Lysophosphatidic acid regulates inflammation-related genes in human endothelial cells through LPA₁ and LPA₃

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Abstract

Lysophosphatidic acid (LPA) is a low-molecular-weight lysophospholipid (LPL), which regulates endothelial cells participating in inflammation processes via interactions with endothelial differentiation gene (Edg) family G protein-coupled receptors. In this study, we attempted to determine which LPA receptors mediate the inflammatory response in human endothelial cells. Introduction of siRNA against LPA₁ significantly suppressed LPA-induced ICAM-1 mRNA, total protein, and cell surface expressions, and subsequent U937 monocyte adhesion to LPA-treated human umbilical endothelial cells (HUVECs). By knock down of LPA₁ and LPA₃ in HUVECs, LPA-enhanced IL-1 β mRNA expression was significantly attenuated. Moreover, LPA₁ and LPA₃ siRNA also inhibited LPA-enhanced IL-1-dependent long-term IL-8 and MCP-1 mRNA expression, and subsequent THP-1 cell chemotaxis toward LPA-treated HUVEC-conditioned media. These results suggest that the expression of LPA-induced inflammatory response genes is mediated by LPA₁ and LPA₃. Our findings suggest the possible utilization of LPA₁ or LPA₃ as drug targets to treat severe inflammation.

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Lysophosphatidic acid (LPA) is a multifunctional bioactive lipid mediator generated by enzymatic cleavage of cell membrane phospholipids, present in human plasma, biological fluids, and tissues [1]. By binding to specific G-protein-coupled receptors (GPCRs), LPA mediates many cellular responses, including cell differentiation, proliferation, and migration and wound healing [2–4]. LPA acts on at least five LPA receptors (LPA_{1–5}). Three of these LPA receptors, LPA₁/Edg2 [5], LPA₂/Edg4 [6], and LPA/Edg7 [7], are members of the endothelial differentia-

tion gene (Edg) receptor family [1,8]. The fourth receptor, P2Y9/LPA₄, is closely related to purinergic receptors [9]. The orphan receptor, GPR92, was recently confirmed to be the fifth LPA receptor (LPA₅), which might have physiological functions distinct from those of LPA_{1–4} [10].

LPA receptors transduce diverse heterologous signal pathways through coupling with multiple G proteins, including G_{q/11}, G_{i/o}, and G_{12/13} [2]. LPA₁ displays weak coupling to G_q but efficiently couples to G_i and G_{12/13}. In contrast, LPA₂ and LPA₃ can effectively couple to G_q, which triggers calcium signaling and activates PKC. In addition, LPA₂ can also couple to G_{12/13} [11]. In the RH7777 cell line, an LPA-unresponsive rat hepatoma cell line, which stably transfects LPA₁, affected inhibition of cAMP accumulation, whereas LPA₂ and LPA₃ mediated

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calcium mobilization. The inhibition of cAMP accumulation is pertussis toxin-sensitive [6]. These results further confirm that LPA₁ is Gi-linked, whereas LPA₂ and LPA₃ are Gq-linked. Our previous studies demonstrated that LPA enhances monocyte–endothelial cell adhesion and monocyte chemotaxis toward endothelial cells through the respective upregulation of ICAM-1 and IL-1-dependent chemokine IL-8 and MCP-1 expressions in endothelial cells, thus promoting inflammation processes [12,13]. However, which LPA receptors mediate these inflammatory-related gene expressions in endothelial cells in terms of modulating inflammation processes remains unclear [14].

Loss-of-function genetic or pharmacologic approaches are required to determine the functions of LPA receptors *in vivo* [15]. However LPA₁^{-/-} mice display various severe phenotypic abnormalities and approximately 50% neonatal lethality due to defective suckling and olfaction dysfunction [16]. There are no available specific pharmacologic tools appropriate for the knock down of these receptors. Therefore, we utilized siRNA technology, which is reported to initiate the mechanism of posttranscriptional gene silencing to determine the roles of LPA receptors in endothelial cells. In this report, by specifically suppressing LPA receptor gene expressions in HUVECs, we present evidence that LPA-upregulated ICAM-1 mRNA, total protein, cell surface expressions, and subsequent cell adhesion of U937 cells with LPA-treated HUVECs are largely mediated by LPA₁ moreover, LPA-enhanced IL-1β mRNA expression, and subsequent IL-1-dependent IL-8 and MCP-1 mRNA expressions in response to LPA are mediated through both LPA₁ and LPA₃. In addition, LPA₁ and LPA₃ also mediate THP-1 cell chemotaxis toward LPA-treated HUVEC-conditioned media. These results suggest that the binding of LPA to LPA₁ and LPA₃ modulates ICAM-1, IL-1β, IL-8, and MCP-1 gene expressions, which contribute to monocyte/endothelium adhesion and chemoattraction toward the endothelium, thus facilitating inflammation processes.

