Lysophosphatidic Acid Stimulates the G-protein-coupled Receptor EDG-1 as a Low Affinity Agonist*

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EDG-1, an inducible G-protein-coupled receptor from vascular endothelial cells, is a high affinity receptor for sphingosine 1-phosphate (SPP) (Lee, M-J., van Brocklyn, J. R., Thangada, S., Liu, C. H., Hand, A. R., Menzelleev, R., Spiegel, S., and Hla, T. (1998) Science 279, 1552–1555). In this study, we show that lysophosphatidic acid (LPA), a platelet-derived bioactive lipid structurally related to SPP, is an agonist for EDG-1. LPA binds to EDG-1 receptor with an apparent $K_D$ of 2.3 μM. In addition, LPA binding to EDG-1 induces receptor phosphorylation, mitogen-activated protein kinase activation, as well as Rho-dependent morphogenesis and P-cadherin expression. These data suggest that LPA is a low-affinity agonist for EDG-1. Activation of the endothelial receptor EDG-1 by platelet-derived lipids LPA and SPP may be important in thrombosis and angiogenesis, conditions in which critical platelet-endothelial interactions occur.

The G-protein coupled receptor (GPR)$^3$ EDG-1 was originally cloned as an immediate-early gene induced during the morphogenetic differentiation phase of angiogenesis (1). We subsequently demonstrated that the EDG-1 binds to heterotrimeric G$_i$ proteins and stimulates G$_i$-dependent mitogen-activated protein (MAP) kinase activation (2). Recently, we showed that human embryonic kidney (HEK293) cells stably transfected with the EDG-1 cDNA exhibit enhanced adherens junction-based cell-cell aggregation, exaggerated cadherin expression and were phenotypically differentiated (3). Interestingly, the induction of morphogenetic differentiation was ligand dependent and we demonstrated that serum-borne sphingosine 1-phosphate (SPP) is a highly potent agonist for the EDG-1 GPR. Indeed, SPP binding to EDG-1-transfected cells was reversible, saturable, specific, and exhibited a high-affinity binding site with apparent $K_D$ of 8.1 nM (3). In addition, SPP induces morphogenetic differentiation and P-cadherin expression as well as G$_i$-dependent MAP kinase activation via the EDG-1 receptor (3). Morphogenesis induced by SPP was inhibited by the C3 exoenzyme which specifically ADP-ribosylates the small G-protein Rho (4). These data allowed us to conclude that EDG-1 is a high-affinity receptor for SPP (3).

SPP, released from activated platelets (5), is recognized as a potent bioactive lipid mediator with multiple biological activities (6–12). SPP, which is also a platelet-derived bioactive lipid mediator (13), is structurally similar to SPP. Like SPP, LPA also regulates a wide range of cellular responses, including proliferation (14–16), platelet aggregation (14–16), stress fiber formation (17), neurite retraction (18, 19), cell rounding (20), tumor cell migration (21), among others. Moreover, it is well documented that LPA regulates these responses by activating specific G-protein-coupled receptors present on the cell surface of numerous cell types (22). Recently, EDG-2/Vzg-1 was shown to be an LPA receptor involved in cell rounding, G$_i$-dependent inhibition of adenylyl cyclase (20), and serum response factor induced transcription (23). Prompted by the fact that (i) EDG-2/Vzg-1 is highly related to EDG-1 and (ii) LPA and SPP are structurally and functionally similar, we investigated in this report whether EDG-1 serves as a receptor for LPA.

EXPERIMENTAL PROCEDURES

Materials—Phospholipases, fatty acid free BSA, LPA, lysophosphatidylinositol, lysophosphatidyleserine, lysophosphatidylethanol, aprotinin, leupeptin, and pepstatin were from Sigma; [3H]LPA (specific activity 56.2 Ci/mmol) from NEN Life Science Products Inc., [35S]methionine, [3H]leucine, [3H]proline, and [3H]cysteine (specific activity 760 MG/mmol) and [γ-32P]ATP (specific activity 3000 Ci/mmol) from Amersham; SPP, sphingomyelin, ceramide, and ceramide 1-phosphate from Biomol; C3 exotoxin, pertussis toxin, and PD 98059 from Calbiochem; anti-P-cadherin antibody was from Transduction Laboratories; anti-M2 antibody from Eastman Kodak; rhodamine-conjugated sheep anti-mouse IgG from Cappel; anti-HA monoclonal antibody, endoglycosylase H, and alkaline phosphatase were from Boehringer Mannheim; G418, LipofectAMINE, and LipofectAMINE Plus reagents were from Life Technologies, Inc.; DMEM, RPMI 1640, and Dulbecco’s phosphate-buffered saline (PBS, pH 7.4) were from Mediatech; trypsin EDTA was from JRH Bioscience; fetal bovine serum (FBS) was from HyClone; mouse mammary leukemia virus-reverse transcriptase from Life Technologies, Inc.; RNasin from Promega; Tntg DNA polymerase from Cetus; and the FastTrackTM 2.0 mRNA isolation kit from Invitrogen.

