CELL BIOLOGY. For the article “Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARγ agonist,” by Thomas M. McIntyre, Aaron V. Pontsler, Adriana R. Silva, Andy St. Hilaire, Yong Xu, Jerald C. Hinshaw, Guy A. Zimmerman, Kotaro Hama, Junken Aoki, Hiroyuki Arai, and Glenn D. Prestwich, which appeared in number 1, January 7, 2003, of Proc. Natl. Acad. Sci. USA (100, 131–136; First Published December 26, 2002; 10.1073/pnas.0135855100), Fig. 4 should have appeared in color. The correct figure and its legend appear below.

![Figure 4](image-url)  
**Fig. 4.** LPA stimulates lipid accumulation, CD36 expression, and oxidized LDL uptake through a PPAR-responsive element. (a) LPA stimulates monocyte uptake of oxidized LDL. Freshly elutriated human monocytes were allowed to interact with an anti-ICAM3-coated well, which leads to rapid PPARγ expression (13), and then stimulated, or not (negative, oxLDL), with oleyl LPA. Some cells were then briefly exposed to oxidized LDL before intracellular lipid stores were visualized with oil red O stain. (b) LPA increases the expression of CD36 on the surface of primary human monocytes. Monocytes engaging anti-ICAM3 were treated or not with LPA, and then recovered by gentle scraping and washing by centrifugation before their surface CD36 was assessed by flow cytometry. (c) LPA and the LPA analogs XY4 and XY8 stimulate CD36 promoter function only when the PPRE is present. RAW264.7 cells were transfected with the human CD36 promoter containing the PPRE (CD36-273) or a reporter that lacks only this element (CD36-261) and then stimulated with oleyl LPA, azPC, XY4, or XY8. Expression of luciferase normalized to β-galactosidase was determined as above. (d) Anti-CD36 blocks LPA-stimulated accumulation of cholesterol from oxidized LDL. Freshly isolated human monocytes were treated as in a, but after being preincubated with a blocking anti-CD36 antibody before exposure to oxidized LDL.

MEDICAL SCIENCES. For the article “A monoclonal antibody recognizing human cancers with amplification/overexpression of the human epidermal growth factor receptor,” by Achim A. Jungbluth, Elisabeth Stockert, H. J. Su Huang, Vincent P. Collins, Keren Coplan, Kristin Iversen, Denise Kolb, Terrance J. Johns, Andrew M. Scott, William J. Gullick, Gerd Ritter, Leonard Cohen, Matthew J. Scanlan, Webster K. Cavenee, and Lloyd J. Old, which appeared in number 2, January 21, 2003, of Proc. Natl. Acad. Sci. USA (100, 639–644; First Published January 6, 2003; 10.1073/pnas.232686499), the author name Webster K. Cavenee should have appeared as Webster K. Cavenee. The corrected author line appears below. The online version has been corrected.


PHARMACOLOGY. For the article “In vivo activation of a mutant μ-opioid receptor by antagonist: Future direction for opiate pain treatment paradigm that lacks undesirable side effects,” by Ping-Yee Law, Jesse W. Yang, Xiaohong Guo, and Horace H. Loh, which appears in number 4, February 18, 2003, of Proc. Natl. Acad. Sci. USA (100, 2117–2121; First Published January 13, 2003; 10.1073/pnas.0334906100), the author name Webster K. Cavenee should have appeared as Webster K. Cavane. The corrected author line appears below.

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Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARγ agonist


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Lysophosphatidic acid (LPA) is a pluripotent lipid mediator acting through plasma membrane-associated LPA receptors that transduce many, but not all, of its effects. We identify peroxisome proliferator-activated receptor γ (PPARγ) as an intracellular receptor for LPA. The transcription factor PPARγ is activated by several lipid ligands, but agonists derived from physiologic signaling pathways are unknown. We show that LPA, but not its precursor phosphatidic acid, displaces the drug rosiglitazone from the ligand-binding pocket of PPARγ. LPA and novel LPA analogs we made stimulate expression of a PPAR-responsive element reporter and the endogenous PPARγ-controlled gene CD36, and induced monocyte lipid accumulation from oxidized low-density lipoprotein via the CD36 scavenger receptor. The synthetic LPA analogs were effective PPARγ agonists, but were poor ones for LPA1, LPA2, or LPA receptor transfected cells. Transfection studies in yeast, which lack nuclear hormone and LPA1 receptors, show that LPA directly activates PPARγ. A major growth factor of serum is LPA generated by thrombin-activated platelets, and media from activated platelets stimulated PPARγ function in transfected RAW264.7 macrophages. This function was suppressed by ectopic LPA-acyltransferase expression. LPA is a physiologic PPARγ ligand, placing PPARγ in a signaling pathway, and PPARγ is the first intracellular receptor identified for LPA. Moreover, LPA produced by stimulated platelet platelets activates PPARγ in nucleated cells.