Methods

Materials. LPA, sphingosine 1-phosphate (S1P), fatty acid-free bovine serum albumin (FAF-BSA), and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO). The human ICAM-1 antibody (clone M19) and FITC-conjugated mouse anti-human ICAM-1 monoclonal antibody (clone 6.5B5) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG was obtained from Boehringer Mannheim (Indianapolis, IN).

Cell culture. HUVECs were isolated from fresh umbilical cords (IRB: 9561709146, National Taiwan University Hospital) as described previously [12] and were cultured in M199 medium supplemented with 10% FBS and 25% EGM (Cell Applications, San Diego, CA). U937 and THP-1 cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% FBS. Cells were maintained under standard cell culture condition at 37 °C in humidified air with 5% CO₂.

RT-PCR analysis. 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. Polymerase chain reaction (PCR) amplification was performed with Taq DNA polymerase (Geneaid, Taoyuan, Taiwan) using the following sets of primers: 5'-GC

AAGCTCCAGTGAAATGCAAAC-3' and 5'-TGTCTACTGACCCC AACCTTGATG-3' (ICAM-1), 5'-AAACAGATGAAGTGCTCCTT CAGG-3' and 5'-TGGAGAACACCACTTGTTGCTCCA-3' (IL-1β), 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' and 5'-TCTCAGCCCTCT TCAAAAACCTTCTC-3' (IL-8), 5'-TCTCAGTGCAGAGGCTCGCGA-3' and 5'-GAGTGAGTGTCAAGTCTTCG-3' (MCP-1), 5'-ACCA CAGTTCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' (GAPDH), 5'-CGGAGACTGACTGTCAGCA-3' and 5'-GGTCCA GAACTATGCCGAGA-3' (LPA₁), 5'-AGCTGCACAGCCGCCTGCC CCGT-3' and 5'-TGCTGTGCCATGCCAGACCTTGTC-3' (LPA₂), and 5'-TTAGCTGCTGCCGATTCTT-3' and 5'-ATGATGAGGAAGGCC ATGAG-3' (LPA₃). PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and then photographed.

Cell transfections. Sequences of used siRNA were UUCUCCGAACGU GUUCACGUdTdT, and ACGUGACACGUUCGGAGAAdTdT (scrambled), and CCGCCGCUUCCAUUUUUCCUdTdT, and AGGAA AAAUGGAAGCGGCGGdTdT (LPA₁). LPA₃ siRNA was purchased from Santa Cruz Biotechnology. HUVECs were transfected using the optimized protocol for electroporation of HUVECs with the Nucleofector apparatus (Amax Biosystems, Cologne, Germany) as described previously [17].

Western blotting for ICAM-1. 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% 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. The blots were performed as described previously [17]. The blots were stripped and reprobed for β-actin to demonstrate uniform loading of proteins.

FACSscan. Transfected HUVEC cultures were serum-starved for 16 h and incubated with LPA or S1P for 8 h at 37 °C. Suspensions of 10⁶ HUVECs in PBS (200 μl) with 0.1% fatty acid-free BSA received 2 μl of FITC-conjugated human anti-ICAM-1 and were incubated for 30 min at 4 °C. Antibody binding of HUVECs with and without stimulation was determined by CyFlow[®] SL (Partec, Münster, Germany) and analyzed by WinMDI version 2.8 software.

In vitro adhesion assay. Transfected HUVECs were cultured in six-well plates and stimulated with 5 μM LPA or S1P for 8 h. After washing with M199 medium, U937 monocytes (1 ml, 5 × 10⁶ cells/ml) were added to the cultures and then incubated at 37 °C for 1 h. Cultures were washed with M199 medium until no visible suspension of U937 cells was observed and then imaged by microscopy. The number of monocytes per culture was counted.

Chemotaxis assay. The transwell migration of human THP-1 cells toward conditioned media was studied in a 48-well microchamber (Neuro Probe, Cabin John, MD) as described previously [13]. Cells migrating across the filter onto the lower surface were photographed using light microscopy. Quantification of the migration results was determined by the color intensity of the spots solubilized by solubilization buffer, and the absorbance was determined at 540 nm.

