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Lysophospholipid Signaling: Beyond the EDGs

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Abstract

As our understanding of the myriads of biological effects caused by lysophospholipids expands we become witnesses to another miracle of nature that has endowed the simplest lysophospholipids with functions seemingly ubiquitous to every mammalian cell. A decade after the discovery of the EDG-family lysophospholipid receptors the field has gained unimaginable impetus explaining the biological effects of sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA). The discovery of LPA receptors in the purinergic G protein-coupled receptor (GPCR) gene cluster refined this picture and added complexity to our concepts of lysophospholipid cell signaling. The intracellular lysophospholipid targets – identified and not yet identified – make us realize the dual – mediator and second messenger – roles of lysophospholipids. In this paper we provide new data obtained concerning LPA-elicited responses using cell lines naturally lacking or intentionally knocked out of many of the known LPA GPCR, widely used by investigators in the field as cells with LPA receptor “null background”. Our observations raise caution about the lack of LPA responsiveness in these cells and underline the unprecedented complexity and redundancy of lysophospholipid-evoked cellular responses.

INTRODUCTION

Until 1996, the mediator role of the lysophospholipids sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) remained tentative due to the lack of an identified cell surface receptor. The identification of the endothelial differentiation gene (EDG)-family of G protein-coupled receptors (GPCRs), the best characterized group of lysophospholipid receptors, provided a huge impetus to the field. This gene family is comprised of eight closely related genes; three receptors for the pluripotent lipid mediator LPA and five receptors for the structurally related lipid mediator S1P. The first EDG-family LPA receptor was identified in 1996, when Hecht and colleagues [1] identified LPA as a ligand for a GPCR that was cloned from the ventricular zone of developing mouse brain tissue and originally designated vzg-1. Because of its high (34%) amino acid sequence homology to endothelial differentiation gene EDG-1, an orphan GPCR cloned as a phorbol ester-induced early response gene in vascular endothelial cells, vzg-1 was later named EDG-2 [2]. In 1998 EDG-1 was shown to be a high affinity receptor for S1P [3]. The other EDG family members were cloned based upon sequence homology shortly thereafter, including two more LPA-specific receptors, EDG-4 [4] and EDG-7 [5], as well as four more S1P-specific receptors, EDG-3 [6] EDG-5 [7], EDG-6 [8],

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and EDG-8 [9]. In 2001 the IUPHAR proposed that the EDG nomenclature of the receptors be changed to reflect the receptors natural ligand and the order of its identification, giving the current receptor nomenclature of LPA_{1–3} and SIP_{1–5}.

Knockout animals have been generated for the three EDG-family LPA receptors, including the LPA_{1&2} double knockout, which although displayed distinct phenotypic alterations, remained largely normal [10–12]. The receptor knockout studies are in sharp contrast with the lysophospholipase D knockout mice. Lysophospholipase D cleaves lysophospholipids to generate LPA, and these animals that are devoid of this essential step in LPA production die in utero due to the lack of vascular stabilization [13,14]. The EDG LPA receptor knockouts do not show defects in vascular development.

More recently GPR23 [15] and GPR92 [16,17] have been identified as LPA receptors, and tentatively renamed LPA₄ and LPA₅, respectively, awaiting the approval of the IUPHAR nomenclature committee. Phylogenetically, LPA₄ and LPA₅ form a subfamily of LPA receptors – they are distantly related to the EDG-family LPA receptors, sharing only 20–24% sequence amino acid homology with the EDGs [15,16], which are 50–57% homologous with each other [15]. LPA₄ and LPA₅ are more closely related to the purino-receptor cluster of GPCRs, and share 31% homology with each other [16]. Perhaps just a coincidence, but the lysophospholipase D is an atypical lipase and belongs to the nucleotide pyrophosphate phosphates (NPP) gene family [18], whose substrates include many purine nucleotides.

