Modulation of Pro-inflammatory Gene Expression by Nuclear Lysophosphatidic Acid Receptor Type-1*

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Lysophosphatidic acid (LPA) is a bioactive molecule involved in inflammation, immunity, wound healing, and neoplasia. Its pleiotropic actions arise presumably by interaction with their cell surface G protein-coupled receptors. Herein, the presence of the specific nuclear lysosphosphatidic acid receptor-1 (LPA1R) was revealed in unstimulated porcine cerebral microvascular endothelial cells (pCMVECs), LPA1R stably transfected HTC4 rat hepatoma cells, and rat liver tissue using complementary approaches, including radioligand binding experiments, electron- and cryomicroscopy, cell fractionation, and immunoblotting with three distinct antibodies. Immunoprecipitation studies in enriched plasmalemmal and immunoblotting with three distinct antibodies. Immuno precipitation studies in enriched plasmalemmal frations of unstimulated pCMVEC showed that LPA1Rs are dually sequestrated in caveolin-1 and clathrin subcompartments, whereas in nuclear fractions LPA1R appeared primarily in cavea. Immunofluorescent assays using a cell-free isolated nuclear system confirmed LPA1R and caveolin-1 co-localization. In pCMVEC, LPA-stimulated increases in cyclooxygenase-2 and inducible nitric oxide synthase RNA and protein expression were insensitive to caveolea-disrupting agents but sensitive to LPA-generating phospholipase A2, enzyme and tyrosine kinase inhibitors. Moreover, LPA-induced increases in Ca2+ transients and/or iNOS expression in highly purified rat liver nuclei were prevented by pertussis toxin, phosphoinositide 3-kinase/Akt inhibitor wortmannin and Ca2+ chelator and channel blockers EGTA and SKF96365, respectively. This study describes for the first time the nucleus as a potential organelle for LPA intracrine signaling in the regulation of pro-inflammatory gene expression.

In the mammalian system, LPA1 signaling cascades regulate important cellular processes, including gene expression, cell proliferation and growth, cell survival and death, and cell motility and secretion (1–3). These plethora of activities are characteristic features of inflammation that occur in various physiological as well as pathological states (e.g. ontogenic change, wound healing, cancer, etc.) (1–3). In humans, physiological responses induced by LPA arise from specific interactions with at least three genetically identified receptors designated LPA1, LPA2, and LPA3 (formerly referred to as EDG2, EDG4, and EDG7 receptors, respectively), which belong to the heptahelical transmembrane-spanning G protein-coupled receptor (GPCR) superfamy (4). These receptors show a broad, virtually distinct distribution and may couple in a cell-dependent manner to numerous heterotrimeric G proteins. In this context, LPA1 and LPA2 receptors have been shown to interact with G13/G12, G13 and G12/13 proteins, whereas the LPA3 receptor combines with G13 and G13/14 proteins (5). Although many responses induced by extracellular LPA can result from its interaction with plasma membrane GPCRs, there is circumstantial evidence for an intracellular mode of action of LPA. For instance, putative biogenesis (e.g. secretory and cytosolic calcium-dependent and -independent phospholipase A2, phospholipase D, and monoacylglycerol kinase) and degradation (e.g. phosphohydrolase and lysophospholipase) pathways for LPA have been detected at the nuclear membrane and/or within the nucleus of targeted cells (6–11). Further support for the functionality of constitutive intracellular LPA receptors, specifically at the cell nucleus, is revealed by adjacent localization of required signaling effectors, which couple to the receptors. These accessory proteins include, among others, G-proteins, ion channels, phospholipases A2, C, D, adenylyl cyclase, MAPKs, and NF-κB