Statistical analysis. Significant differences between control and treatment groups were determined by ANOVA. Each experiment was repeated at least three times. The results are expressed as means ± SE. A value of *p* < 0.05 was considered statistically significant.

Results

LPA-induced ICAM-1 mRNA expression is mediated through LPA₁, while IL-1- and IL-1-dependent IL-8 and MCP-1 mRNA expressions are mediated through LPA₁ and LPA₃ in HUVECs

We first determined the LPA receptor expression profiles in HUVECs by RT-PCR. Our results indicated that

Fig. 1. Effects of LPA receptors siRNAs on ICAM-1, IL-1β, IL-8, and MCP-1 mRNA expressions in HUVECs. (A) cDNA of HUVECs was analyzed by RT-PCR to determine the LPA receptor expression profiles. The expected PCR products were 397, 888, and 392 bp for LPA_{1–3}, respectively. HUVECs were transfected with scrambled, LPA₁, LPA₃ or LPA₁+LPA₃ siRNA. Twenty-four hours after transfection, HUVECs were starved and treated with 5 μM LPA or S1P for 4 h (B) or 16 h (C). ICAM-1, IL-1β, IL-8, and MCP-1 mRNA levels were assessed by RT-PCR and shown in the figure as indicated. The expression level of LPA₁ or LPA₃ in each panel was used to evaluate the efficiency of each siRNA. The amount of GAPDH was used as a loading control. C represents the untreated control.

Fig. 2. LPA_1 siRNAs suppressed total and cell surface ICAM-1 protein expressions in HUVECs. HUVECs were transfected with scrambled, LPA_1 , LPA_3 , or LPA_1+LPA_3 siRNA. Transfected HUVECs were starved and treated with 5 μ M LPA or SIP for 8 h. (A) Total cell lysates were isolated and ICAM-1 protein expression levels were monitored by Western blotting using a human monoclonal anti-ICAM-1 antibody. A human β -actin antibody was used as the loading control. (B) Treated cells were dissociated by trypsinization and incubated with an FITC-labeled anti-human ICAM-1 monoclonal antibody for 30 min and analyzed by FACscan. C represents the untreated control.

at least LPA_1 and LPA_3 were expressed in HUVECs (Fig. 1A). Therefore, we first investigated mediation of LPA-induced ICAM-1, IL-1 β , IL-8, and MCP-1 mRNA expressions by these LPA receptors. siRNA was used to knock down the expression level of a given LPA receptor. HUVECs were transfected with scrambled, LPA_1 , LPA_3 , or LPA_1+LPA_3 siRNA. We used SIP, a structural and functional homolog of LPA, as a control in the following experiments. HUVECs transfected with siRNA specific for each LPA receptor showed significantly decreased expression of each LPA receptor mRNA in LPA- and SIP-treated, and untreated samples (Fig. 1B and C). The expression patterns of GAPDH, which was used as the loading control, did not significantly differ in LPA- or SIP-treated, or untreated samples. After 4 h of treatment, decreases in LPA- but not SIP-induced ICAM-1, IL-8, or MCP-1 mRNA expressions were observed in HUVECs transfected with LPA_1 siRNA but not in those transfected with scrambled siRNA. Moreover, LPA- but not SIP-induced IL-1 β mRNA expression in HUVECs was significantly suppressed by transfection with LPA_1 or LPA_3 siRNA (Fig. 1B). However, LPA-induced IL-8 and MCP-1 mRNA expressions at 16 h in HUVECs were partially sup-

pressed by transfection with LPA_1 or LPA_3 siRNA (Fig. 1C). These results demonstrated that LPA-regulated ICAM-1 mRNA expression is mediated by LPA_1 , while LPA-regulated IL-1 β mRNA expression is mediated by both LPA_1 and LPA_3 , therefore subsequently modulating long-term IL-8 and MCP-1 mRNA expressions in an LPA_1 and LPA_3 dependent mechanism in HUVECs.

LPA-induced ICAM-1 protein expression is mediated through LPA_1 in HUVECs

Since LPA_1 's mediation of LPA-induced ICAM-1 mRNA expression was examined, we further investigated if LPA-elevated mRNA levels of ICAM-1 mediated through LPA_1 were also correlated to protein expression levels. Total ICAM-1 protein levels were detected by Western blotting. Decreases in LPA- but not SIP-induced ICAM-1 total protein expression were observed in HUVECs treated with LPA_1 siRNA but not those treated with LPA_3 siRNA. Co-transfection of LPA_1 and LPA_1 further totally abrogated LPA-induced ICAM-1 protein expression in HUVECs (Fig. 2A). The expression patterns of β -actin, which was used as the loading control, did not

significantly differ in LPA- or S1P-treated, or untreated samples. According to the FACScan analysis, which detects the antigenic activities of proteins on cell surfaces, we also observed a significant decrease in LPA- but not S1P-induced ICAM-1 cell surface expressions in HUVECs treated with LPA₁ and LPA₁+LPA₃ siRNA, but not in those treated with LPA₃ siRNA (Fig. 2B). These results indicate that the enhancement effects of LPA on ICAM-1 expression mediated through LPA₁ also occurred with total protein and cell surface expression in HUVECs.

