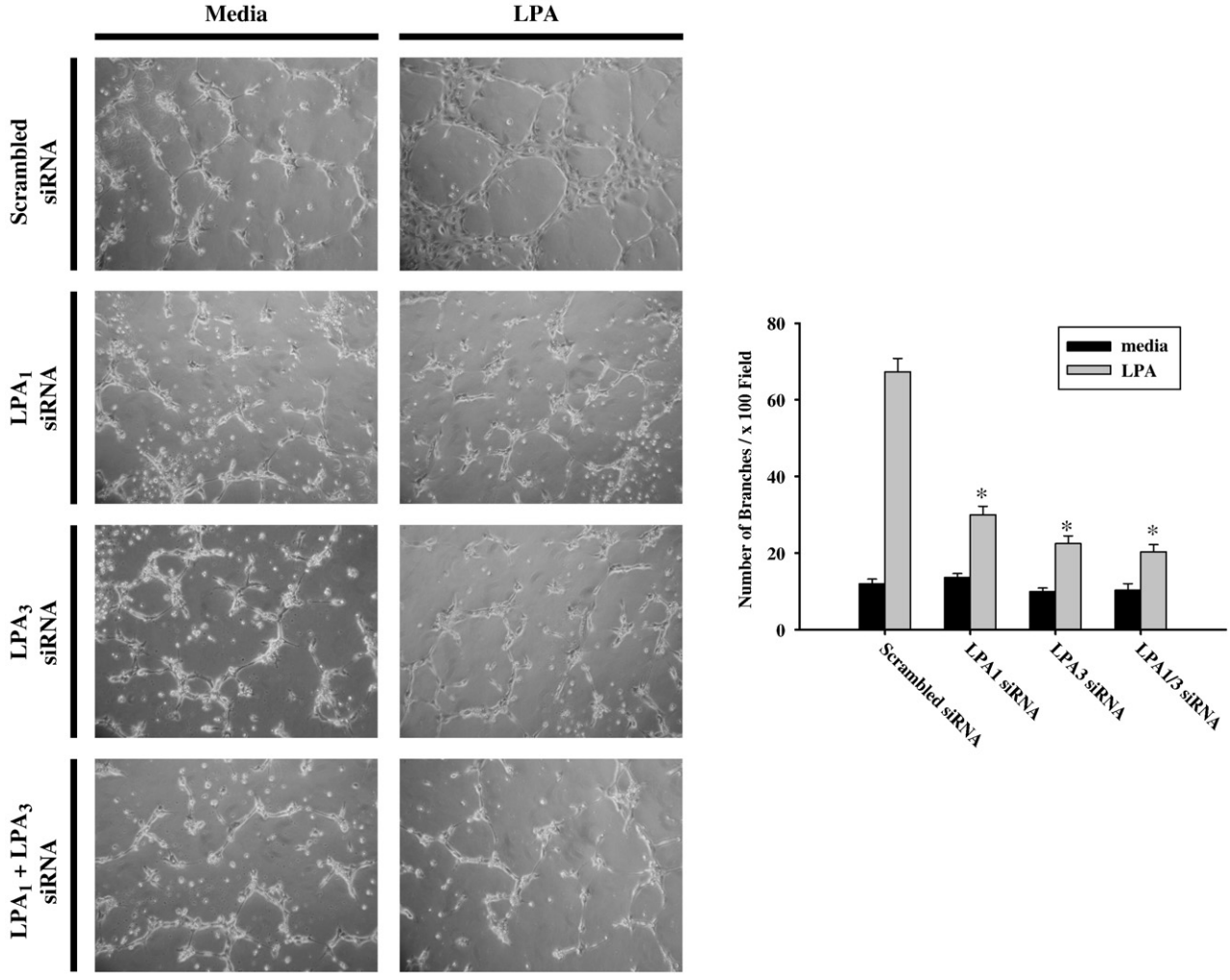

Fig. 2. Both LPA_1 and LPA_3 mediate LPA-induced endothelial cell tube formation *in vitro*. (A) HUVECs were transfected with scrambled, LPA_1, LPA_3, or LPA_1+LPA_3 siRNA. Twenty-four hours after transfection, HUVECs were starved and treated with medium or 5 μM LPA for 8 h and then seeded onto Matrigel-coated plates. Images were taken at 6 h after plating and visualized by phase-contrast microscopy. The histogram represents branches from each cell which was counted from three representative 100× fields/well. *Statistically different as compared to the scrambled siRNA-transfected sample treated by LPA ($p<0.05$). Similar experiments were repeated three times, and a representative result is shown in the figure.