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1 The abbreviations used are: LPA, lysophosphatidic acid; GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; MEK, MAPK/Erk kinase; PNgase F, peptide N-glycosidase F; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; PTH, parathyroid hormone; R, receptor; RTK, receptor tyrosine kinase; PDVF, polyvinylidene difluoride; COX-2, cyclooxygenase-2; INOS, inducible nitric oxide synthase; PTX, pertussis toxin; PAF, platelet-activating factor; IL-1β, interleukin-1β; RT, room temperature; RT-PCR, reverse transcription-PCR; BSA, bovine serum albumin; PBS, phosphate-buffered saline; pCMVEC, porcine cerebral microvascular endothelial cell; PM, plasma membrane; WN, whole nuclei; NE, nuclear envelope; BSAaq, bovine serum albumin fatty acid-free; cLPA, oleoyl-lysophosphatidic acid; sLPA, stearoyl-lysophosphatidic acid; pLPA, palmitoyl-lysophosphatidic acid.
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(see Ref. 12 for review). Alternatively, LPA may exert intracellular actions by generating its own formation, inferring possible active intracellular binding sites. Along this line of thought, LPA signaling at discrete subcellular domains may be provided in part by intracellular conveyors such as gelsolin and the fatty acid-binding protein (8), by uptake of extracellular LPA bound to albumin (via albindin receptor; gp60) (13), and/or to lipocalins (14).

Lastly, several established pathways of GPCR regulation and desensitization driven by extracellular agonists have also been implicated in their intracellular relocation, which results in delayed complementary signaling cascades (15, 16). In this context, accumulating evidence suggests that nuclear translocation of peptide growth factors (e.g. angiogenin, PDGF, basic FGF, EGF, and PTH) and/or their integral membrane receptors, is mandatory for gene transcription associated with proliferating/growth events (17). Because most of these latter receptors (e.g. EGF, PDGF, and PTH receptors) co-localize within caveolae (18), we speculated that translocation to this specific organelle proceeds via receptor endocytosis through the caveolar compartment. Whether this phenomenon holds true for the transmission of LPA gene responses (1, 2) is not yet known.

The biochemical mechanisms by which GPCRs, including LPA-Rs, modulate gene transcription are complex and not fully understood (19). Some mechanisms recently uncovered to explain GPCR-mediated gene induction implicate an endocytosis-associated β-arrestin-c-Src interaction leading to downstream activation of Ras and mitogen-activated protein kinases (MAPKs) through possibly metallloprotease-dependent transactivation of receptor tyrosine kinases (RTK) involving de novo release of their ligand (19). Herein, we postulated that endogenous LPA stimulates gene expression, specifically the proinflammatory genes cyclooxygenase-2 (COX-2) and inducible nitric-oxide synthase (iNOS), through a formerly undescribed mechanism, which involves the activation of nuclear LPA receptors that pre-exist at the nuclear envelope or originate from the internalization/endocytosis of plasmalemmal LPA receptors. In the present study, we focused on endothelial cells, which are known to express predominantly LPA_R (20) and consolidated our findings on LPA_R stably transfected HTC4 rat hepatoma cells (21) and rat liver tissue specimens. Our in vitro and in vivo findings support the existence of constitutive LPA_R at the cell nucleus, which upon stimulation mediates calcium transients and transcriptional signals of immediate-early response genes. Our results also suggest that (i) LPA-induced PLA_2-dependent COX-2 expression is not reliant on prostaglandin, leukotriene, or epoxide production and (ii) contrary to common peptide growth hormone receptors, the sequestration and transcellular transport of LPA-R via caveolae to the nucleus is not a prerequisite for LPA-R activity on gene expression. This study unravels an as yet undescribed mechanism by which LPA modulates gene expression.

EXPERIMENTAL PROCEDURES

Chemical Reagents and Antibodies—Materials and chemicals were obtained from the following sources: oleoyl, stearoyl, palmitoyl-lysophosphatidic acids (LPA); dioleoyl-phosphatidic acid, oleoyl-lysophosphatidylcholine, oleoyl-lysophosphatidylethanolamine (Avanti Polar Lipids Inc., Birmingham, AL); (C16)-PAF (Cayman); 3-aminopropyltriethoxysilane, Nonidet P-40, filipin, thapsigargin, methyl-β-cyclodextrin, thapsig; EGTA, pertussis toxin (PTX), forsk-2, wortmannin, ionomycin, MRS86, cystidine-5-diphosphocholine, mesapicine, tyrophostin AG 1478, PD 98059, and tunicamycin (Calbiochem); SK&F96385, CV 3988, and methylcarbamyl platelet-activating factor (C-PAF) (BIOMOL); ketoconazole (ICN Biociences Inc.); fluo-4-AM (Molecular Probes); FNGase F assay kit (New England BioLabs); brain microvesSEL endothelial growth media (BioWhittaker); Dulbecco's modified Eagle's medium (Invitrogen); [3H]HOLPA (PerkinElmer Life Sciences); RNA guard RNase inhibitor (Amersham Biosciences); and recombinant human interleukin-1β (IL-1β) (BIOSOURCE International). All other chemicals were analytical reagents and were purchased from Fisher Scientific (Montréal, Quebec, Canada).