Cell Culture, Transfection, and Morphogenetic Differentiation Assay—Culture of HEK293 cells (ATCC CRL-1573) and transfection of EDG-1 cDNA was performed as described (2, 3). Stably transfected cells were subcloned twice to isolate pure clones. Four independently isolated EDG-1-transfected clones and two vector transfected clones were used in this study. Morphogenetic differentiation assay of EDG-1 expressed cell clones (HEK293-EDG-1) was carried out essentially as described (3). Briefly, cells (7.5 × 10$^5$ cells/ml) were plated into 6-well tissue culture plates (Falcon) in DMEM, 10 mM HEPES pH 7.4 containing 10% (v/v) charcoal-stripped lipop-depleted FBS (CBS) with the indicated supplements. To examine the effect of serum-borne bioactive lipids on differentiation, FBS was treated with specific phospholipases (1 unit/500 μl) at 25 °C for 24 h. As a control, FBS was incubated with heat-denatured (95°C for 1 h) phospholipases.

The human HEL 92.1.7 erythroleukemia (ATCC TIB-180) cell line was cultured in RPMI 1640 medium supplemented with 10% FBS. The transfection of these cells were performed with LipofectAMINE Plus
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Cell Rounding Assay—HEK293-EDG-1 cells were plated in 10% charcoal-stripped FBS (0.8 × 10^5 cells/5 mm) containing indicated concentrations of bioactive lipid ligands. The C3 exotxin was used at 5 μg/ml. Three days later, cells were washed twice with PBS and permeabilized with 0.12% (v/v) Triton X-100 for 5 min. Cells were then incubated with anti-M2 antibody or with anti-M2 as described previously (2, 3).

Reverse Transcriptase-Polymerase Chain Reaction and Northern Analysis—mRNAs were isolated from cultured cells with FastTrack® mRNA isolation kit 2.0 as per the manufacturer’s instructions. The full-length coding region of EDG-1 was labeled with [α-32P]dCTP by the random primer method and used as a probe for Northern hybridization analysis. One microgram of isolated poly(A)^+ RNA in each lane was electrophoresed on 1% agarose/formaldehyde gel and transferred onto a Zeta Probe membrane (Bio-Rad). Membrane was hybridized at 65 °C for 16 h with [32P]-labeled probe in Church-Gilbert’s solution (7% SDS, 0.5 M NaH2PO4, 0.5% BSA, 40 mM EDTA, 20% formaldehyde). Subsequently, membrane was washed with 2 × SSC, 0.05% SDS for 30–40 min at 65 °C with several changes. After the last wash with 0.1 × SSC, 0.1% SDS for 40 min at 65 °C, the filter was exposed to Kodak X-Omat film and autoradiographed. Total RNA from HUVEC cells (5 μg/lane) was used as a control to show the endogenous EDG-1. For reverse transcriptase-polymerase chain reaction analysis, total RNA from cultured cells was purified as described (1). RNA was then converted to cDNA by treatment with 200 units of mouse mammary leukemia virus-reverse transcriptase in 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 15 mM NaCl, 3 mM MgCl2, 1 unit of RNasin, 0.2 μg of random hexamer primers, 0.5 μM dNTPs and incubated at 37 °C for 1 h. The reaction was terminated by heating at 95 °C for 10 min and diluted to 1 ml with distilled water. Enzymatic amplification was conducted with a 10-μl aliquot of the cDNA mixture. Polymerase chain reaction was performed in 50 mM Tris-HCl (pH 8.0), 1.5 mM MgCl2, 10 mM KCl, 0.2 mM dNTPs, 0.5 μM of each primers for EDG-2/EDG-1 and 2.5 units of Taq DNA polymerase. The reaction mixture was heated at 94 °C for 1 min, annealed at 55 °C for 2 min, and extended at 72 °C for 3 min for 30 repetitive cycles. The primers used were 5'-GGTCGACGAATCAC-CCGGAG-3' (sense) and 5'-GCTTATTGGACACGCGTGA-3' (antisense) to amplify EDG-2/EDG-1 mRNA.

