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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

Chi-Iou Lin [a], Chiung-Nien Chen [c,d,g], Miao-Tzu Huang [e], Shyh-Jye Lee [a,b], Chih-Hsin Lin [a], Cheng-Chi Chang [f], Hsinyu Lee [a,b,c,d,*]

[a] Institute of Zoology, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 106, Taiwan
[b] Department of Life Science, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 106, Taiwan
[c] Angiogenesis Research Center, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 106, Taiwan
[d] Department of Surgery, National Taiwan University Hospital, No.7, Chung San South Rd., Taipei 100, Taiwan
[e] Department of Pediatrics, National Taiwan University Hospital, No.7, Chung San South Rd., Taipei 100, Taiwan
[f] Laboratory of Molecular and Cellular Toxicology, Institute of Toxicology, National Taiwan University College of Medicine, Jen Ai Rd., Sec. 1, Taipei 100, Taiwan
[g] Division of Mechanics, Research Center for Applied Sciences, Academia Sinica, No. 128, Academia Rd., Sec.2, Taipei 115, Taiwan

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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

* Corresponding author. Department of Life Science, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 106, Taiwan. Tel.: +886 2 3366 2499; fax: +886 2 2363 6837.
E-mail address: hsinyu@ntu.edu.tw (H. Lee).

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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