Antibodies and their sources are: Anti-murine iNOS monoclonal antibody, anti-CD51 monoclonal antibody, and anti-human caveolin-1 monoclonal antibody (Transduction Laboratory); anti-human COX-2 polyclonal antibody (Cayman); anti-human β-actin polyclonal antibody; anti-human Von Willebrand factor polyclonal antibody (Dako, Denmark); anti-phospho-MAPK (Erk1/2) polyclonal antibody (Promega); anti-MAPK (Erk1/2) polyclonal antibody (Upstate Biotechnology); anti-phospho-Akt (Ser^473) and anti-Akt polyclonal antibodies (New England BioLabs); anti-alkaline phosphatase polyclonal antibody (Abcam); anti-cytochrome c monoclonal antibody (BD PharMingen); horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (Pierce); rabbit polyclonal anti-human LPA_R-C antibody (raised against a C-terminal epitope consisting of amino acids 328–344) and its cognate peptide antigen (Upstate Biotechnology); and rabbit polyclonal and mouse monoclonal anti-human LPA_R-N receptor antibodies (N-terminal epitope consisting of amino acids 6–25; Dr. E. J. Gotel, University of California, CA). The specificity of anti-LPA_R antibodies has been fully established elsewhere (22–25). Animals—Experiments were performed on endothelial cells derived from Yorkshire piglet brain microvasculature (Fermes Ménard, Quebec, Canada) and hepatocytes from adult Sprague-Dawley male rats (Charles River, Quebec, Canada). Animal housing and experimental protocols were carried out in accordance with regulations set by the Canadian Council of Animal Care Committee and were approved by the Sainte-Justine Hospital Animal Care Committee.

Cell Culture and Fractionation—Primary endothelial cells obtained from porcine cerebral microvessels (26) and stably transfected, Geneti- resistant LPA_R-HTC4 rat hepatoma cells (21) were cultured and passaged, as previously reported. Cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin G, 1% streptomycin and used between passages 6–12 at 90% confluence unless otherwise stated. Primary cells were serum-starved overnight. Isolation of nuclei was achieved by cell fractionation using the hypotonic/Nonidet P-40 lysis method (27). Nuclear envelopes were prepared by incubating nuclear suspensions (8 × 10^6 nuclei/ml) with DNase I (800 units, pancreas type II, Roche Applied Science) and RNase A (32 mg/ml, Promega) for 30 min at 37 °C (28). Supernatants of homogenized cells (in lysis buffer) were sequentially centrifuged at 10,000 × g for 15 min then 120,000 × g for 60 min to obtain mitochondrial and plasma membrane fractions, respectively. Plasma and nuclear membranes and intact nuclei were stored at −80 °C unless otherwise stated. Protein concentration was determined by Bradford protein assay using BSA as standard. The morphological integrity and purity (>90%) were routinely assessed by light microscopy after trypan blue staining and confirmed by electron microscopy (27) (Fig. 1D, inset). The plasma membrane marker 5′-nucleotidase activity (Sigma assay kit) in nuclear versus plasma membrane fraction was less than 7%.

Isolation of Subcellular Fractions of Rat Liver—Hepatocytes were harvested following digestion of liver with collagenase (type II, 0.05%), Sigma, as described (29). Isolation of liver nuclei was carried out by ultracentrifugation through a sucrose gradient according to Nicotera et al. (30). Plasma membrane microsomes and nuclear envelopes were prepared, and morphological integrity and purity were ascertained as indicated above; 5′-nucleotidase activity endowed in the isolated nuclear fraction was less than 2% of that of plasma membrane fraction.