Cell Rounding Assay—HEK293-pcDNA and HEK293-EDG-1 cells (25,000/35-mm culture dish) were starved in 10% FBS for 24 h, and the media containing the desired agents were added. Phase-contrast photographs of cellular morphology were taken at the indicated times.
As controls, other lysophospholipids (i.e. lysophosphatidylserine, lysophosphatidylethanolamine, lysophosphatidylinositol, and lysophosphatidylcholine) were used.

RESULTS

We have established several human embryonic kidney 293 fibroblasts (HEK293) clones which stably express the FLAG epitope-tagged EDG-1 GPR (3). Immunostaining with anti-FLAG antibody (anti-M2) shows that the expressed EDG-1 GPR is predominantly located on the plasma membrane (Fig. 1). Immunostaining of vector-transfected clones did not express immunofluorescence signals.

To determine if LPA is an agonist for EDG-1, we employed a radioligand [3H]LPA binding assay on HEK293 stable transfectants. As shown in Fig. 2A, specific [3H]LPA binding was markedly enhanced in four independent HEK293-EDG-1 clones compared with the vector-transfected and parental HEK293 cells. Specific LPA binding was time-dependent and was observed in both EDG-1-transfected and vector-transfected cells. However, EDG-1-transfected cells bound significantly more [3H]LPA (Fig. 2B). Specific LPA binding was competed by unlabeled LPA but not by other lysophospholipid and sphingolipid analogs (Fig. 2C). Moreover, specific [3H]LPA binding was suppressed by the anti-M2 antibody in a dose-dependent manner only in HEK293-EDG-1 cells (Fig. 2D). This implies that LPA binds directly to the EDG-1 GPR. Furthermore, LPA binding was saturable; Scatchard analysis of [3H]LPA binding on HEK293-EDG-1 cells indicated two types of binding sites; apparent Kd = 178 nM, Bmax = 313 fmol/10⁵ cells as well as apparent Kd = 2.3 μM, Bmax = 6.23 pmol/10⁵ cells (Fig. 2E). In contrast, there is only one type of binding site detected on HEK293 parental cells with an apparent Kd = 212 nM and Bmax = 420 fmol/10⁵ cells (Fig. 2E). To determine whether the N-terminal FLAG tag does not alter the LPA binding properties of EDG-1.