Expanding the complexity of LPA signaling, a member of the nuclear hormone receptor superfamily, the peroxisome proliferator-activated receptor gamma (PPAR γ), which is a lipid-activated transcription factor, has been identified as an intracellular LPA receptor [19–21]. Extracellular LPA has long been known to have access to the intracellular compartment but only when applied in a carrier (albumin)-free manner [22]. LPA was shown to stimulate a PPAR-responsive reporter element, stimulate transcription of PPAR γ target genes and displace rosiglitazone, a man-made high affinity agonist, from the ligand binding pocket of PPAR γ . Mutational studies have revealed that LPA and rosiglitazone bind to partially overlapping residues in the ligand binding pocket of PPAR γ [21].

In light of the pleiotropic growth factor-like effects of LPA the field faces is dilemma: how many LPA receptors are there and which receptor mediates a particular cellular response? Inherent in this question lies another question: are there any cells that are not activated by LPA? Here we provide a critical analysis of LPA signaling based on experiments utilizing cells that lack the EDG-family receptors and often used in the field as LPA receptor “null” cellular hosts. Our studies indicate that these cells display many of the cellular responses that have been associated with the activation of the EDG-family GPCR.

MATERIALS AND METHODS

Materials

Alkyl-glycerophosphate (AGP 16:0 and 18:1) and lysophosphatidic acid (LPA 16:0, 18:0, and 18:1) were purchased from Avanti Polar Lipids (Alabaster, AL). Pertussis toxin (PTX) was purchased from Biomol Laboratories, Inc. (Plymouth Meeting, PA). *Clostridium botulinum* exoenzyme C3 (C3 toxin) was purchased from List Biological Laboratories (Campbell, CA). The Rac1 inhibitor NSC23766 was purchased from Calbiochem (San Diego, CA). SMARTpool siRNAs were purchased from Dharmacon (Lafayette, CO). The following antibodies and sources were used: rabbit anti-phospho-ERK (Thr202/Tyr204) and rabbit anti-phospho-MLC2 (Ser19) (Cell Signaling, Beverly, MA), rabbit anti-phospho-p38 (Thr180/Tyr182) (Promega, Madison, WI), and mouse monoclonal anti- β -actin (Calbiochem). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were

purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488-conjugated goat anti-rabbit IgG F(ab')₂ fragments were purchased from Molecular Probes (Eugene, OR).

Cell culture

RH7777 cells, B103 cells, PC3 cells and LPA_{1&2} double knockout animal-derived mouse embryonic fibroblasts (DKO MEFs) were grown in Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 10 µg/ml streptomycin, and 2 mM glutamine.

Receptor profiling

Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA). Reverse-transcription polymerase chain reaction was done using the Superscript III kit (Invitrogen), and a total of 31 cycles were performed using 400 ng of RNA as template; the products were separated on agarose gels and visualized with ethidium bromide. The following gene- and species-specific primers were used: LPA₁: forward (human) 5'-

CCGCCGCTTCCATTTTCCTATTTA-3', forward (rat and mouse) 5'-

CCGCCGCTTCCATTTCCCTATTTA-3', reverse (human, rat and mouse) 5'-

CAGTTCCAGCCCACACTGGGTAT-3'; LPA₂: forward (human) 5'-

CACTCCTGGCACTGCCTCTGTG-3', forward (mouse and rat) 5'-

CACTTCTGGCACTGCCTCTGTG-3', reverse (human and mouse) 5'-

TGCGGCGCATCTCAGCATCTCG-3', reverse (rat) 5'-

TACGGGCGCATCTCAGCGTCTCG-3'; LPA₃: forward (human, mouse and rat) 5'-

CACATGTCAATCATGAGGATG-3', reverse (human and rat) 5'-

CCTAAGACAGTCATCACCGTCTTC-3', reverse (mouse) 5'-

CCTAAGACGGTCATCACTGTCTTC-3'; LPA₄: forward (human, mouse and rat) 5'-

GAAGGCTTCTCCAAACGTGTCTG-3', reverse (human, mouse and rat) 5'-

TTCAGAGTTGCAAGGCACAAGG-3'; LPA₅: forward (human, mouse and rat) 5'-

CTGATGCTCATCAACGTGGACC-3', reverse (human, mouse and rat) 5'-

TAGGGCACGAAGCACAGCAG-3'; PPAR_γ: forward (human) 5'-

TGATATCAAGCCCTTCACTACT-3', forward (mouse) 5'-

TGACATCAAGCCCTTTACCACA-3', forward (rat) 5'-

TGACATCAAACCCTTTACCACG-3', reverse (human, mouse and rat) 5'-

GCTTTGGTCAGCGGGAAGGAC-3'; β-actin: forward (human, mouse and rat) 5'-

GACAACGGCTCCGGCATGTG-3', reverse (human, mouse and rat) 5'-

GGCTGGGGTGTGAAGGTCTCAA-3'.