Purity of subcellular fractions was further substantiated by means of immunological methods using the specific organelle marker antigens alkaline phosphatase and CD51 (plasma membrane), lamin A/C (nuclei), and cytochrome c (mitochondria, cytosol), as depicted below in Fig. IA. Fig. 1B exhibits electron micrographs of highly purified nuclei and envelope fractions (right panel), appearing as intact spheres, following this isolation procedure.

Radioligand Binding Assays—Cells were seeded into 24-well plates (500 µl of media/well) and allowed to reach 90% confluency (~70,000 cells/well) before beginning experiments. Quiescent cells were washed three times with PBS containing BSA (0.1%), soybean trypsin inhibitor (1 µg/ml), and phenylmethylsulfonyl fluoride (10 µM), then incubated in the same buffer at 4 °C for 90 min with 10 nM radioactive tracer [3H]HOLPA (50 Ci/mmol). Thereafter, cells were rinsed twice with cold incubation buffer (1 ml), lysed with sodium hydroxide (0.1 N), and transferred into scintillation vials. Nonspecific binding was determined
in the presence of unlabeled LPA (10 mM). Radioactivity of samples was measured with a beta-counter. In parallel, cells from untreated

Subcellular Fractions—Binding assays were conducted on either whole nuclei or nuclear envelopes originating from porcine EC and rat

Western Blot Analysis of LPA 1R—Equal amounts (25 µg) of plasma membrane (PM), whole nuclei (WN), and derived nuclear envelope (NE) protein from pCMVEC, HTC4, and hepatocyte cells were solubilized in Laemmli buffer, separated by 12% SDS-PAGE, and electroblotted to PVDF membranes. Membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline-Tween 20 (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) (TBST) and subsequently incubated overnight at 4 °C either with a monoclonal anti-LPA-R-N (1:100), a polyclonal anti-LPA-R-N (1:2000), or a polyclonal anti-LPA-R-C (1:2000). Thereafter, membranes were washed with TBST and incubated for 1 h at RT with secondary antibodies (monoclonal, 1:250; polyclonal, 1:5000) conjugated to horseradish peroxidase. Finally, membranes were

Electron and Cryomicroscopic Immunohistochemistry of LPA 1R—Male Sprague-Dawley rats (250–300 g) were used. Rat liver tissue sectioning and pre-embedding immunogold staining was done as described in detail previously (33). Vibriome sections (50 µm) of liver were incubated with the primary antibody (a rabbit anti-LPA-R-C antibody) (1:50) overnight at 4 °C followed by another overnight incubation with goat anti-rabbit gold (10 nm)-conjugated IgG (1:50) (British Biocell International). In control sections, the primary antibody was incubated with goat anti-rabbit AlexaFluor 488-conjugated IgG (2 µg/ml) and a chicken anti-mouse AlexaFluor 647-conjugated IgG (10 µg/ml) as indicated above. The nuclear suspension was then placed on poly-l-ornithine-treated glass coverslips (25 mm) and examined with a laser-scanning confocal microscope. Samples were imaged using a 60× differential interference contrast oil immersion objective lens on a Nikon TE300 microscope with a Bio-Rad Radiance 2000 confocal accessory. The two images were collected by using the same Z values and were merged using a Silhouette graphic (SGI) software. Nuclear staining was realized at the end of the experiment using the DNA dye Syto-11 (100 mM) (Molecular Probes). For negative controls the primary antibodies were omitted.

Immunoprecipitation of LPA-R and Western Blotting—Cellular and nuclear extracts (500 µg) of pCMVEC were lysed, immunoprecipitated with antibody anti-LPA-R-N polyclonal antibody (4 µg), and separated by SDS-PAGE (12%) and transferred onto nitrocellulose membrane. Immunoblotting was performed with either an anti-LPA-R-N polyclonal antibody (serving as positive control) (1:2000), anti-clathrin (1:1000), or caveolin-1 (1:1000) monoclonal antibody. Cellular and nuclear extracts of EC were subjected to extraction in Triton X-100 followed by sucrose density gradient centrifugation to isolate caveolae (35). Caveole-enriched fractions (low density and Triton X-100-insoluble materials) were verified by SDS-PAGE and probed for caveolin-1. Equal amounts of proteins from caveolae and non-caveolar membranes were then immunoblotted for LPA-R as indicated above.