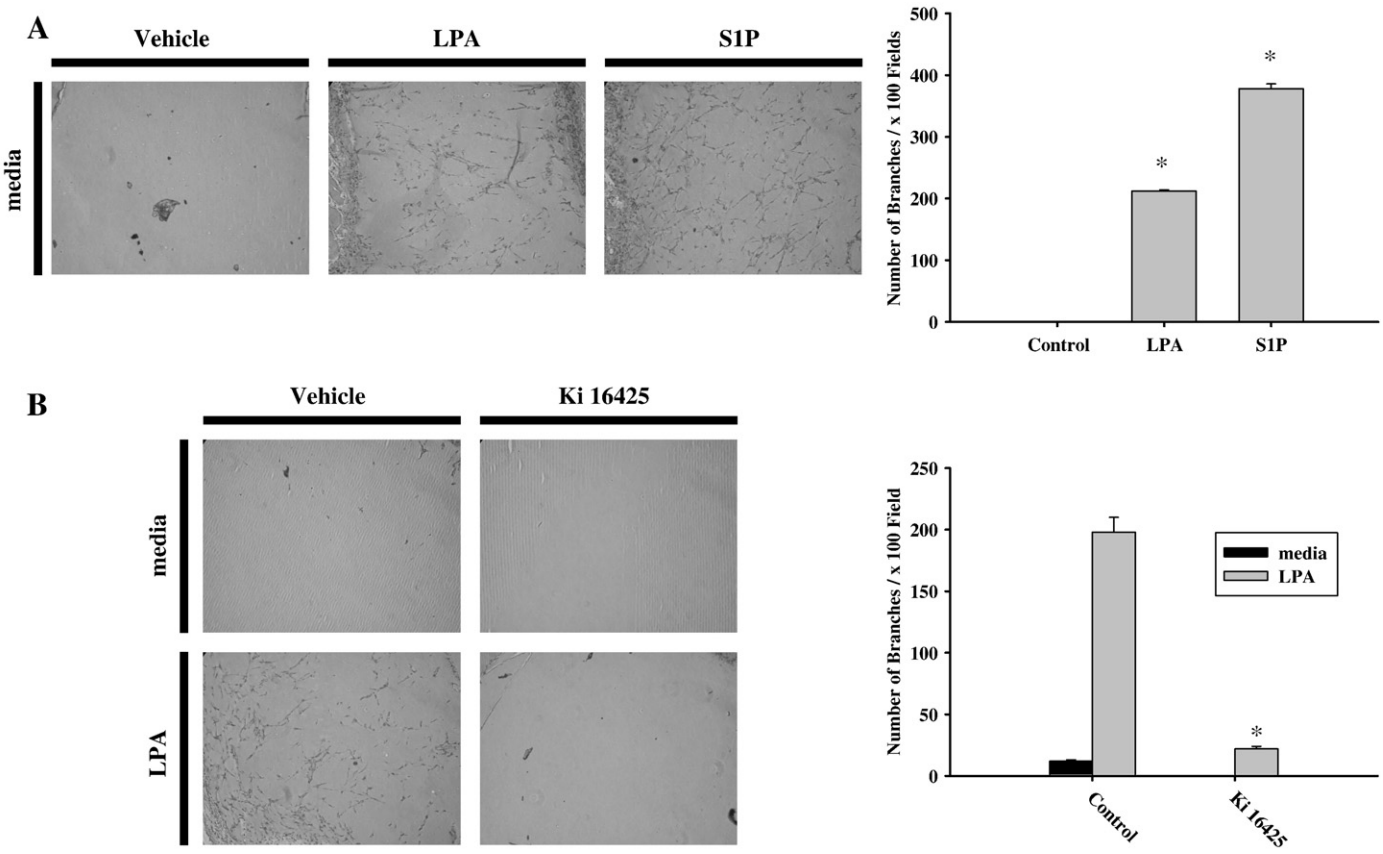

Fig. 3. Both LPA_1 and LPA_3 mediate LPA-induced endothelial cell tube formation *in vivo*. (A) Matrigel plugs were generated by a subcutaneous injection of Matrigel-containing medium, LPA (5 μM), or S1P (5 μM). The plugs were removed 7 days later and processed for hematoxylin and eosin (H&E) staining. The histogram represents branches from each cell which was counted from three representative 40× fields/well. Statistically different as compared to the control (*$p<0.05$). (B) H&E staining of Matrigel plugs containing medium or LPA (5 μM) supplemented with vehicle or 10 μM Ki16425. All images were visualized by fluorescence microscopy. The histogram represents number of branches counted. Similar experiments were repeated three times, and a representative result is shown in the figure. *Statistically different as compared to the media-incubated sample treated by LPA ($p<0.05$).