Western blotting

Cells were grown in six-well plates and changed to serum-free medium 6 h before treatment. Cells were collected on ice and lysed in 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 5 mM β-glycerophosphate and 1% protease inhibitor cocktail (Sigma, St. Louis, MO). The lysates were

cleared by centrifugation ($10,000 \times g$) for 15 min at 4°C, and the supernatants were collected. Protein concentrations were determined using a BCA Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% (w/v) bovine serum albumin dissolved in 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 (TBS-T) for 1 h and incubated with primary antibody in TBS-T containing 0.1% nonfat dried milk and 0.1% bovine serum albumin overnight at 4°C. Blots were washed in PBS and incubated with the appropriate HRP-conjugated secondary antibodies, and immunoreactive bands were visualized using the SuperSignal West Pico chemiluminescence reagent (Pierce Biotechnology).

Light microscopy

Cells were seeded in 35 mm dishes, and medium was changed to serum-free medium 6 h before treatment. In some cases cells were pretreated overnight with 100 μ M NSC23766, 10 μ g/ml C3 toxin, or 250 ng/ml PTX. The cells were kept in a humidified incubator at 37°C during treatment with LPA or vehicle, and images were captured every minute using an Olympus CK2 light microscope.

Fluorescence microscopy

RH7777 cells were grown on poly-D-lysine coated coverslips. 6 h before treatment, cell medium was replaced with serum-free medium. After treatment with vehicle or LPA, cells were fixed in 10% formalin for 15 min, rinsed in phosphate-buffered saline (136 mM sodium chloride, 8 mM sodium phosphate, 2.7 mM potassium chloride, 1.5 mM potassium phosphate, pH 7.3), and permeabilized in phosphate-buffered saline that contained 0.02% triton-X-100 for 5 min. Cells were blocked in phosphate-buffered saline/1% bovine serum albumin for 1 h, and incubated with primary antibody (anti-phosphorylated MLC2 at a 1:100 dilution) overnight at 4°C. The cells were washed with phosphate-buffered saline and incubated with secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin) at a 1:200 dilution for 1 h at room temperature. The secondary antibody solution also contained Texas Red-conjugated phalloidin (5 U/ml) to visualize filamentous actin distribution. Cells were washed and mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Fluorescence was visualized using a Nikon Eclipse 80i fluorescence microscope.

siRNA gene knockdown

SMARTpool siRNAs directed against PPAR γ or a control non-targeting pool were transfected into RH7777 cells at a final siRNA concentration of 40 nM using Lipofectamine 2000 reagent (Invitrogen). Cells were replated 24 h after transfection, and cells were treated with LPA 48 h after replating. Medium was changed to serum-free medium 6 h before treatment. Cellular lysates were collected and analyzed by Western blotting.

RESULTS

Receptor profiling

Several cell lines have previously been reported to lack EDG-family LPA receptors. The McArtl rat hepatoma cell line 7777 (RH7777) cells are frequently used in studies utilizing the ectopic expression of LPA receptors, since these cells do not express the EDG-family LPA receptors and do not respond to LPA through any endogenous receptors as judged by calcium mobilization or inhibition of cAMP accumulation [15,23,24]. Other cell lines previously reported to be devoid of EDG-family receptors are HTC4 [25], LPA_{1&2}^{-/-} double knock-out mouse embryonic fibroblasts (DKO MEFs) [11] and the B103 rat neuroblastoma cell line [26]. Reverse-transcription PCR analysis confirmed that DKO MEFs, RH7777, and B103 cell