Western Blot of COX-2 and iNOS Proteins—pCMVEC were seeded into 6-well dishes, rendered confluent by scraping, and treated for 6 h with either the vehicle (bisdiluted and deionized water), sLPA (1–10 µM) or human IL-1β (10 ng/ml). Cells were then washed twice with cold PBS (5 ml) and harvested in the same buffer by gentle scraping. Sedimented cells were then lysed by brief sonification in buffer consisting of Tris-HCl (10 mM), pH 7.4, EDTA (5 mM), and complete protease mixture inhibitor and then centrifuged at 200 × g for 10 min at 4 °C. The supernatant was collected and submitted to an ultracentrifugation (100,000 × g for 60 min at 4 °C). Resulting crude membrane and cytosolic extracts were concentrated (Centricon Plus 20, Millipore) and analyzed for protein content. Denatured membrane proteins (50 µg) were resolved by SDS-PAGE on 7.5% gel and transferred onto PVDF membrane (Bio-Rad), which was incubated with either an anti-human Von Willebrand factor polyclonal antibody (1:2000), an anti-COX-2 polyclonal antibody (1:1000), or an anti-β-actin polyclonal antibody (1:500). Denatured cytosolic proteins (30 µg) were processed in a similar way and immunoblotted using a mouse anti-iNOS monoclonal antibody (1:200). Relative quantities of proteins were expressed in terms of a densitometric ratio to β-actin. Densitometric analysis was carried out by integrating the intensity of all pixels in a representative area, excluding background, using Image-Pro+ software (Version 4.1; Media Cybernetic, Silver Spring, MD).

Gene Induction and Semi-quantification of iNOS and COX-2 mRNA—pCMVEC seeded into 10-cm dishes were treated for 2 h at 37 °C with either the vehicle (bisdiluted and deionized water), sLPA (1–10 µM), or human IL-1β (10 ng/ml) in the presence or absence of caveole-disrupting agents filipin (5 µg/ml) and methyl-β-cyclodextrin (5 mM), non-selective phospholipase A2 inhibitors quinacrine (20 µM), and cycloheximide (5 µg/ml), which was incubated with either an anti-human iNOS gene expression was also studied in a cell-free nuclear system. For this purpose, isolated rat liver nuclei (10¹⁰ nuclei; assay: ~250 µg of protein) were placed in buffer of the following composition: Tris-HCl (10 mM), pH 7.5, KCl (10 mM), MgCl₂ (3 mM), CaCl₂ (100 mM), ATP, UTP, GTP, and CTP (500 µM), and Superase RNase inhibitor (10 units, 100 units). iNOS gene expression was also studied in a cell-free nuclear system. For this purpose, isolated rat liver nuclei (10¹⁰ nuclei; assay: ~250 µg of protein) were placed in buffer of the following composition: Tris-HCl (10 mM), pH 7.5, KCl (10 mM), MgCl₂ (3 mM), CaCl₂ (100 mM), ATP, UTP, GTP, and CTP (500 µM), and Superase RNase inhibitor (10 units, 100 units). iNOS gene expression was also studied in a cell-free nuclear system. For this purpose, isolated rat liver nuclei (10¹⁰ nuclei; assay: ~250 µg of protein) were placed in buffer of the following composition: Tris-HCl (10 mM), pH 7.5, KCl (10 mM), MgCl₂ (3 mM), CaCl₂ (100 mM), ATP, UTP, GTP, and CTP (500 µM), and Superase RNase inhibitor (10 units, 100 units). iNOS gene expression was also studied in a cell-free nuclear system. For this purpose, isolated rat liver nuclei (10¹⁰ nuclei; assay: ~250 µg of protein) were placed in buffer of the following composition: Tris-HCl (10 mM), pH 7.5, KCl (10 mM), MgCl₂ (3 mM), CaCl₂ (100 mM), ATP, UTP, GTP, and CTP (500 µM), and Superase RNase inhibitor (10 units, 100 units).
Amilor, total reaction medium volume of 60 μl). Nuclei were incubated at 37° C for 60 min with sterile water vehicle or oLPA (1 μM) in the presence or absence of Ca2+- chelator EGTA (100 μM), pre-activated PTX (20 μg/ml, 60-min preincubation), or phosphoinositide 3-kinase/Akt (PI 3-kinase) inhibitor wortmannin (100 nM) (27). Total RNA from purified nuclei was isolated by the standard guanidine isothiocyanate method (40). iNOS and COX-2 mRNA were quantified by reverse transcriptase-polymerase chain reaction (RT-PCR) method as described elsewhere (29, 41, 42), with slight modifications. Briefly, 1 μg of total RNA was transcribed with Moloney murine leukemia virus reverse transcriptase (40). The PCR products were 32P-labeled, separated on a 6% polyacrylamide gel, and quantified with a PhosphorImager (Amersham Biosciences).