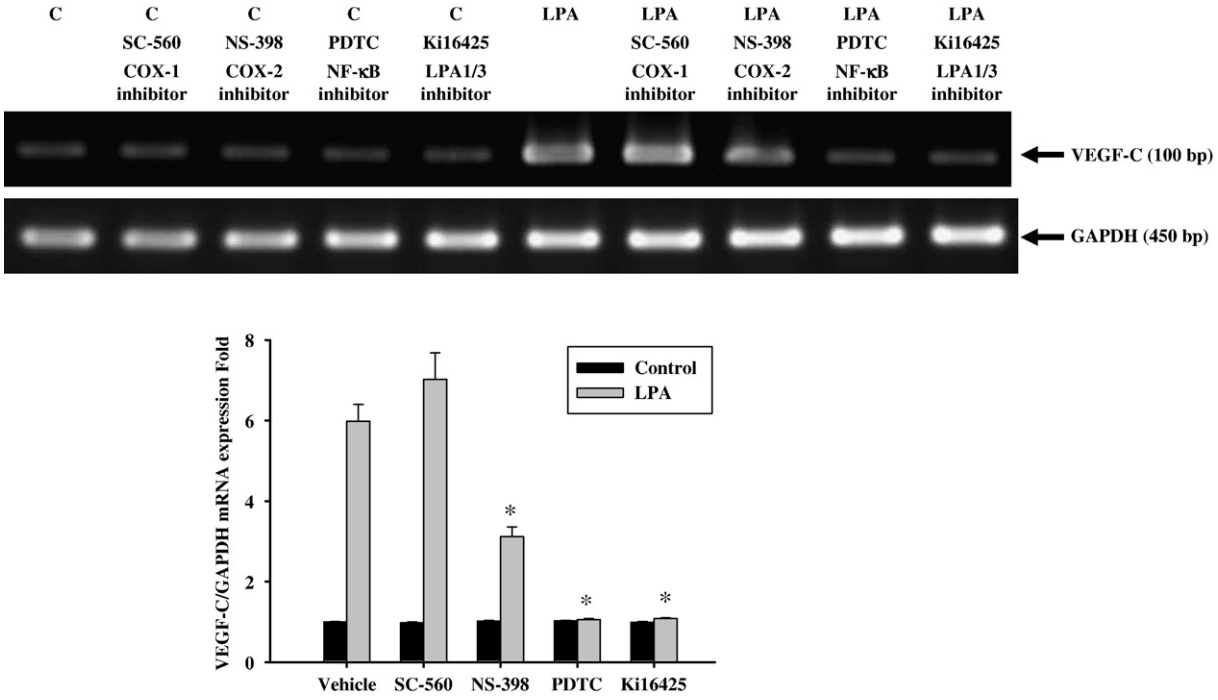

Fig. 4. Effects of a COX-1 inhibitor (SC-560), COX-2 inhibitor (NS-398), NF-κB inhibitor (PDTC), and $LPA_{1/3}$ inhibitor (Ki16425) on LPA-stimulated VEGF-C mRNA expression in HUVECs. (A) HUVECs were pretreated with 5 μM of SC-560, 50 μM of NS-398, 10 μM of Ki16425, or 200 μM of PDTC for 1 h, followed by LPA (5 μM) treatment for an additional 2 h. The VEGF-C mRNA expression level was monitored by RT-PCR. All data are relative multiples of expression compared to untreated cells. Histograms represent quantification of RT-PCR corrected with GAPDH analyzed by PhosphoreImager® using ImageQuaNT® software. All data are relative multiples of expression compared to untreated cells. Similar experiments were repeated three times, and a representative result is shown in the figure. *Statistically different as compared to vehicle-incubated sample treated by LPA ($p<0.05$).

we showed that LPA-induced new vessel formation was profoundly suppressed in the presence of Ki16425 (Fig. 3B). These results suggest that LPA-induced mouse endothelial cell tube formation *in vitro* and *in vivo* might be mediated by both LPA_1 and LPA_3.

3.3. Blockers of NF-κB, LPA_1/LPA_3, and COX-2 suppress LPA-enhanced VEGF-C mRNA expression in HUVECs

Since both LPA_1 and LPA_3 mediate LPA-induced VEGF-C mRNA expression in HUVECs (Fig. 1), we further investigated through which downstream signal transduction molecule the effects of LPA on VEGF-C expression are mediated. Pretreatment with Ki16425, a chemical inhibitor known to reduce LPA binding activity of LPA_1 and LPA_3 [38], PDTC, an inhibitor of the NF-κB-dependent pathway, SC-560, an inhibitor of COX-1, and NS-398, an inhibitor of COX-2, for 1 h had no significant effects on the basal level of VEGF-C expression in HUVECs. However, the stimulatory effects of 5 μM LPA on VEGF-C expression in HUVECs were totally suppressed by 10 μM of Ki16425 and 200 μM of PDTC treatment. On the other hand, pretreatment with 50 μM of NS-398 partially inhibited LPA-enhanced VEGF-C expression in HUVECs. However, pretreatment with 5 μM of SC-560 had no effects on LPA-induced VEGF-C expression in HUVECs (Fig. 4). These results suggest that the enhancement effects of LPA on VEGF-C expression are LPA_1/LPA_3- and NF-κB-dependent and may partially be regulated by COX-2.

3.4. LPA-induced HUVEC tube formation in vitro is mediated through EGFR transactivation

Various GPCR ligands, such as gastrin, can induce endothelial cell tube formation via an EGFR transactivation mechanism [39]. Accumulating evidence has demonstrated that matrix metalloproteinases (MMPs) play essential roles in the EGFR transactivation mechanism [40,41]. Moreover, we also found that LPA induces EGFR transactivation and subsequent cell proliferation in HUVECs [42]. Thus we further verified if LPA-stimulated endothelial cell tube formation is mediated through EGFR transactivation. GM 6001, a broad-spectrum MMP