FIG. 2. LPA Binds to EDG-1. A, specific [3H]LPA binding on four different HEK293-EDG-1 clones, HEK293-pCDNA, and parental HEK293 cells. B, time dependence of specific LPA binding. C, the competition of specific LPA binding on HEK293-EDG-1 cells with various lipid analogs (20 μM each). Nonspecific LPA binding was defined as binding in the presence of 50 μM LPA. D, indicated concentration of the antibody (μg/ml) was preincubated with the cells for 1 h at 4 °C and [3H]LPA binding was performed as described. mlgG, irrelevant mouse IgG. E, Scatchard plot of [3H]LPA binding to HEK293-EDG-1 and HEK293-pCDNA cells. Note that there are two types of LPA-binding sites in HEK293-EDG-1 cells, whereas only a single type of binding site in parental HEK293 cells. All panels are derived from a representative experiment which was repeated at least two times. Experiments on panels B-E were conducted on HEK293-pCDNA and HEK293-EDG-1 (#1) clones. F, homologous competition of [3H]LPA binding on HEL 92.1.7 cells which were transiently transfected with N-terminal FLAG-tagged (EDG-1) or C-terminal green fluorescent protein-tagged (EDG-1-GFP) EDG-1 cDNAs. Note that the N-terminal FLAG tag does not alter the LPA binding properties of EDG-1.
Gi-linked MAP kinase pathway and Rho-dependent morphogenesis transduces two distinct intracellular signaling pathways: the receptor. As shown previously (2, 3), the activation of EDG-1 that both LPA and SPP are capable of binding to EDG-1. LPA binding to HEK293-4 °C indicated that both LPA and SPP inhibited subsequent sphingomyelin (10% v/v) aaGi-coupled mechanism (25). We therefore tested whether netic differentiation (3). LPA is known to activate MAP kinase. The activation is completely inhibited in the presence of pertussis ERK-2 activity was assayed as described. Note that both LPA and SPP, in a dose-dependent man ner, inhibited [3H]LPA-binding sites in HEK293-EDG-1 cells. SPMM, sphingomyelin (10 μM); LPS, 10 μM. low affinity receptor with an apparent $K_d$ of 2.3 μM for LPA. Although unlabeled LPA competed effectively with [3H]LPA binding (IC50 3 μM), unlabeled SPP competed less effectively. At 100 μM SPP, 10 μM [3H]LPA binding was competed ~47% (Fig. 3A). However, a 15-min pretreatment at 37 °C with LPA or SPP, followed by wash out and [3H]LPA binding analysis at 4 °C indicated that both LPA and SPP inhibited subsequent LPA binding to HEK293-EDG-1 cells (Fig. 3B). This suggests that both LPA and SPP are capable of binding to EDG-1. Next, we investigated whether LPA can signal via the EDG-1 receptor. As shown previously (2, 3), the activation of EDG-1 transduces two distinct intracellular signaling pathways: the G1-linked MAP kinase pathway and Rho-dependent morphogenetic differentiation (3). LPA is known to activate MAP kinase via a G1-coupled mechanism (25). We therefore tested whether LPA binding to EDG-1 also activates MAP kinase. The EDG-1 cDNA was co-transfected into COS-7 cells with the HA epitope-tagged ERK-2 construct (2) and stimulated with LPA. As shown in Fig. 4A, LPA-induced MAP kinase activity was greatly potentiated by EDG-1 expression. LPA induced approximately 2–3-fold increase of MAP kinase activity in the absence of EDG-1 whereas it induced more than a 10-fold increase in MAP kinase activity when EDG-1 was overexpressed. This assay was also conducted in HEL92.1.7 cells which do not respond to LPA. LPA treatment markedly activates MAP kinase in HEL 92.1.7 cells overexpressed EDG-1 GPR, and this activation is completely inhibited in the presence of pertussis toxin (Fig. 4B). Together, these data show that LPA activates MAP kinase via the EDG-1 GPR and G1 pathway. Ligand binding to GPR results in the rapid activation of intracellular signaling components, which is accompanied by the phosphorylation of the cytosolic domains of the receptor (26). Many cytosolic kinases, such as the β-adrenergic receptor kinase family, participate in the ligand-activated receptor phosphorylation responses (27). Such responses are thought to be important in GPR desensitization (26, 27). We next investigated if LPA is capable of inducing phosphorylation of EDG-1 GPR. As shown in Fig. 5A, LPA stimulated EDG-1 phosphorylation whereas the biologically inactive lipids such as lysophosphatidylglycerol did not. SPP, which is a high affinity agonist for EDG-1, induces EDG-1 phosphorylation more potently. Also, FBS, which contains LPA and SPP, stimulates the EDG-1 phosphorylation. The deduced molecular mass of nascent EDG-1 polypeptide is approximately 42 kDa. Interestingly, ligand stimulation results in immunoreactive EDG-1 polypeptides which migrate in SDS-PAGE at the approximate 52-kDa position (Fig. 5, A and B). This is most likely due to the combination of glycosylation and phosphorylation of EDG-1, since deglycosylation or dephosphorylation of EDG-1 results in mob-
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In this report, we show that: (i) LPA binding is enhanced in EDG-1-transfected cells and the enhancement is suppressed specifically by anti-FLAG antibody (Fig. 2, A–D). (ii) LPA is able to activate MAP kinase via the EDG-1 GPR (Fig. 4). (iii) LPA induces rapid phosphorylation of EDG-1 receptor (Fig. 5). (iv) LPA induces, in a Rho-dependent manner, morphogenetic differentiation and P-cadherin expression in HEK293-EDG-1 cells (Fig. 6). Collectively, these data indicate that LPA directly binds to EDG-1 and activates EDG-1-mediated signaling events, and therefore suggests that EDG-1 can serve as a plasma membrane-localized receptor for the serum-borne bioactive lipid LPA.

Furthermore, Scatchard analysis indicates that there are two types of LPA-binding sites present in HEK293-EDG-1 cells (apparent \( K_d \) values = 178 nM and 2.3 \( \mu \)M), whereas a single type of binding site present in parental HEK293 cells (apparent \( K_d \) = 212 nM) (Fig. 2E). It is possible that the \( B_{\text{max}} \) values for LPA-binding sites may be an overestimated due to the lipophelic nature of LPA. Nevertheless, these data suggest that EDG-1 is a low affinity receptor for LPA. In agreement with this notion, micromolar LPA is required to induce EDG-1-mediated differentiation (Fig. 6A, P-cadherin induction (Fig. 6D), and MAP kinase activation (Fig. 4). SPP is more potent than LPA in inducing EDG-1 signaling (3). The physiological concentration of LPA in serum was estimated to be approximately 5 \( \mu \)M (28). Therefore, the apparent \( K_d \) is well within in vivo concentrations. Thus, EDG-1 might be activated by LPA under physiological or pathophysiological conditions.