LPA-enhanced adhesive properties of HUVECs toward U937 cells are mediated through LPA₁

In our previous study, we demonstrated that LPLs' enhancement of monocyte adherence to endothelial cells is mediated through upregulating ICAM-1 expression in endothelial cells [12]. In the present study, we found that the enhancement of ICAM-1 mRNA expression by LPA in HUVECs was mediated by LPA₁ (Fig. 2). We next attempted to investigate if LPA-induced monocyte adhesion toward endothelial cells is mediated through LPA₁. By an *in vitro* adhesion assay, treatment with scrambled siRNA showed no effects on LPS-activated U937 cell adherence to LPA- and S1P-treated HUVECs. However, treatment with LPA₁ but not LPA₃ siRNA significantly inhibited U937 cell adherence to LPA- but not S1P-treated HUVECs (Fig. 3). These results illustrate that the enhancement effect of LPA on the adhesive property of endothelial

cells toward monocytes is only mediated by activation of LPA₁. Our results suggest that LPA₁ might mediate LPA-induced ICAM-1 expression in HUVECs and subsequent monocyte–endothelium adhesion.

LPA-enhanced chemotactic activity of HUVECs toward THP-1 cells is mediated through LPA₁ and LPA₃

Our previous study indicated that the enhancement effect of LPA on chemotactic activity of endothelial cell is mediated through upregulation of the expressions of IL-8 and MCP-1 [13]. In addition, LPA-enhanced IL-8 and MCP-1 mRNA expressions were mediated through LPA₁ and LPA₃ (Fig. 1). Therefore, we further tested the hypothesis that LPA's enhancement of the chemotactic activity in endothelial cells is mediated by LPA₁ and LPA₃. By a chemotaxis assay, treatment with scrambled siRNA showed no effects on THP-1 cells' transwell migration towards conditioned media of LPA- or S1P-treated HUVECs. However, conditioned media collected from LPA₁, LPA₃, or LPA₁+LPA₃ siRNA-transfected HUVECs significantly inhibited LPA- but not S1P-enhanced THP-1 cell transwell migration (Fig. 4). These results imply that the enhancement effects of LPA on the chemotactic activity of endothelial cell are mediated by LPA₁ and LPA₃ activation. Our results suggest that LPA₁ and LPA₃ might mediate LPA-induced IL-8 and MCP-1 expressions in HUVECs, subsequently chemottracting monocytes toward the endothelium.

Fig. 3. LPA₁ siRNAs suppressed LPA- but not S1P-enhanced ICAM-1-mediated U937/HUVEC adhesion. (A) HUVECs were transfected with scrambled, LPA₁ or LPA₃ siRNA. Transfected cells were treated with medium, 5 μ M of LPA or S1P for 8 h and then co-incubated with 1 μ g/ml LPS-stimulated U937 cells for 1 h. (B) Histogram represents quantification of adherent U937 cell counts (* p < 0.05).

Fig. 4. LPA₁ and LPA₃ siRNAs inhibit the chemotactic activity of conditioned media from LPA- but not S1P-treated HUVECs. (A) HUVECs were transfected with scrambled, LPA₁, LPA₃ or LPA₁+LPA₃ siRNA, followed by medium, 5 μ M of LPA or S1P treatment for 24 h. The chemotactic activity was determined using a 48-well micro chemotaxis chamber. THP-1 cells were added to the upper chambers and allowed to migrate for 4 h through 8- μ m porous membranes towards the lower chambers which contained the harvested conditioned media which had been added as indicated. Cells migrated to the lower chambers were fixed, stained, and photographed. (B) Histogram represents quantification results of migrating THP-1 cells (* $p < 0.05$). All data are relative multiples of expression compared to untreated cells.