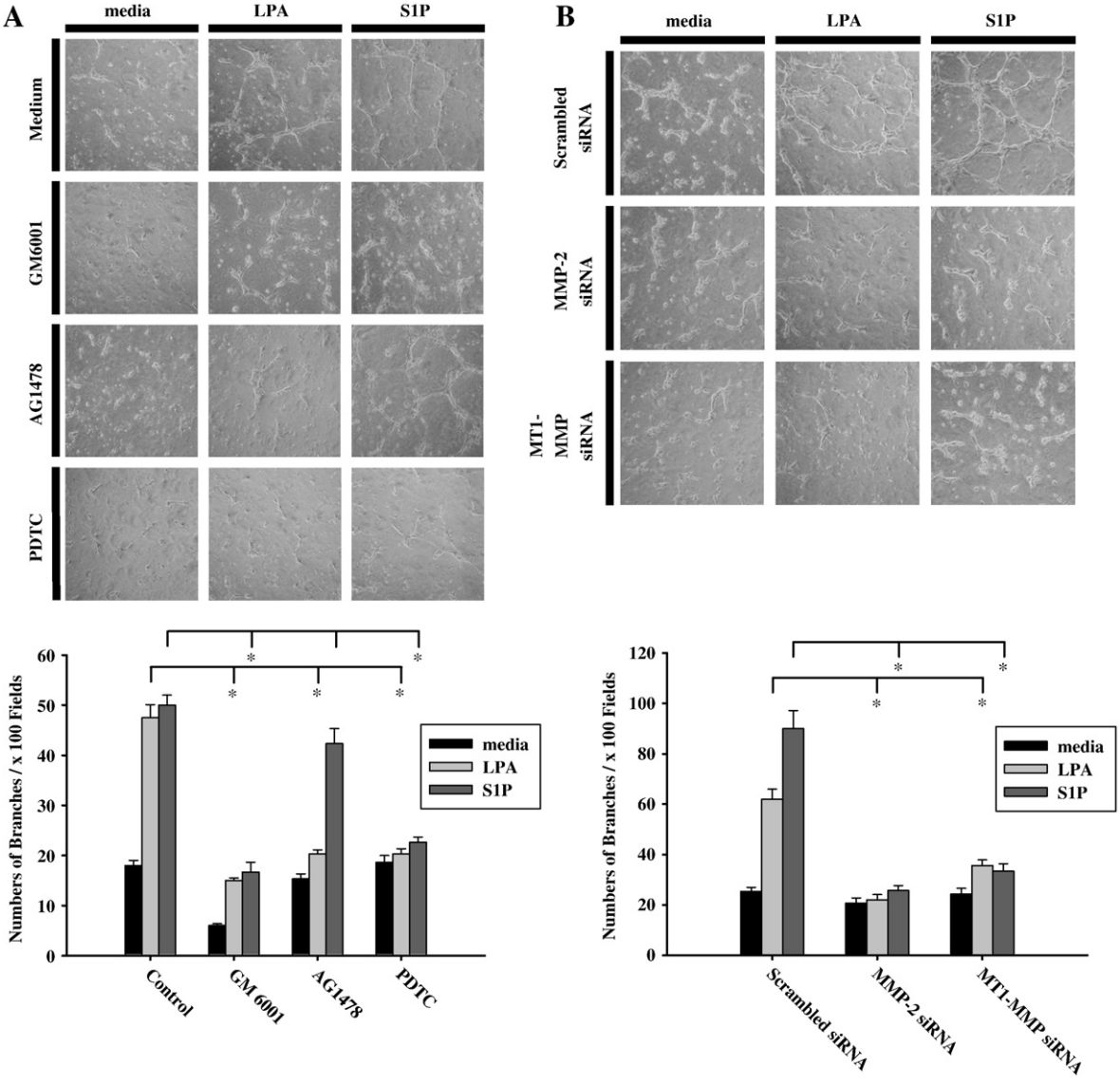

Fig. 5. LPA-induced human endothelial cell tube formation *in vitro* and VEGF-C mRNA expression in an EGFR transactivation-, NF-κB-, MMP-dependent mechanism. (A) HUVECs were pretreated with GM 6001 (10 μM), AG1478 (1 μM), or 200 μM of PDTC for 1 h, followed by LPA (5 μM) treatment for an additional 8 h, and then cells were seeded onto Matrigel-coated plates. (B) HUVECs were transfected with scrambled, MMP-2, or MT1-MMP siRNA. Twenty-four hours after transfection, HUVECs were starved and treated with medium or 5 μM LPA for 8 h and then seeded on Matrigel-coated plates. All images were taken 6 h after plating and visualized by phase-contrast microscopy. The histogram represents branches from each cell which was counted from three representative 100× fields/well. Similar experiments were repeated three times, and a representative result is shown in the figure. Error bars indicate $*p<0.05$.

inhibitor [43], and AG1478, an EGFR tyrosine kinase inhibitor [44], are commonly used inhibitors for investigating EGFR transactivation [45,46]. Therefore we used these two inhibitors to investigate if EGFR transactivation mediates LPA-stimulated endothelial tube formation. In *in vitro* Matrigel tube formation assay, pretreatment with AG1478 (250 μM), GM 6001 (10 μM), or PDTC (200 μM) significantly suppressed LPA-induced HUVEC tube formation *in vitro*. However, S1P-induced HUVEC tube formation *in vitro* was not suppressed by treatment with AG1478 (Fig. 5A). To identify which MMP molecule mediates LPA-induced HUVEC tube formation *in vitro*, HUVECs were transfected with scrambled, MMP-2 or MT1-MMP siRNA and treated with medium and 5 μM of LPA or S1P, and then subjected to *in vitro* Matrigel tube formation assay. Results showed that the introduction of MMP-2 and MT1-MMP siRNA profoundly suppressed both LPA- and S1P-enhanced HUVEC tube formation *in vitro* (Fig. 5B). These finding suggest that both MT1-MMP and MMP-2 play essential roles in enhancing human endothelial cell tube formation *in vitro*.

3.5. LPA-induced VEGF-C mRNA expression in HUVECs is mediated through EGFR transactivation

Since LPA-induced HUVEC tube formation *in vitro* depends on EGFR transactivation, the effect of EGFR transactivation on the elevation of VEGF mRNA expression was also evaluated. As shown in Fig. 6A, pretreatment with both GM 6001 and AG1478 significantly abrogated LPA-induced VEGF-C mRNA expression in HUVECs (Fig. 6A). In addition, LPA-induced EGFR phosphorylation in HUVECs was blocked by a COX-2 inhibitor. However, pretreatment with a COX-1 inhibitor and an NF-κB inhibitor had no effects on the elevation of EGFR phosphorylation by LPA (Fig. 6B). These results imply that LPA might stimulate COX-2-mediated EGFR transactivation, therefore affecting NF-κB activation and subsequent VEGF-C expression in HUVECs.

3.6. LPA-induced EGFR transactivation, activation of COX-2 and NF-κB in HUVECs are mediated by LPA_1 and LPA_3

Since both LPA_1 and LPA_3 mediate LPA-induced VEGF-C mRNA expression and endothelial cells tube formation *in vitro* and *in vivo*, we further investigate if LPA-stimulated EGFR transactivation and activation of COX-2 and NF-κB in HUVECs in a LPA_1 and LPA_3-dependent manner. Similar to the Western blot results in Fig. 6B, we observed that treatment of 5 μM of LPA for 2 min significantly enhanced EGFR phosphorylation in HUVECs by phospho-EGFR ELISA. Moreover, these enhancement effects were profoundly suppressed by the introduction of LPA_1, LPA_3 and LPA_1 + LPA_3 siRNA in HUVECs