Lysophosphatidic acid (LPA) is a pluripotent lipid mediator controlling growth, motility, and differentiation (1, 2). It is the ovarian cancer-activating factor that is elevated in the serum of ovarian cancer patients (3), and it controls adipogenesis (4). LPA also is generated during platelet activation (5) to become a major growth factor of serum. LPA stimulates three G protein-linked, plasma membrane-associated receptors [LPA1, LPA2, and LPA3, formerly edg2, edg4, and edg7 (6)] that recognize extracellular LPA (7). However, control of complex processes including growth and differentiation is difficult to reconcile with these receptors (8), suggesting that undiscovered receptors for LPA may exist. LPA is a central component of cellular phospholipid metabolism and, because a role for intracellular LPA beyond this is unknown, the plasma membrane separates signaling LPA from metabolic LPA.

The transcription factor peroxisome proliferator-activated receptor γ (PPARγ) regulates genes that in general control energy metabolism (9). PPARγ, like other members of its extended nuclear hormone receptor superfamily, is activated by binding an appropriate lipid ligand (10). Synthetic compounds, including the widely prescribed drug rosiglitazone, target PPARγ and activate transcription with high affinity. Anionic fatty acids and their oxidized derivatives also bind and activate PPARγ (11), but they do so through low-affinity interactions (12). Although a number of biologic ligands for PPARγ are known, these ligands do not provide signal amplification because the products are about as potent as their precursors.

PPARγ agonists arising from physiologic signaling pathways remain elusive, but a pathologic agonist is known. Uncontrolled free radical oxidation of low-density lipoprotein (LDL) fragments its phospholipids, and one of these phospholipid oxidation products, hexadecyl azelaic phosphatidylcholine (azPC), binds and activates PPARγ as potently as the drug rosiglitazone (13). azPC differs from its inactive phospholipid precursor in that it has a shortened sn-2 residue that is also anionic. Oxidatively fragmented phospholipids are found in atherosclerotic lesions (14) and in the circulation after exposure to primary (15) or second-hand (16) cigarette smoke, but are unlikely to constitute PPARγ ligands under normal conditions. Knowing that an anionic phospholipid was a PPARγ agonist, we examined other anionic phospholipids integrated in signaling pathways for this property.

Materials and Methods

Binding Analysis. [3H]azPC and [3H]rosiglitazone were synthesized and then incubated with immobilized Hist12-PPARγ in the presence or absence of a 20-fold molar excess (6.7 μM) of the stated phospholipids (Avanti Polar Lipids, Alabaster, AL) or oxidized fatty acids (Cayman Chemical, Ann Arbor, MI) as described (13).

Transfection Assays. RAW264.7 monocytic cells (American Type Culture Collection) were transiently transfected (13) for 4 h with 0.1 μg of a simian virus 40 (SV40)-β-galactosidase reporter and 1 μg of an acyl-CoA oxidase-luciferase, a CD36-273-luciferase reporter with its peroxisomal proliferator-responsive element (PPRE), or a CD36-261 reporter that lacks just this element before stimulation overnight with 5 μM agonist. Some experiments included 0.25 μg of empty pcDNA1, pcDNA1 expression plasmids encoding LPA acyltransferase (17), or PPARγ (13). Saccharomyces cerevisiae was transfected with lithium acetate and rat acyl-CoA oxidase PPRE-β-galactosidase reporter, PPARγ, and retinoid X receptor (RXR) before selection in nutrient-deficient media (18). Overnight cultures were supplemented with the stated lipid for 16 h before harvesting and analyzing β-galactosidase levels with o-nitrophenyl β-D-galactopyranoside.

LPAγ Receptor Activation. LPA receptor activation was evaluated by examining Ca2+ flux in fura-2-loaded S99 cells expressing

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Abbreviations: LPA, lysophosphatidic acid; PPARγ, peroxisome proliferator-activated receptor γ; PPRE, peroxisomal proliferator-responsive element; RXR, retinoid X receptor; LDL, low-density lipoprotein; azPC, hexadecyl azelaic phosphatidylcholine; 3V40, simian virus 40.

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LPA1, LPA2, or LPA3 by excitation at 340 and 380 nm with emission monitored at 500 nm as described (19).