lines lack mRNA for the EDG-family LPA receptors LPA₁, LPA₂, and LPA₃ (Fig. 1), although a very weak signal could be detected for LPA₂ in RH7777 cells upon overexposure of the gel. We also designed primers and tested these cell lines for the non-EDG LPA GPCRs LPA₄ and LPA₅, as well as the intracellular LPA receptor and transcription factor PPAR γ . PPAR γ transcripts were present in RH7777 and DKO cells, but not in B103 cells [21]. PPAR γ mRNA was also detected in the human prostate cancer cell line PC3, which was included as a positive control for the EDG-family LPA receptors. LPA₄ transcripts were detected in DKO MEFs and B103 cells, but not in RH7777 cells or PC3 cells. Modest amounts of LPA₅ transcripts were detected only in RH7777 cells. That is in agreement with a previous report which originally identified LPA₅ as a LPA receptor [17], in which RH7777 cells but not B103 cells were found to contain LPA₅ mRNA. Although those authors detected LPA₅ transcripts in RH7777 cells, stress fiber formation in response to LPA could not be detected unless LPA₅ was ectopically expressed, suggesting that functionally relevant LPA₅ protein may not be expressed in RH7777 cells despite the presence of its mRNA.

EDG receptor-independent mitogen-activated kinase activation by LPA

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that integrate extracellular signals into diverse cellular responses that include gene expression, migration, mitosis, survival, and apoptosis [27]. LPA has been shown to activate the MAPKs extracellular signal-regulated kinase (ERK) 1/2 and p38 in many cell types. We examined MAPK activation in response to LPA in RH7777 cells and DKO MEFs, by immunoblotting with phospho-specific antibodies that react only with active, phosphorylated ERK1/2 or p38. Treatment with 3 μ M LPA 18:1 stimulated a transient activation of ERK and p38 MAPK in RH7777 cells that was maximal at 5 minutes after treatment and had returned to basal levels by 15 minutes (Fig. 2A). In extracts from DKO MEFs, a similarly transient activation of ERK1/2 and p38 was also observed (Fig. 2B). In RH7777 cells the relative activation of p38 was greater than the more modest ERK activation we observed; in DKO MEFs the relative order of activation was reversed with ERK being more strongly activated than p38 MAPK. RH7777 cells responded to both LPA 18:1 and AGP 18:1 in a dose dependent fashion, with the two LPA species being approximately equal in potency at inducing MAPK activation (Fig. 2C and 2D). This result argues against any involvement of EDG-family LPA receptors in this response, since Bando et al showed that in SF9 cells transfected with LPA₁, LPA₂ or LPA₃, AGP 18:1 was nearly a hundred times less potent in inducing calcium transients than LPA 18:1 [5].

LPA₄ and LPA₅ are capable of activating many cellular signaling pathways, but the involvement of these receptors in MAPK activation is currently unknown. LPA₄ has been reported to mediate calcium transients, Rho-dependent morphological changes, and cAMP accumulation [15,28]; and LPA₅ has been reported to mediate phosphoinositide hydrolysis, cAMP accumulation, calcium mobilization, electrophysiological currents, neurite retraction, and stress fiber formation [16,29]. The potential involvement of LPA₄ (in DKO MEFs) or LPA₅ (in RH7777 cells) in mediating LPA-induced ERK1/2 and p38 MAPK activation warrants further investigation.

RH7777 cells and DKO MEFs respond with cytoskeletal changes to LPA

LPA is known to cause cytoskeletal changes, including neurite retraction and stress fiber formation [30]. We found that 3 μ M LPA 18:1 caused rapid cytoskeletal changes in RH7777 and DKO cells; these changes were characterized by rapid retraction of cellular processes and transient blebbing of the plasma membrane. In contrast, B103 cells, which also lack EDG-family LPA receptors and LPA₅ but express LPA₄, did not undergo any detectable cytoskeletal changes in response to 3 μ M LPA 18:1 (Fig. 3). Cellular blebbing is a widespread phenomenon that occurs in vitro and in vivo, although its function is not well understood. It has been

associated with certain cellular processes including motility, mitosis and differentiation [31–33]. Morphologically, cellular blebbing consists of protrusion followed by retraction of blebs on the plasma membrane; cellular blebbing should be distinguished from blebbing that occurs when cells undergo apoptosis, in which the blebs do not retract back into the cell.