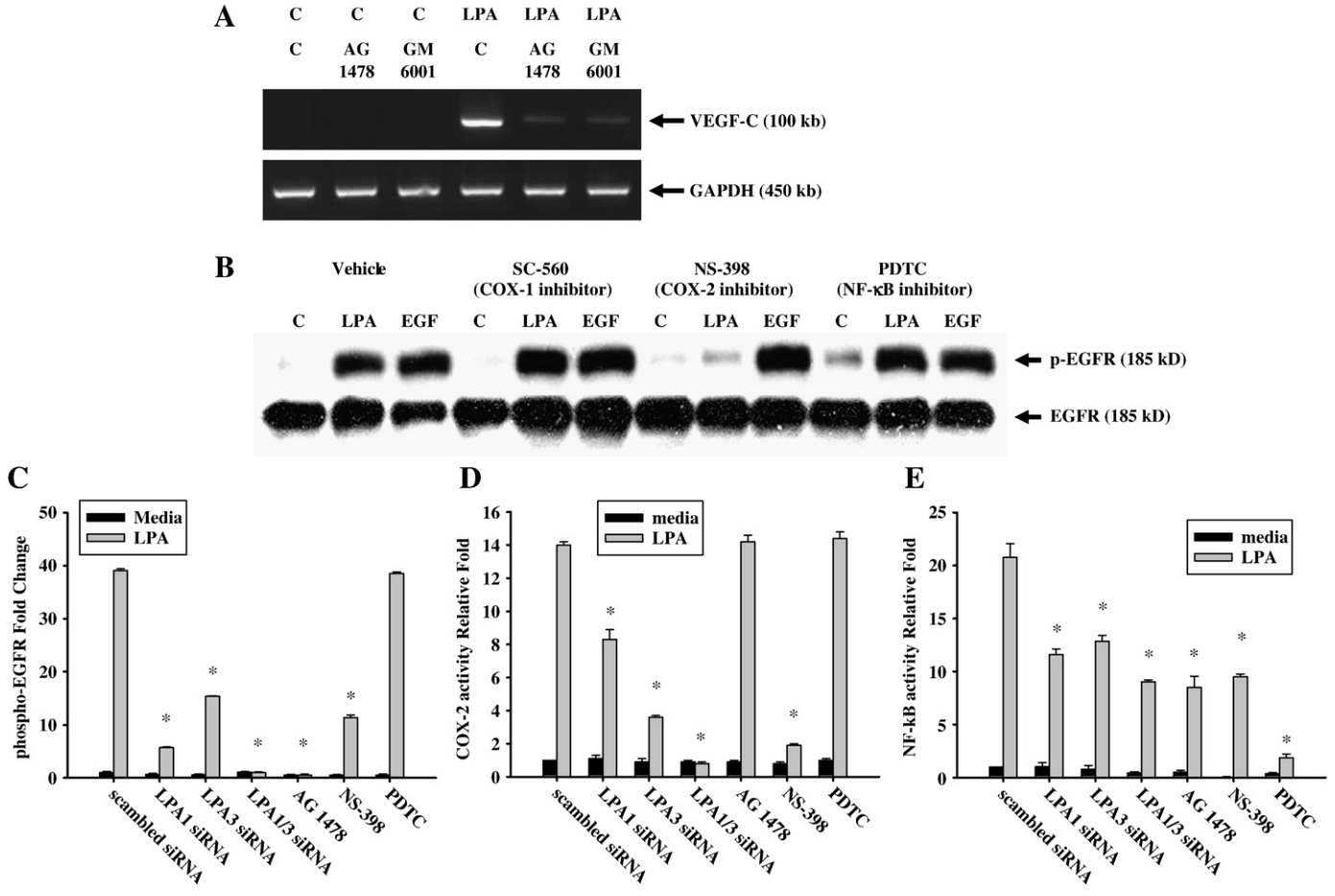

Fig. 6. LPA-induced VEGF-C mRNA expression in HUVECs in an EGFR transactivation-dependent mechanism. (A) HUVECs were pretreated with GM 6001 (10 μM) or AG1478 (1 μM), followed by LPA (5 μM) treatment for an additional 2 h. The VEGF-C mRNA expression level was monitored by RT-PCR. All data are relative multiples of expression compared to untreated cells. Similar experiments were repeated three times, and a representative result is shown in the figure. (B) HUVECs were pre-incubated with SC-560 (5 μM), NS-398 (50 μM), or PDTC (200 μM) for 1 h and treated with control medium, LPA (5 μM), or EGF (10 ng/ml) for 2 min. Proteins were immunoblotted with an anti-p-EGFR antibody. Re-probing against EGFR was done to ensure equal protein loading amounts. (C) Scrambled, LPA_1, LPA_3, or LPA_1 + LPA_3 siRNA-transfected HUVECs were starved and treated with media or 5 μM LPA. Untrasfected HUVECs were also pretreated with AG1478 (1 μM), NS-398 (50 μM) and PDTC (200 μM) for 1 h and then treated with media or 5 μM LPA. Cells were collected 2 min after treatment and subjected to ELISA for phosphor-EGFR as described in Materials and methods. Treated cells were also collected 30 min after treatment and subjected to ELISA for COX-2 activation (D) and NF-κB activation (E). All ELISA analysis data are expressed as the mean ± S.E. Similar experiments were repeated three times, and a representative result is shown in the figure. *Statistically different as compared to the scrambled siRNA-transfected sample treated by LPA ($p < 0.05$).

(Fig. 6C). By COX-2 and NF-κB activity assay, we found that treatment of LPA (5 μM) for 30 min also upregulated COX-2 and NF-κB activities. In addition, LPA_1, LPA_3 and LPA_1+LPA_3 siRNA significantly inhibited LPA-stimulated COX-2 and NF-κB activity elevation in HUVECs (Fig. 6D and E). These findings further revealed that both LPA_1 and LPA_3 play important roles on EGFR transactivation and the activation of COX-2 and NF-κB in human endothelial cells. Furthermore, our present data showed that NS-398 could inhibit LPA-enhanced EGFR phosphorylation and NF-κB activities, while AG1478 could inhibit LPA-enhanced NF-κB but not COX-2 activities in HUVECs. However, PDTC had no effects on LPA-enhanced EGFR phosphorylation and COX-2 activities in HUVECs (Fig. 6C–E). These findings revealed that COX-2 activation might located upstream of EGFR transactivation, whereas the NF-κB activation is downstream of EGFR transactivation in HUVECs.