Chemical Synthesis. Hexadecyl lysophosphatidylcholine was prepared as described (20). Translocase-3 (Tris[2-(2-naphthalene-sulfonamido)ethyl]amine) was prepared as described (21). Total synthesis of enantiomerically pure XY4 [1,1-difluorodeoxy-(2R)-palmitoloy-sn-glycero-3-phosphate] was achieved in nine steps, with 21% overall yield, from protected d-mannitol. XY4 is an analog of sn-2-palmitoyl LPA where the sn-1-hydroxyl group is replaced by two electron-negative fluorine atoms to prevent acyl migration. Its synthesis (22) involves difluorination of an aldehyde intermediate, selective deprotection of a glyceryl methylysil bromide, after neutralization the free phosphoric acid was obtained and characterized by 13C-NMR and by high-resolution mass spectrometry. XY8 is an analog of sn-1-palmitoyl-LPA. Synthesis of enantiomerically pure XY8 [1-palmitoyl-(2R)-fluorodeoxy-sn-glycero-3-phosphate] involves selective protection of the primary alcohol and deoxyfluorination of the secondary alcohol; after deprotection as with XY4, an ion exchange step afforded the sodium salt after seven steps in 18% overall yield from (R)-isopropyldieneglycerol (Y.X., L. Qian, and G.D.P., unpublished work).

Monocyte Manipulation. Monocytes were elutriated from human blood (23). For oxidized LDL accumulation, the cells were immobilized on anti-ICAM3 (BBA29, R & D Systems) on plastic chamber slides for 2 h as before (24) to induce PPARγ, and then stimulated, or not, with 5 μM oleoyl LPA for 16 h. After this, the cells were exposed to 50 μg/ml oxidized LDL (25) for 30 min before fixation and staining with oil red O. Total cellular cholesterol was determined with a Sigma60 cholesterol oxidase kit (Sigma), whereas protein content was determined with Coomassie blue (Pierce). Flow cytometry of surface CD36 emission monitored at 500 nm as described (19).

Platelet Stimulation. Platelet-rich plasma was isolated from the blood of healthy volunteers by centrifugation for 20 min at 200 × g, and 3 × 10⁸ platelets per ml were then stimulated with 0.1 units/ml thrombin. Prostaglandin E₁ (0.1 μM) was added to control platelets to avoid activation. After this, platelets were removed by centrifugation at room temperature for 10 min at 500 × g, and the supernatant was tested on transfected RAW264.7 cells at a dilution of either 1:10 or 1:100.

Results

LPA Binds and Activates PPARγ. We used competition binding to test for PPARγ ligands. We immobilized recombinant human PPARγ on Ni⁺ chelate beads and allowed [3H]rosiglitazone to bind [crystallography shows it immobilized in the ligand binding pocket (26, 27)] in the absence or presence of a 20-fold molar excess of various unlabeled lipids. Unlabeled rosiglitazone and azPC, a synthetic anionic oxidized phospholipid that is a PPARγ ligand (13), displaced [3H]rosiglitazone from PPARγ (Fig. 1a). We found that the anionic phospholipid LPA also competed with [3H]rosiglitazone for binding to PPARγ, but that neither sphingosine 1-phosphate nor the water-soluble phospholipid platelet-activating factor did so. We obtained similar results when we tested for ligand competition using [3H]azPC. LPA effectively displaced [3H]azPC from immobilized PPARγ (Fig. 1b), with a preference for the more soluble oleoyl homolog. The diacyl phospholipid phosphatidic acid did not compete for [3H]rosiglitazone binding, nor did the zwitterionic lipids lysoPAF (hexadecyl phosphatidylcholine) or sphingosine 1-phosphate. Neither 9-nor 13-hydroxyoctadecadienoic acid was an effective competitor, at just a 20-fold excess, as anticipated from their affinity relative to rosiglitazone.

We determined whether the binding of 1-oleoyl LPA to PPARγ initiated transcription from a PPRE. We transiently transfected the RAW264.7 macrophage-like cell line with a luciferase reporter gene controlled by the canonical acyl-CoA oxidase PPRE to find (Fig. 1c) that LPA stimulated this reporter. The effect of LPA depended on its concentration and was maximally effective by 2 μM, a concentration well below that found in serum (28). There was a slight preference for the sn-1 ether over the ester homolog, but both LPAs were agonists. The PPAR subfamily contains PPARα and PPARβ/δ that also act on PPREs, but only cotransfection with PPARγ enhanced the effect of LPA or rosiglitazone (data not shown).
Support for an intracellular site of action for LPA was provided by a converse approach in which we suppressed intracellular levels of LPA through the overexpression of LPA acyltransferase (17). This metabolic enzyme enhances the flux of LPA to phosphatidic acid, a lipid that was not a PPARγ ligand (vide supra), by catalyzing the condensation of LPA with intracellular acyl-CoA. LPA acyltransferase expression completely suppressed LPA, but not rosiglitazone or azPC, stimulation of the acyl-CoA oxidase PPRE reporter (Fig. 2c).