RH7777 cells were incubated with several molecular species of LPA to assess the structure-activity relationship of the blebbing phenomenon. LPA 18:1 and AGP 18:1 induced membrane blebbing in RH7777 cells, but the saturated species tested, LPA 18:0, AGP 18:0, and AGP 16:0, did not (Fig. 4). Hooks et al [34] previously reported that RH7777 have a mitogenic response to LPA, that required micromolar concentrations of LPA and was inconsistent with the pharmacology of LPA_{1–3} but consistent with the structure-activity relationship of the LPA-induced platelet aggregation response [35–37]. That report suggested the LPA-receptor activity in RH7777 cells pharmacologically resembled the mechanism that mediates platelet aggregation in response to LPA. Although the blebbing response in RH7777 cells occurs independently of LPA₁, LPA₂, and LPA₃, and requires micromolar amounts of LPA for a robust response, the structure-activity relationship of blebbing we observed in RH7777 cells is not consistent with the structure-activity relationship reported by Tokumura et al for platelet aggregation [38]. LPA 18:0, AGP 16:0 and AGP 18:0 were ineffective at inducing membrane blebbing in our assays, whereas LPA 18:1 and AGP 18:1 were the most potent of the LPA species we tested. In contrast, Tokumura et al reported AGP 16:0, AGP 18:0, and LPA 16:0 were all more potent at inducing platelet aggregation than LPA 18:1. Tokumura et al posited the possible existence of at least two distinct novel receptors on platelets responsible for aggregation in response to LPA, one with a specificity for acyl-linked LPAs and one specific for alkyl-linked LPAs [38]. AGP appears to be the preferred ligand of LPA₅ (Yanagida, K., Ishii, S. and Shimizu, T., personal communication), and human platelets express abundant LPA₅ transcripts (Tigyi, G. and Siess, W., unpublished data), raising the intriguing possibility that a shared LPA₅ or a LPA₅-like activity contributes to the LPA-responsiveness of both RH7777 cells and platelets.

In order to elucidate the signal transduction pathways involved in cellular blebbing of RH7777 cells in response to LPA, RH7777 cells were pretreated with various signaling inhibitors before treatment with LPA. Pretreatment with the Rac inhibitor NSC23766 or the Rho inhibitor C3 toxin blocked cellular blebbing in response to LPA, but pretreatment with the G_{αi} inhibitor PTX had no effect (Fig. 5). This suggests that the elusive LPA receptor responsible for blebbing activates the Rho and Rac GTPases, like LPA₁ and LPA₂ have been shown to do [23,25,39–42].

Phosphorylation of myosin light chain 2 (MLC2) is associated with LPA-dependent membrane blebbing in RH7777 cells

LPA had previously been shown to mediate cellular blebbing in NB2a rat neuroblastoma cells in a myosin light chain kinase-dependent manner [33]. We examined LPA-dependent phosphorylation of MLC2, a target of phosphorylation by myosin light chain kinase and a protein known to be involved in membrane blebbing [43,44]. In RH7777 cells treated with 3 μM LPA 18:1, phosphorylation of MLC2 was maximal at 5 min and decreased to baseline levels by 15 min (Fig. 6A). Phosphorylation of MLC2 was tested in response to several concentrations of LPA 18:1, and was found to be maximal in response to 3 μM LPA (Fig. 6B). To further examine the role phosphorylated MLC2 played in cellular blebbing in response to LPA, RH7777 cells were examined by immunofluorescence microscopy using an antibody that reacts with phosphorylated MLC2 (Fig. 7). After 5 min treatment with LPA 18:1, a time when cellular blebbing was maximal, the cell membrane of LPA-treated cells, but not vehicle treated cells, showed strong immunoreactivity with an antibody recognizing phosphorylated

MLC2. Regions of the cell membrane undergoing blebbing reacted the most strongly with the antibody.