3.7. LPA-enhanced expressions of the lymphatic markers, Prox-1, LYVE-1, and podoplanin, in HUVECs, which were mediated through EGFR transactivation- and $LPA_{1/3}$-dependent mechanism

Since LPA was shown to induce VEGF-C mRNA expression in human endothelial cells (Fig. 1), we next determined whether LPA-induced VEGF-C mRNA expression contributes to lymphangiogenesis. By a Cyflow analysis, we observed that the protein expression levels of the three lymphatic markers, Prox-1, LYVE-1, and podoplanin, were upregulated by treatment with 5 μM of LPA at 8 h (Fig. 7A). On the contrary, pretreatment with AG1478 (250 μM), GM 6001 (10 μM), and Ki16425 (10 μM) for 1 h significantly suppressed the enhancement effects of LPA on Prox-1, LYVE-1, and podoplanin protein expressions in HUVECs (Fig. 7A). These results indicated that LPA's enhancement of Prox-1, LYVE-1, and podoplanin expressions in endothelial cells is EGFR transactivation- and $LPA_{1/3}$-dependent, further implying that LPA's regulation of endothelial cells' participation in the lymphangiogenesis process is mediated through activation of $LPA_{1/3}$ and a subsequent EGFR transactivation mechanism.

3.8. EGFR siRNA suppressed LPA-induced HUVEC tube formation and Prox-1 expression in vitro

To further verify if EGFR transactivation mechanism plays a role on LPA-enhanced endothelial tube formation, HUVECs were transfected with scrambled or EGFR siRNA and treated with media or 5 μM of LPA, and then subjected to *in vitro* Matrigel tube formation assay. Our data showed that introduction of EGFR siRNA significantly suppressed LPA-enhanced HUVEC tube formation *in vitro* (Fig. 7B). By immunostaining