**Synthetic LPA Analogs Activate PPRE Function.** The tumor promoter ovarian cancer-activating factor is sn-2 LPA (29) rather than the more common sn-1 LPA isomer we used in the experiments above. sn-2 LPA activates LPA2 and LPA1 receptors (19), but studies of positional specificity are complicated by a rapid chemical equilibrium favoring the sn-1 isomer by almost 6-fold. To circumvent this intramolecular rearrangement, we synthesized sn-1 and sn-2 LPA analogs where one hydroxyl group was conservatively changed to a fluoro moiety to give LPA analogs XY4 and XY8 (Fig. 2a) that cannot undergo acyl migration or further acylation. We found (Fig. 2b and c) that the fluoroacyl LPA analogs induced luciferase expression from the acyl-CoA oxidase PPRE reporter. We established that the effective concentrations of XY4, XY8, and LPA were equivalent (data not shown), so substitution of fluorine for the hydroxyl of LPA was an effective strategy to create stabilized LPA mimetics. There was no positional specificity for the acyl chain because both the sn-1-like (XY8) and sn-2-like (XY4) analogs were equally effective PPARγ agonists. We also found oleoyl analogs of XY4 and XY8 were effective PPARγ agonists (data not shown), so there was no specificity for the acyl residue. The Tris-sulfonamid translocase enhanced the effect of XY4 and XY8 in stimulating the PPRE reporter (Fig. 2b), as anticipated from the free phosphoryl group of XY4 and XY8. XY4 and XY8 cannot be acylated, so intracellular expression of LPA acyltransferase did not change the response to either of these two stable LPA analogs (Fig. 2c).

**LPA Is a Direct, Rather Than Indirect, PPARγ Agonist.** We sought a way to show that LPA directly acts on PPARγ, rather than stimulating the formation of an unknown endogenous ligand through an LPA1 receptor. We also sought a way to circumvent the possibility that other nuclear hormone receptors, which might physically interact with PPARγ, might bind LPA and then indirectly stimulate PPARγ function. We accomplished these goals in two ways; the first was through the use of S. cerevisiae, an organism that lacks all nuclear hormone receptors and contains only a single G protein-linked receptor for mating factor. Yeast will accumulate intact anionic phospholipids from their media (30) and can be stably transfected with a PPARγ reporting system (18). We found (Fig. 3a) that yeast engineered to contain PPARγ, RXR, and the β-galactosidase PPRE reporter expressed the reporter in response to the PPARγ agonists rosiglitazone and azPC, and they expressed the reporter in response to LPA. Strains transfected with just the PPRE reporter, or the reporter and just PPARγ or just RXR, showed no basal reporter expression. The three strains lacking the PPARγ/RXR heterodimer also did not respond to rosiglitazone, azPC, or LPA, confirming the lack of confounding receptors in S. cerevisiae.

We next tested whether G protein-linked receptors for LPA could be excluded from a role in PPARγ activation. We stably transfected S9 insect cells, derived from an organism that lacks all G protein-linked LPA receptors, with messages for LPA1, LPA2, or LPA3. The transfected cells responded to LPA with a transient intracellular Ca2+ flux as anticipated (Fig. 3b). In contrast, the migration-stabilized LPA analogs XY4 and XY8 were not effective agonists for these receptors and were two to
four orders of magnitude less active than oleoyl LPA. Because XY4 and XY8 were effective PPARγ agonists, these LPA analogs did not act through LPAx receptors.

**LPA Induces CD36 Expression and Foam Cell Formation.** LPA accumulates in atherosclerotic lesions (31), a location enriched with PPARγ (32), and PPARγ is required for regulated CD36 expression (33, 34). Monocyte accumulation of oxidized LDL by CD36 (35, 36) leads to intracellular lipid droplet accumulation and foam cell formation that are characteristic of these lesions. We found that human monocytes stimulated with LPA and then exposed to oxidized LDL as a cholesterol source rapidly accumulated excessive intracellular neutral lipid stores that stained with oil red O (Fig. 4a). Human monocytes express a low level of CD36 on their surface, which increases after exposure to troglitazone (33, 34) or azPC (13). We found (Fig. 4b) that LPA also stimulated the expression of CD36 on the surface of human monocytes. The CD36 promoter contains a functional PPRE (13, 35, 37); and a reporter construct containing this PPRE, but not a truncated version lacking just this element, responded to LPA stimulation (Fig. 4c). The LPA analogs XY4 and XY8 also induced expression of the luciferase reporter controlled by the CD36 promoter when it contained the PPRE, but failed to do so when the construct lacked this element (Fig. 4c). Chemical quantitation of cellular cholesterol levels showed that LPA or rosiglitazone treatment doubled cellular cholesterol content (Fig. 4d), and that a blocking anti-CD36 antibody prevented this increase.