Measurement of Ca2+ Signals by LPA in Isolated Nuclei—Nuclear Ca2+ signals were measured by fura-2-AM technique as essentially described (32) with minor modifications. Briefly, isolated nuclei (1–5 × 106 nuclei/ml) placed in incubation buffer (Hepes (25 mM), pH 7.2, KCl (125 mM), K2HPO4 (2 mM), MgCl2 (4 mM), CaCl2 (400 μM)), with an osmolality adjusted with glucose to 300 mosm, were loaded in the dark with fura-2-AM (7 μM) (45 min at 4°C). Fura-2-loaded nuclei were then diluted (1:10), centrifuged at 600 × g for 10 min, and resuspended with the incubation buffer. Leakage of fura-2 from nuclei was minimal (<2%) during the time frame of experiments as reported (31). The [Ca2+]i, nuclear was determined in 1 ml of fura-2-loaded nuclei (~5 × 106 nuclei/ml) at 37°C under constant stirring using the ratio of excitation wavelengths 350 and 380 nm, with emission set at 500 nm. Nuclear calcium signals were measured by means of a spectrophotometer (model LS50, PerkinElmer Life Sciences, UK). Calibration of maximal (Rmax) fluorescence signals was determined by sequential addition of ionomycin (5 μM) and calcium (1 mM), whereas that of minimal (Rmin) fluorescence signals was obtained with Triton X-100 (1%) followed by EGTA (25 mM). The [Ca2+]i was calculated according to Grynkiewicz et al. (43) by the equation: [Ca2+]i = Kd × (F – Rmin)/(Rmax – Rmin) – Kd, where Kd is the dissociation constant (224 nM for fura-2) and

RESULTS

[3H]oLPA Binding to Subcellular Fractions—Both intact nuclei and nuclear envelopes (Fig. 1B) from rat liver (Fig. 1C) and pCMVEC (Fig. 1D) displayed binding sites for [3H]oLPA (10 nM) specific for LPA surrogates (oLPA, sLPA, and pLPA), because lipids structurally distinct from LPA were significantly less efficient or not able to displace the radioactive tracer. pCMVEC and derived nuclei contain 10- to 20-fold more binding sites for LPA than rat liver nuclei.

Western Blot Analysis of LPA1R—Immunoprobning of subcellular fractions from native and recombinant cell systems with an anti-LPA1R-R-N polyclonal antibody revealed a prominent immunoreactive band at ~49 kDa (Fig. 2A), in agreement with Zheng et al. (25). This is suggestive of a receptor with putative carbohydrate adducts, at the nucleus and plasma membrane of the cell types investigated. Similar results were obtained with the anti-LPA1R-C polyclonal antibody. The presence of glycan moieties comprised in LPA1R was established by assessing the
The intracellular localization of LPA1R