The intracellular LPA receptor PPAR γ does not mediate p38 and MLC2 activation in RH7777 cells in response to LPA

Our receptor profiling studies indicated that the intracellular LPA receptor PPAR γ is abundantly expressed in RH7777 cells and DKO MEFs, but not B103 cells. This potentially suggested that PPAR γ might be involved in the blebbing response. Since phosphorylation of MLC2 also seemed to be linked with this response and p38 has also been linked to cell blebbing [45,46], we determined the effect of PPAR γ gene knockdown on MLC2 phosphorylation and p38 activation in response to LPA (Fig. 8). When a validated siRNA reagent was applied 72 h before LPA treatment to knock down PPAR γ gene expression, p38 and MLC2 were still as strongly activated by LPA as in cells that received a control siRNA, arguing against a direct role for PPAR γ in the blebbing response.

Discussion

The present studies explored some of the signaling pathways activated by LPA independent of the EDG-family and potentially of the purinergic-like family LPA receptors. We found that in RH7777 and DKO cells, LPA activates MAPK and elicits a transient blebbing response linked to activation of Rac and Rho, and MLC2 phosphorylation. Profiling of the expression pattern of transcripts encoding the five known LPA GPCRs and PPAR γ does not support an unequivocal correlation and assignment of these responses to any of the known LPA targets, since siRNA knockdown of PPAR γ did not affect p38 activation or MLC2 phosphorylation (Table 1).

All three EDG-family LPA GPCRs activate MAPK [47–50]. The transient MAPK activation observed in RH7777 cells and DKO cells in response to LPA temporally and pharmacologically resembles the cellular blebbing response; it seems likely these two processes are coupled, although the exact relationship requires further clarification. The blebbing response we observed was similar to the mitogenic response to LPA in RH7777 described by Hooks et al [34] in that relatively high, micromolar amounts of LPA are required for a robust response, but differs in that the blebbing response we observed, unlike the mitogenic response they described, was not PTX sensitive. Those authors suggested that the G α i/o proteins responsible for the PTX sensitivity of the mitogenic response may not couple directly to a LPA receptor per se, but may be required in a downstream process of the mitogenic response. Thus, the LPA responses we describe in this paper and the one they described may be mediated by a common mechanism.

There are at least two LPA responses that cannot be explained by the EDG family GPCR. The Aoki group [14] reported that in cultured allantois explants, LPA stabilizes developing blood vessels independently of the EDG-family LPA receptors. These authors found trace amounts of LPA₄ and LPA₅ transcripts, making the latter two receptors highly unlikely targets of this LPA action. Although those authors have not evaluated the possible contributions of PPAR γ to this response, this finding in itself hints that additional LPA targets exist that are responsible for the role of LPA in early tissue development. Whether this elusive target is a specific receptor or an enzyme whose function is regulated by extracellularly applied LPA, as has been shown for lysophospholipase D [51], remains to be determined. In the allantois, this target is not lysophospholipase D since LPA rescues the survival of the allantois from knockout embryos. The other unusual LPA response is observed in platelets. Platelets are 50 – 100 times more responsive to AGP compared to LPA [35,38,52]. N-acetyl-serine phosphoric acid, a weak agonist of the EDG-family LPA GPCR [53], effectively inhibits platelet responses to LPA as well as the LPA-activated oscillatory chloride currents in *Xenopus* oocytes [54]. Whether a

receptor with an LPA₅-like structure-activity relationship is responsible for these effects remains to be tested.

In summary, while the discovery of the EDG family over a decade ago and the more recent identification of LPA_{4&5} have contributed greatly to our understanding concerning the physiological role and pathological effects of lysophospholipids, we remain afar from a comprehensive assignment of the cellular responses to these LPA targets. As our understanding of the myriads of biological effects expands we become witnesses to another miracle of nature that has endowed the simplest lysophospholipids with functions seemingly ubiquitous to every mammalian cell.

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Fig. 1. Characterization of LPA receptor expression

RNA was extracted from RH7777, B103, DKO MEFs, and PC3 cells, and reverse transcription-PCR was performed for 31 cycles. The products were separated on agarose gels and visualized with ethidium bromide staining.