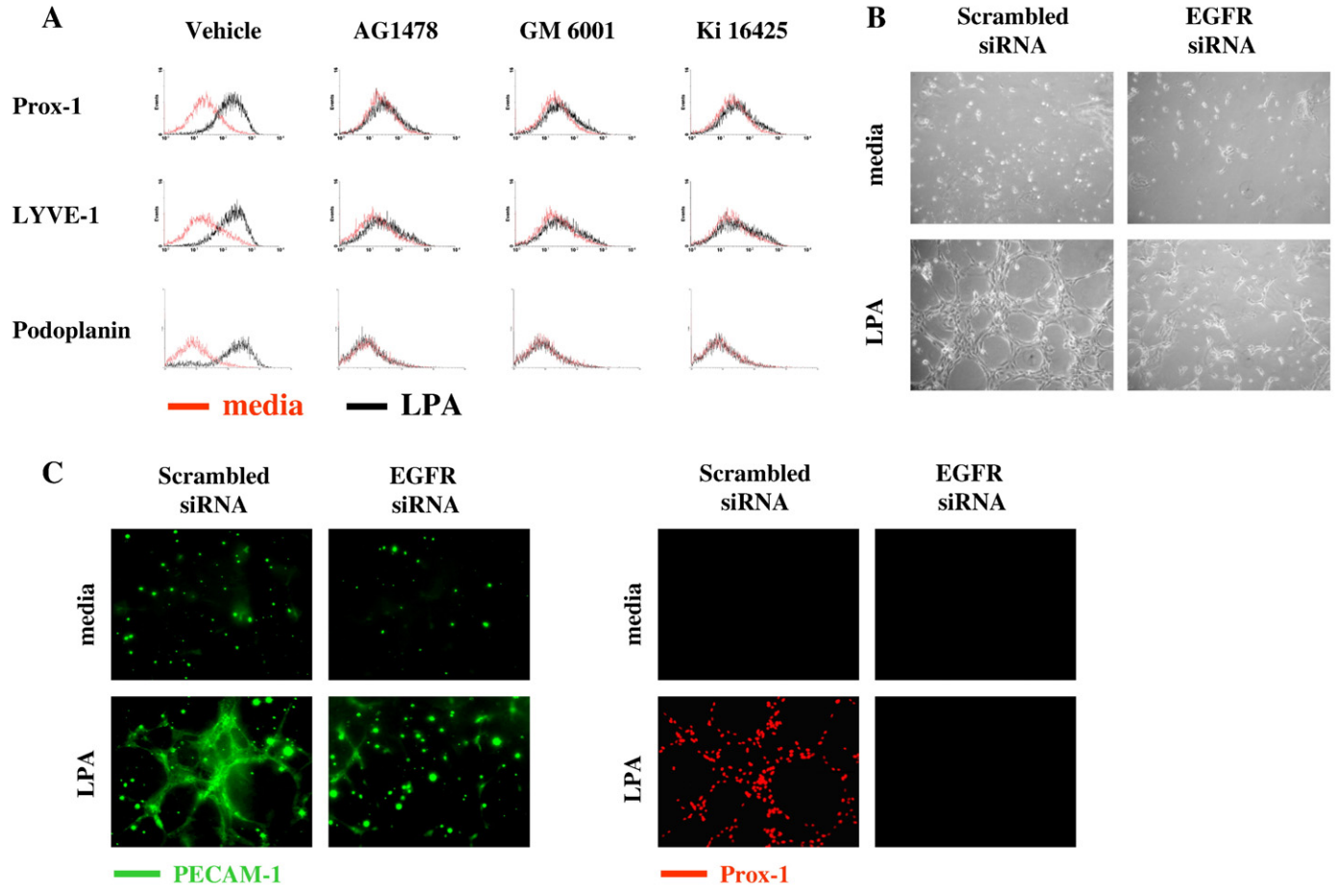

Fig. 7. Effects of an EGFR kinase inhibitor (AG1478), broad-spectrum MMP inhibitor (GM 6001), and $LPA_{1/3}$ inhibitor (Ki16425) on LPA-induced Prox-1, LYVE-1, and podoplanin protein expressions in HUVECs. (A) HUVECs were treated with DMSO (vehicle), GM 6001 (10 μM), AG1478 (1 μM), or Ki16425 (10 μM) for 1 h. Treated cells were then treated with control medium or 5 μM of LPA for 8 h. Cells were dissociated by trypsinization and fixed by a 4% paraformaldehyde solution. Fixed cells were incubated with a rabbit anti-human Prox-1 antibody for 1 h at 4 °C, then treated with an FITC-conjugated secondary antibody for 30 min at 4 °C and analyzed by Cyflow. Trypsinized cells were also incubated with goat anti-human LYVE-1 or mouse anti-human podoplanin antibody at 4 °C, then treated with an FITC-conjugated secondary antibody for 30 min at 4 °C, and analyzed by Cyflow. (B) HUVECs were transfected with scrambled or EGFR siRNA. Twenty-four hours after transfection, HUVECs were treated with media or LPA (5 μM) for 8 h and then seeded on Matrigel-coated plates. Images were taken 6 h after plating and visualized by the phase-contrast microscopy. (C) Matrigel of each experiment was permeablized with −20 °C methanol and subjected to an immunocytochemical assay. Staining of a mouse anti-human PECAM-1 or a goat anti-human Prox-1 primary antibody followed by an FITC-conjugated anti-mouse or an Alexa-555-conjugated donkey anti-goat secondary antibody is shown in the figure. Color figures are available online.

with PECAM-1, a well-known endothelial cell marker, expression levels were enhanced in LPA-treated samples as compared to the media-treated control. Furthermore, introduction of EGFR siRNA profoundly suppressed PECAM-1 signals in both LPA- and media-treated samples. These results further indicated that LPA-stimulated HUVEC branch formation, and these enhancement effects are EGFR transactivation-dependent (Fig. 7C, left panel). By immunostaining with Prox-1, we found that Prox-1 signals in cell nucleus were elevated in tube-forming HUVECs in response to LPA. However, these enhancement effects were also suppressed by EGFR siRNA (Fig. 7C, right panel). These results further confirmed that LPA-stimulated endothelial tube formation and lymphatic marker expressions are mediated though EGFR transactivation, which is consistent with the results in Figs. 5 and 7A.

4. Discussion

In our previous study, we demonstrated that LPA enhances IL-1β and IL-8 mRNA expressions in HUVECs, and LPA-upregulated IL-8 expression is IL-1-dependent [13]. Moreover, these enhancement effects are mediated by the activation of LPA_1 and LPA_3 [14]. In addition, IL-1β stimulates VEGF-C expression in HUVECs in an NF-κB-dependent mechanism [35], and IL-8 expression is highly correlated with VEGF-C levels in human circulatory system [23]. We further revealed that LPA upregulated VEGF-C mRNA expression in HUVECs in $LPA_{1/3}$- (Fig. 1) and NF-κB-dependent manners (Fig. 4). These findings clarify that LPA_1 and LPA_3 possibly mediate LPA-induced IL-1β and subsequent IL-8 expression, therefore transducing NF-κB activation and then upregulating VEGF-C expression in human endothelial cells.

Our previous studies indicated that LPA_1 and LPA_3 but not LPA_2 expressed in HUVECs [1,12]. Moreover, both LPA_1 and LPA_3, but not other LPA receptors, play important roles in endothelial cells' participation in physiological functions such as inflammation [14]. In the present study, we showed that LPA_1 and LPA_3 are both critical for LPA's ability to upregulate VEGF-C mRNA expression in HUVECs (Fig. 1) and subsequent endothelial cell tube formation *in vitro* and *in vivo* (Figs. 2 and 3). LPA_1 and LPA_3 also mediate LPA-enhanced EGFR transactivation and the activation of COX-2 and NF-κB in HUVECs (Fig. 6). This is possibly due to a combination effect of the complex downstream signaling events triggered by these two receptors. Recent study reported that LPA_1 and LPA_3 could dimerize into heterozygous complex, and LPA receptors dimerization may affect downstream signaling pathways [47]. These results implicated that LPA_1 and LPA_3 dimerization may play a role on LPA-transduced signaling pathways in human endothelial cells, which might explain our observations. A previous study indicated that $LPA_1^{(-/-)}$ mice showed 50% survival [48] and LPA_3-deficient mice failed to survive during pregnancy due to abnormal embryo implantation [49]. Moreover, lymphatic endothelial formation and the lymphangiogenesis process are necessary for embryo implantation [50]. Those findings imply that both LPA_1 and LPA_3 mediate the effects of LPA on VEGF-C expression in endothelial cells, therefore facilitating the lymphangiogenesis process.

LPA is a potent agonist for GPCR-mediated EGFR transactivation [40]. Two EGFR family members, EGFR and Her2/Neu, have been reported to be necessary for VEGF-C expression in different cancer cells [30,31,51]. We demonstrated that LPA-induced VEGF-C expression in human endothelial cells (Fig. 6A) and subsequent *in vitro* endothelial cell tube formation could be suppressed by AG1478, an EGFR inhibitor (Fig. 5). In addition, LPA-enhanced EGFR phosphorylation in HUVECs (Fig. 6B). These results suggest that activation of the EGFR plays a role in LPA-mediated VEGF-C expression in HUVECs. Since AG1478 attenuates the activities of both EGFR and Her2/Neu [52,53], the involvement of Her2/Neu in LPA-induced VEGF-C expression and function requires further investigation.