Electron Microscopy

A

B

C

D

E

F

Fig. 2. A, Western blot of LPA1R in cell and nuclear fractions of different cell types. Proteins were extracted from: whole nuclei (WN), nuclear envelopes (NE), and plasma membranes (PM) from rat livers (RL), porcine cerebral endothelial cells (EC), and HTC4 rat hepatoma cells. Proteins (25 μg) were separated on SDS-PAGE (12%) and probed for LPA1R using a rabbit polyclonal anti-LPA1R antibody as described under “Experimental Procedures.” B, immunoblots of rat liver plasma membrane (RLPM) treated or not with PNGase F. Proteins (50 μg) were treated as in A. C, immunoblots of total cellular extract of EC pretreated or not with tunicamycin. Proteins were separated on SDS-PAGE (9%) and probed for LPA1R as described above. Note the increase of immunoreactivity of antibody toward its N-terminal epitope in the absence of N-linked glycans at LPA1R N-terminal segment. Results are representative of three to four experiments.

electromobility shift of LPA1R protein to the predicted theoretical molecular mass (~38 kDa) (31) following the treatment of rat liver cell membrane with PNGase F (Fig. 2B), or pCMVEC with tunicamycin (Fig. 2C); these oligosaccharide moieties account for ~37% of the overall LPA1R molecular weight. In pCMVEC, two glycoforms (47 and 49 kDa) of LPA1R were detected by performing a low concentration SDS-PAGE (9%) (Fig. 2C). These results conformed to the quasi-universal post-translational modifications occurring in the GPCR family (45), including that of lysosphopholipid receptor system (46).

In Situ Detection of Perinuclear and Intranuclear LPA1R by Electron Microscopy—The intracellular localization of LPA1R was ascertained in vivo by high resolution immunogold electron microscopy and cryomicroscopy on rat liver tissue sections. LPA1Rs were identified at the plasma membrane of hepatocytes (Fig. 3A) and endothelial cells (Fig. 3B), as expected, and notably intracellularly, including the perinuclear and nuclear regions (Fig. 3B) mostly confined to euchromatin structures; these nuclear associated receptors account for 7–15% of overall cellular immunoreactivity. Negative controls revealed no specific labeling when the primary antibody was pre-absorbed with its cognate peptide (Fig. 3C) or was omitted (Fig. 3D).

Ultrastructural localization of immunoreactive LPA1R in rat liver sections was further substantiated by electron cryomicroscopy using polyclonal rabbit anti-LPA1R-C (Fig. 3E) and anti-LPA1R-N antibodies (Fig. 3F), which target two distinct epitopes. In both cases, a broad and similar distribution pattern for LPA1R was revealed namely at the hepatic plasmalemma (not shown), the endoplasmic reticulum, the perinuclear envelope (arrowheads), as well as inside the nucleus (Fig. 3E and F). Sporadic cytoplasmic detection of LPA1R was also observed at higher magnification, presumptively in vesicular compartments (not shown).

Co-localization of LPA1R and Caveolin-1 in pCMVEC-isolated Nuclei—Many endocytic and transcytotic transport processes involve subcellular redistribution of transmembrane proteins, including possibly GPCRs, which are mainly mediated via either the clathrin or caveolae membrane system (16, 18). Therefore, we sought to investigate which of these endocytic systems may be monopolized for the nuclear translocation of cell surface LPA1R, if indeed it is occurring. Immunoprecipitation experiments demonstrated that LPA1R is dually retrieved in cell membrane clathrin-coated pits and caveolae while being only present in the caveolar compartment in the nucleus (Fig. 4A). Further support for the nuclear co-localization of LPA1R with caveolae in pCMVEC was obtained from Western blot experiments depicting the presence of LPA1R primarily in cholesterol-rich detergent-resistant caveolar domains of purified nuclei (Fig. 4B) and from immunofluorescence studies describing the double labeling of LPA1R and caveolin-1 in more than 90% of nuclear organelles examined. LPA1R co-localized with caveolin-1, as anticipated not exclusively.