Fig. 2. MAPKs are activated in response to LPA in RH7777 cells and DKO MEFs

The kinetics of MAP kinase activation in response to LPA was measured in (A) RH7777 cells and (B) DKO MEFs. Cells were serum-starved for 6 h before treatment with 3 μ M LPA 18:1. Cellular lysates were separated by SDS-PAGE and immunoblotted with antibodies that recognize phospho-ERK, phospho-p38, or β -actin. Immunoreactive bands were measured by densitometry and normalized against β -actin. C & D. Dose response of MAPK activation in RH7777 cells. Cells were serum-starved for 6 h before treatment with different concentrations of LPA 18:1 (panel C) or AGP 18:1 (panel D), and analyzed as above.

Fig. 3. Membrane blebbing occurs in RH7777 cells and DKO MEFs, but not B103 cells, in response to LPA

Cellular membrane blebbing was examined by phase-contrast microscopy in RH7777 cells, DKO MEFs, and B103 cells. Cells were serum starved for 6 h before addition of 3 μ M LPA 18:1. Phase-contrast light microscopy images were captured at 0, 5, and 15 min of LPA treatment.

Fig. 4. The structure-activity relationship of LPA-induced blebbing in RH7777 cells

Cellular blebbing in response to various LPA species was tested in cells deprived of serum for 6 h, then treated for 5 min with 3 μ M LPA 18:0, AGP 18:0, LPA 18:1, AGP 18:1, or AGP 16:0. Phase-contrast light microscopy images were captured at 0 and 5 min of LPA treatment.

Fig. 5. Inhibitors of Rac and Rho, but not G α i, inhibit blebbing in response to LPA in RH7777 cells
 RH7777 cells were pretreated overnight with 100 μ M NSC23766, 10 μ g/ml C3 toxin, or 250 ng/ml PTX before treatment with 3 μ M LPA 18:1. Phase-contrast light microscopy images were captured at 0 and 5 min of LPA treatment.

Fig. 6. MLC2 is phosphorylated in response to LPA in RH7777 cells

The time course (panel A) and dose dependence (panel B) of LPA-stimulated MLC2 phosphorylation was measured in RH7777 cells. Cells were serum-starved for 6 h before treatment with LPA 18:1. Cellular lysates were separated by SDS-PAGE and immunoblotted with phospho-specific antibodies that recognize activated MLC2. Membranes were also probed with anti- β -actin as a loading control.

Vehicle

LPA 18:1

Fig. 7. Phospho-MLC2 localizes with cellular membrane blebs in response to LPA

Phosphorylation of MLC2 in LPA treated RH7777 cells was examined by immunofluorescence microscopy. Cells were grown on coverslips, deprived of serum for 6 h, treated with 3 μ M LPA 18:1 or vehicle for 5 min, and fixed in 10% formalin. Cells were probed with antibodies that recognize phospho-MLC2 (green), and filamentous actin (red) was detected with Texas Red-conjugated phalloidin. Nuclei were stained with DAPI (blue).

Fig. 8. PPAR γ knock-down does not inhibit p38 and MLC2 phosphorylation in response to LPA RH7777 cells were transfected for 72 h with a pool of siRNAs that target PPAR γ or with a control, non-targeting siRNA pool. Cells were serum-starved for 6 h before treatment with 3 μ M LPA 18:1. Cellular lysates were separated by SDS-PAGE and immunoblotted with phospho-specific antibodies that recognize activated p38 or phospho-MLC2. Lysates were also probed with anti-PPAR γ to confirm gene knock-down, and anti- β -actin as a loading control.

Table 1

LPA receptor profiles and LPA responses in RH7777 cells, DKO MEFs, and B103 cells.

Cell line	LPA receptors	MAPK activation	MLC2 phosphorylation	Blebbing
RH7777	LPA ₅ , PPAR γ	p38>ERK	yes	yes
DKO MEF	LPA ₄ , PPAR γ	ERK>p38	not tested	yes
B103	LPA ₄	none	not tested	no