**LPA Is a Transcellular Lipid Mediator.** Aggregating platelets generate LPA through the release of lipases that act on plasma lipids, creating the micromolar levels of LPA in serum (28). We stimulated human platelets in platelet-rich plasma with thrombin, or not, and then collected cell-free supernatants by centrifugation. RAW264.7 monocytic cells previously transfected with the PPRE-containing CD36 reporter responded to these platelet supernatants by expressing the luciferase reporter just as they did when stimulated with synthetic LPA or rosiglitazone (Fig. 5a). None of these agents stimulated reporter expression when the CD36 regulatory region lacked the PPRE. The supernatants collected from activated platelets also stimulated reporter expression from the acyl-CoA oxidase reporter (Fig. 5b), and the formation of this PPARγ agonist from activated platelets was rapid; the PPARγ agonist ap-
with the supernatant from unactivated or thrombin-activated platelets as in a. Accumulation of a PPARγ effective PPARγ agonist(s) in platelet-rich plasma is time-dependent. Human platelet-rich plasma was treated with activated thrombin for the stated times before addition, at a 100-fold dilution, to RAW264.7 cells transfected with acyl-CoA oxidase PPRE luciferase and SV40-β-galactosidase reporters. (c) LPA acyltransferase expression suppresses the response of a PPRE reporter to the supernatants of thrombin-activated platelets. RAW264.7 cells were transfected with acyl-CoA oxidase PPRE and SV40-β-galactosidase reporters and, for some cells, with an LPA acyltransferase (LPAAT) expression construct. These cells were treated with the supernatant from unactivated or thrombin-activated platelets as in a.

**Discussion**

PPARγ is not an orphan nuclear hormone receptor, but neither is it currently integrated in a pathway displaying signal amplification. The primary mode of PPARγ activation is the conformational change induced by a lipid ligand, and many lipids that bind and stimulate PPARγ function are known, but all are low-affinity ligands that are unlikely to be bona fide ligands (10). Thus various arachidonate and linoleate oxygenated metabolites bind and activate PPARγ, but because their Kd values are in the same low micromolar range (38) as their unmodified parent compounds there is little signal amplification achieved by their modification (12). Cellular metabolism of LPA (to free fatty acids, for instance) was not responsible for the PPARγ activation we observed, because a phospholipase A1-insensitive sn-1 ether homolog of LPA was an effective PPARγ agonist, as were LPA analogs unable to undergo acyl migration or acylation to phosphatidic acid.

LPA is formed and released from cells in response to physiologic signals, and, because its precursor phosphatidic acid was not a PPARγ ligand, provides signal amplification for PPARγ activation just as it does for LPAα receptors. We found PPARγ, like the LPA receptors LPA1 and LPA3 (19), was activated by either sn-1 or sn-2 LPA homologs but also by alkyl LPA with an sn-1 ether bond. PPARγ also did not display specificity for an unsaturated acyl residue, so it appears that an anionic phosphoryl group is a primary determinant for PPARγ activation by LPA. PPARγ, therefore, responds to a wider range of LPA homologs than any single LPAα receptor (19).

Our experiments show extracellular LPA has access to nuclear PPARγ, so externally generated LPA can affect PPARγ-controlled genes in surrounding cells. LPA is produced extracellularly by lipoprotein oxidation (31) or by the action of secretory phospholipases A2 on microvesicles released from activated cells (39). LPA also is produced in plasma by thrombin-activated platelets (3) through the stimulated release of phospholipase A1 (5, 40), phospholipase A2 (5), and lysophospholipase D (autotaxin) (41, 42), which act on plasma lipids or secreted lysophosphatidylcholine (42). Whereas plasma contains undetectable amounts of LPA, its concentration in serum is several micromolar (28). Serum levels of LPA increase over gestation (43) and in ovarian tumorigenesis (44), although this also may reflect platelet activation and attendant LPA production (45). Extracellular LPA also derives from stimulated ovarian cancer cells (29) and potentially other cancer cells (42). LPA, therefore, can couple activated tumor cells or platelets (28) to PPARγ stimulation and gene regulation in neighboring as well as distal target cells.

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