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Lysophosphatidic acid regulates inflammation-related genes in human endothelial cells through LPA_1 and LPA_3

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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 LPA_1 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 LPA_1 and LPA_3 in HUVECs, LPA-enhanced IL-1β mRNA expression was significantly attenuated. Moreover, LPA_1 and LPA_3 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 LPA_1 and LPA_3. Our findings suggest the possible utilization of LPA_1 or LPA_3 as drug targets to treat severe inflammation.

Keywords: LPA; LPA_1; LPA_3; Endothelial cells

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, LPA_1/Edg2 [5], LPA_2/Edg4 [6], and LPA/Edg7 [7], are members of the endothelial differentiation gene (Edg) receptor family [1,8]. The fourth receptor, P2Y9/LPA_4, is closely related to purinergic receptors [9]. The orphan receptor, GPR92, was recently confirmed to be the fifth LPA receptor (LPA_5), 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]. LPA_1 displays weak coupling to Gq but efficiently couples to Gi and $G_{12/13}$. In contrast, LPA_2 and LPA_3 can effectively couple to Gq, which triggers calcium signaling and activates PKC. In addition, LPA_2 can also couple to $G_{12/13}$ [11]. In the RH7777 cell line, an LPA-unresponsive rat hepatoma cell line, which stably transfects LPA_1, affected inhibition of cAMP accumulation, whereas LPA_2 and LPA_3 mediated

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calcium mobilization. The inhibition of cAMP accumulation is pertussis toxin-sensitive [6]. These results further confirm that LPA_1 is Gi-linked, whereas LPA_2 and LPA_3 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 $LPA_1^{-/-}$ 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 LPA_1 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 LPA_1 and LPA_3. In addition, LPA_1 and LPA_3 also mediate THP-1 cell chemotaxis toward LPA-treated HUVEC-conditioned media. These results suggest that the binding of LPA to LPA_1 and LPA_3 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% CO_2.

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′-GCAAGCTCCCAGTGAAATGCAAAC-3′ and 5′-TGTCTACTGACCCCAACCCTTGATG-3′ (ICAM-1), 5′-AAACAGATGAAGTGCTCCTTCAGG-3′ and 5′-TGGAGAACACCACTTGTTGCTCCA-3′ (IL-1β), 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′ and 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′ (IL-8), 5′-TCTCAGTGCAGAGGCTCGCGA-3′ and 5′-GAGTGAGTGTTCAAGTCTTCG-3′ (MCP-1), 5′-ACCACAGTTCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ (GAPDH), 5′-CGGAGACTGACTGTCAGCA-3′ and 5′-GGTCCAGAACTATGCCGAGA-3′ (LPA_1), 5′-AGCTGCACAGCCGCCTGCCCCGT-3′ and 5′-TGCTGTGCCATGCCAGACCTTGTC-3′ (LPA_2), and 5′-TTAGCTGCTGCCGATTTCTT-3′ and 5′-ATGATGAGGAAGGCCATGAG-3′ (LPA_3). PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and then photographed.

Cell transfections. Sequences of used siRNA were UUCUCCGAACGUGUUCACGUdTdT, and ACGUGACACGUUCGGAGAAdTdT (scrambled), and CCGCCGCUUCCAUUUUUCCUdTdT, and AGGAAAAUGGAAGCGGCGGGdTdT (LPA_1). LPA_3 siRNA was purchased from Santa Cruz Biotechnology. HUVECs were transfected using the optimized protocol for electroporation of HUVECs with the Nucleofector apparatus (Amaxa 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.

FACscan. Transfected HUVEC cultures were serum-starved for 16 h and incubated with LPA or S1P for 8 h at 37 °C. Suspensions of 10^6 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, 5×10^6 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 LPA_1, while IL-1- and IL-1-dependent IL-8 and MCP-1 mRNA expressions are mediated through LPA_1 and LPA_3 in HUVECs

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