COX-2 activation is highly correlated with VEGF-C expression [54]. Downregulation of COX-2 with siRNA significantly suppresses VEGF-C expression in breast cancer cells [55]. In addition, downstream signaling molecules have also been identified which are transduced by COX-2 to upregulate VEGF-C in cancer cells [29,31]. Her2/Neu transactivation-mediated VEGF-C expression is dependent on the activation of NF-κB in a breast cancer cell line [32], and activation of NF-κB is responsible for the elevation in COX-2-mediated VEGF-C expression [56]. As supported by these findings, we further demonstrated that LPA upregulated VEGF-C mRNA expression in human endothelial cells through activation of COX-2, EGFR, and NF-κB (Figs. 4 and 6A). In our previous study, we showed that LPA induces MMP-2 activity elevation in HUVECs [11]. Moreover, inhibition of COX-2 activity has been reported to suppress MMP-2 enzymatic activity in cancer cells and subsequent cancer cell invasion and migration [57,58]. In this study, we showed that inhibition of MMPs abrogated LPA-

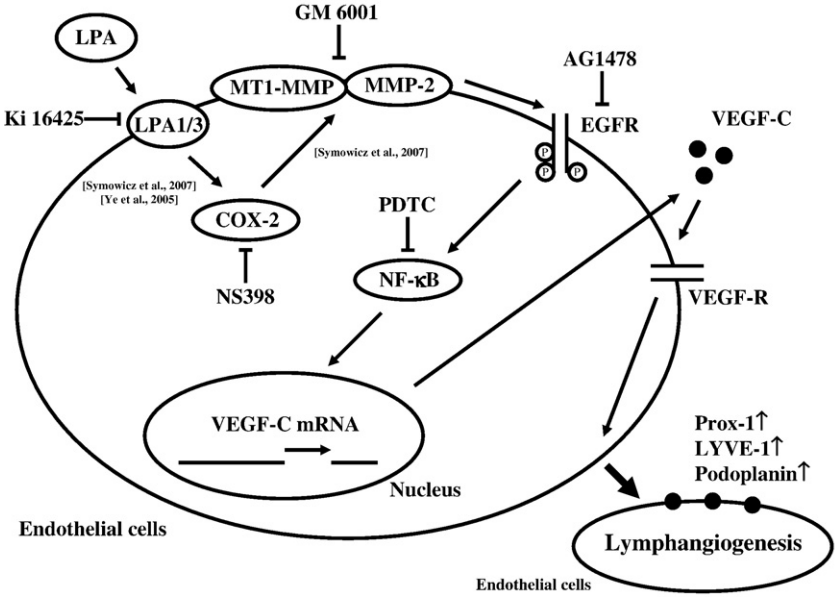

Fig. 8. Mechanism of lysophosphatidic acid's (LPA) regulation of endothelial cell lymphangiogenesis.

induced VEGF-C expression in HUVECs (Fig. 6A) and subsequent *in vitro* tube formation (Fig. 5). These findings further confirm that LPA induces MMP-2 activation in human endothelial cells in a COX-2 dependent manner, thus promoting endothelial cell tube formation and participation in the lymphangiogenesis process.

Much evidence has revealed that an elevated COX-2 expression level is required for EGFR transactivation in colon cancer cells [59,60] Moreover, Shida et al. showed that LPA induces COX-2 expression in an EGFR transactivation-dependent manner in colon cancer cells, thereby promoting colorectal cancer metastasis [61]. Those findings suggest that EGFR transactivation acts as an upstream mechanism for COX-2 expression. In contrast, our results indicated that LPA-induced EGFR transactivation in HUVECs could be blocked by NS-398, an inhibitor of COX-2 (Fig. 6B), implying that EGFR transactivation is a downstream mechanism of COX-2 activation in human endothelial cells. One recent study also showed that NS-398 inhibits EGFR transactivation in colon cancer cells [62]. These observations reveal that the connection between COX-2 activation and EGFR transactivation plays an important role in LPA-mediated cancer metastasis. Our data further clarified that LPA might induce COX-2 activation, thereby stimulating EGFR transactivation and subsequent VEGF-C expression in human endothelial cells, thus promoting endothelial tube formation. Ye et al. suggested that LPA_3 might mediate LPA-induced embryo implantation in female mice in a COX-2-dependent manner [49]. Our results suggest that LPA_3 might be essential for LPA's upregulation of COX-2 and subsequent VEGF-C expression, thereby enhancing lymphangiogenesis. These results suggest that the LPA_3-COX-2 axis might be necessary for lymphangiogenesis and embryo implantation. It would be interesting to determine whether LPA regulates embryo implantation by modulating lymphangiogenesis.

In the *in vitro* tube formation assay, we observed that LPA- but not S1P-enhanced HUVEC tube formation was mediated by EGFR transactivation (Fig. 5). These results show that LPA but not S1P regulates endothelial cell tube formation through EGFR transactivation. Langlois's group reported that S1P has no effects on BAEC tube formation, while stimulation of MT1-MMP-overexpressing BAEC tube formation is dependent on EGFR transactivation [63]. However, a previous study demonstrated that S1P enhances HUVEC tube formation [64]. Our results further confirmed that S1P-stimulated HUVEC tube formation is not dependent on EGFR transactivation (Fig. 5A). These results suggest that lysophospholipids might regulate endothelial cell tube formation through different signaling mechanisms among different physiological states.

In summary, this study first demonstrates that LPA possibly enhances the lymphangiogenesis process through upregulating VEGF-C mRNA expression in human endothelial cells. By activating LPA_1 and LPA_3, LPA regulates VEGF-C mRNA expression in COX-2-, EGFR transactivation-, and NF-κB-dependent manners. These effects might contribute to endothelial cell differentiation and lymphatic vessel formation (Fig. 8). Our study first clarifies the role of LPA in lymphangiogenesis, and these findings also suggest some potential targets for anti-lymphangiogenesis therapies.

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