Discussion

The RT-PCR analysis revealed that not all LPA receptors are expressed in HUVECs. LPA₂ (Fig. 1A) was not expressed in HUVECs, which is consistent with previous reports [2,14]. In this study, we observed that the effects of LPA treatment for 4 h on elevating ICAM-1 mRNA expression were mediated by LPA₁ but not LPA₃, while IL-1 β mRNA expression was mediated by both LPA₁ and LPA₃ in HUVECs. However, LPA-induced IL-8 and MCP-1 mRNA expressions at 4 h were mediated by LPA₁, while these enhancement effects at 16 h were both mediated by LPA₁ and LPA₃ (Fig. 1). Our previous study demonstrated that LPA's induction of IL-8 and MCP-1 mRNA expressions for 16 h is IL-1-dependent [13]. Our present results further indicate that LPA-induced IL-8 and MCP-1 mRNA expressions were prolonged to 16 h due to LPA₁- and LPA₃-mediated IL-1 production. However, LPA₁ but not LPA₃ mediated early direct LPA-induced and IL-1-independent IL-8 and MCP-1 mRNA expressions in human endothelial cells.

A recent study reported that LPA profoundly upregulates IL-8 expression, chemotactic activity, and cell adhesion in DLD1 cells, a cell line exclusively expressing LPA₁ [18]. Those results further confirm our observation that the LPA-induced adhesive property (Fig. 3) and chemotactic activity (Fig. 4) in endothelial cells were mostly mediated by LPA₁ activation. In addition, Zhang et al. reported that the induction of cell proliferation and LPA autocrine in human corneal epithelial cells by LPA or various proinflammatory agonists may be inhibited by pre-treatment with pertussis toxin and Ki16425 [19], a selective antagonist of LPA₁ and LPA₃, which implies that the effect is mainly dependent on LPA₁. Gobeil et al. reported that various proinflammatory gene expressions are modulated by nuclear LPA₁, implying that either membrane- or nuclear-located LPA₁ regulates inflammation processes in response to extracellular or intracellular LPA [20]. These results imply that LPA₁ is the most important receptor mediating LPA regulation of various cell functions in different cell types which promote the inflammation process.

LPA regulates multiple cellular physiological behaviors through binding with LPA receptors. However, not many papers have identified which of these receptors contributes to LPA-regulated cell functions. Our data showed that LPA's induction of IL-8 and MCP-1 mRNA expressions and subsequent chemotactic activities in HUVECs were LPA₁ and LPA₃ dependent (Figs. 2 and 4). Consistently, LPA's induction of IL-8 expression was also completely mediated by LPA₁ and LPA₃, but not LPA₂, in human bronchial epithelial cells [21]. In addition, LPA's induction of MCP-1 expression was mainly mediated through LPA₁ and LPA₃ in human smooth muscle cells [22]. However, LPA₂ is the most effective receptor in LPA-induced IL-8 expression in ovarian [23] and colon [24] cancer cells. These results imply that LPA₁ and LPA₃ might play important roles in IL-8 and MCP-1 expressions, thus regulating inflammation processes, while only LPA₂ is essential for IL-8 expression and subsequent cancer cell chemotaxis.

LPA₁ and LPA₃ expressed on platelets are essential for LPA's presence in the core region of atherosclerotic plaques which triggers rapid platelet activation [25]. Moreover, LPA₁ and LPA₃ also mediate LPA-regulated eosinophil activation, and the initiation of subsequent inflammation events [26]. Herein, we further report that LPA₁ is essential for LPA's regulation of monocyte–endothelial cell adhesion (Fig. 3), and LPA₁ and LPA₃ are critical for monocyte chemoattraction to the endothelium (Fig. 4), which implies that LPA₁ and LPA₃ are necessary for endothelial cell interactions with monocytes during inflammation processes. This evidence strongly suggests that LPA₁ and LPA₃ play critical roles in inflammation process progression, and LPA₁ and LPA₃ antagonists might also be new targets for anti-inflammation therapeutic drugs.

In summary, our results clearly indicate that LPA increases ICAM-1 mRNA, total protein, and cell surface expressions in human endothelial cells largely mediated by the respective activation of LPA₁. Furthermore, LPA-enhanced monocytes adhering to endothelial cells are also in an LPA₁ dependent mechanism. Through activation of both LPA₁ and LPA₃, LPA enhanced IL-1 β mRNA expression, thus regulating long-term LPA-induced IL-8 and MCP-1 mRNA expression, and subsequent monocyte chemotaxis toward endothelial cells. Our results suggest that LPA₁ and LPA₃ might be essential receptors for LPA's modulation of monocyte–endothelial cell interactions, which provides valid information necessary for developing new therapeutic drugs against the inflammation process and atherosclerosis formation.

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