Phospholipase B treatment (which is specific for glycerol-based lipids and not sphingoid-based lipids) destroyed the differentiation inducing activity of serum (Fig. 6B), suggesting that LPA is an important component of serum required for EDG-1-dependent morphogenetic differentiation. However, it requires 20–40 times more LPA to mimic the effect induced by 10% serum (Fig. 6A). Moreover, we were unable to completely reconstitute the differentiation inducing activity of serum by adding 1 \( \mu \)M LPA and SPP together (data not shown). Thus, there may be unidentified factor(s) present in serum which may induce EDG-1-mediated differentiation synergistically with serum-borne SPP and LPA, or stabilize the serum-borne...
SPP and LPA. In agreement, a recent report suggests that LPA induced Rho activation and cytoskeletal changes require the activity of the EGF receptor (29).

Data presented in this report suggest that EDG-1, a high affinity receptor for SPP (3), is a low affinity receptor for LPA. Intriguingly, the binding of \(^{3}H\)LPA was competed poorly by unlabeled SPP (Fig. 3A). However, pretreatment of HEK293-EDG-1 at 37 °C for 15 min with SPP or LPA, but not with control lipids (e.g., LPS and SPM), reduced the subsequent \(^{3}H\)LPA binding in a dose-dependent manner (Fig. 3C). Previously, we have shown that SPP treatment resulted in EDG-1 GPR trafficking into intracellular compartments (3). One plausible explanation for these observations is that SPP and LPA bind to EDG-1 at distinct sites. However, this assumption awaits definitive assignment of binding sites by site-directed mutagenesis studies of EDG-1. A common binding site for SPP/LPA was described in human platelets (11). It should be noted that EDG-1 possesses distinct properties than the platelet SPP/LPA receptor. First, SPP and LPA bind to a common site on platelets (11), whereas SPP and LPA may bind to two

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**Fig. 6.** LPA induces morphogenetic differentiation in HEK293-EDG-1 cells. A, HEK293-EDG-1 (left column) and HEK293-pCDNA (right column) cells were plated in the indicated medium (DMEM plus indicated supplements) and phase-contrast photographs of cellular morphology are shown. Control, 10% lipid-depleted, charcoal-stripped FBS (CFBS); LPA, 5–50 μM LPA in CFBS; anti-M2, anti-FLAG antibody (50 μg/ml) in 20 μM LPA; mIgG, irrelevant mouse IgG (50 μg/ml) in 20 μM LPA; LPS, 20 μM. Note that LPA induced morphogenetic differentiation specific in HEK293-EDG-1 cells. The scale bar represents 86 μm. B, Phospholipase B treatment destroys the FBS-derived differentiation inducing activity. Active, FBS was incubated with 1 unit of phospholipase A2 (PLA2) or phospholipase B (PLB) at 25 °C for 16 h; boiled, boiled enzymes (95 °C, 30 min) were used as controls. Treated FBS were used at the concentration of 10% (v/v) in the morphogenetic differentiation assay using HEK293-EDG-1 cells as described. The scale bar represents 92 μm. C, HEK293-EDG-1 cells were plated in DMEM containing 10% CFBS supplemented with 20 μM LPA. For C3 treatment, cells were pretreated for 48 h with 5 μg/ml toxin prior to the morphogenetic assay. The scale bar represents 85 μm. D, top panel, HEK293-EDG-1 cells were treated with indicated concentrations of LPA in medium containing 10% charcoal-stripped FBS (CFBS). Bottom panel, HEK293-EDG-1 cells were plated in 20 μM LPA in 10% CFBS. Some wells were received indicated amounts of anti-M2 antibody or the mouse IgG (mIgG) (μg/ml) or the C3 exotoxin. Cell extracts were analyzed for the P-cadherin expression by immunoblot analysis. Data are from a representative experiment which was repeated at least two times.
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distinct sites on EDG-1. Second, EDG-1 has a single type of binding site for SPP (apparent Kd = 8.1 nM), whereas platelets have two types of binding sites for SPP (Kd = 110 nM and 2.6 μM).

Recently, EDG-2/Vzg-1, which is highly related to EDG-1 (40% sequence identity over 309 residues), was shown to bind to LPA and transduce signals for cell rounding via a non-Gi/Go pathway (20). Two independent reports confirmed that EDG-2/Vzg-1 indeed respond to LPA (23, 30). Indeed, we observed that parental HEK293 cells express the EDG-1 mRNA and that LPA stabilized endothelial junctions and decreased permeability (38). Also, the role of EDG-1 and its ligands SPP and LPA in the vascular system needs to be addressed in the future.

In conclusion, our data suggest that EDG-1 is a low affinity LPA receptor. Furthermore, our data support the concept that multiple receptors exist for LPA each of which regulate specific biological functions. Third, EDG-1 represents a GPR which interacts with both LPA and SPP, albeit at different affinities.

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