Role of Caveolae and Phospholipase A2-derived Products in LPA-induced COX-2 and iNOS Gene and Protein Expression—The induction of immediate-early response genes COX-2 and iNOS by LPA has recently been reported for various cell types. For instance, LPA-induced transcriptional up-regulation of COX-2 enzyme has been shown in renal mesangial cells (47), fibroblasts (48), and astrocytes (49), whereas iNOS has been induced in cardiomyocytes (50). We extended these observations by showing that sLPA (as well as oLPA) increases, in a concentration-dependent manner, COX-2 and iNOS protein expression in brain vascular endothelia (Fig. 5A), whereas the classic endothelial cell antigen von Willebrand factor was unaffected. The concentration-dependent effects of LPA on COX-2
gene expression were further substantiated by semi-quantitative RT-PCR (Fig. 5B). Based on the abovementioned results demonstrating co-localization of LPA_{1R} with caveolin-1 more so in nuclear than plasma microsomal fractions (Fig. 4, A–C), we sought to explore whether caveolae was instrumental in LPA_{1R}-mediated COX-2 expression by facilitating its transport to the cell nucleus. The caveolae-disrupting agents, filipin and methyl-β-cyclodextrin, both used at effective inhibitory concentrations (37, 38), failed to modify LPA activity. Similarly, inhibition of clathrin-dependent endocytosis by means of sucrose hypertonic solution (15) did not affect COX-2 expression (not shown). Conversely, LPA-induced COX-2 gene transcription was entirely blocked by the PLA2 inhibitors mepacrine and cytidine-5′-diphosphocholine suggesting a role for endogenous arachidonic acid and/or LPA in this phenomenon; partial impairment (–50%) was also observed with RTK inhibitor tyrphostin AG 1478 and MEK inhibitor PD 98059 (Fig. 5B). The participation of cyclooxygenase-, lipoygenase-, and epoxygenase-derived products of arachidonic acid and PAF was ruled out based on the inefficiency of corresponding specific enzyme inhibitors to block LPA responses (Fig. 5B). Thus, it appears that LPA signaling in inflammatory gene expression proceeds, independent of caveolae, via LPA_{1R}-triggered intracellular messengers, possibly LPA per se, acting conceivably on perinuclear/nuclear receptor sites (see Figs. 1–3). To corroborate this premise, we examined whether a direct stimulation of isolated nuclei with LPA would reproduce or mimic gene responses evoked by extracellular LPA. Results from these series of experiments are presented in the following section.

**Signaling Effectors of LPA-stimulated Calcium Transients and iNOS Expression in Highly Purified Rat Liver Nuclei**—It is now becoming clear that the nucleus possesses autonomous calcium signaling machinery (e.g., ion channels, protein kinases, and phospholipases) where the nuclear envelope serves as the nuclear calcium pool (12). Furthermore, nuclear calcium is instrumental in regulating many processes, including gene transcription (12). We therefore examined whether changes in nuclear Ca^{2+} could be elicited by LPA_{1R} stimulation by means of spectrofluorometry and live imaging confocal microscopy using different fluorescent Ca^{2+}-sensitive probes (fura-2/AM and fluo-4/AM). Incubation of isolated hepatic nuclei with sLPA induced a concentration-dependent increase in nuclear Ca^{2+} levels (Fig. 6A); indistinguishable responses were seen with LPA analogues (1 µM) comprising distinct fatty acid species (sLPA and pLPA). Similar responses were observed in LPA_{1R}-HTC4 transformed hepatic cell-derived nuclei. Moreover, the Ca^{2+} chelator EGTA and putative receptor-operated Ca^{2+} channel blocker SK&F96365 prevented LPA-induced Ca^{2+} transients, as seen with PTX suggestive of G_{i/o} coupling, whereas the Ca^{2+} ATPase pump inhibitor thapsigargin was inoperative (Fig. 6A). Rapid nuclear calcium mobilization in response to LPA was visualized in a single nucleus by confocal microscopy (Fig. 6B). Live scan imaging clearly illustrated a progressional transient Ca^{2+} movement across the nucleus (Fig. 6B, third panel). Notably, these discrete nucleoplasmic Ca^{2+} responses evoked by LPA were concentration-dependent (with an apparent affinity in the nanomolar range) (Fig. 6C), reversible and seemingly desensitizable (Fig. 6D) suggestive of a receptor-operated mechanism. Alternatively, this latter fading of organelle responsiveness upon repeated challenges of high concentration of LPA (1 µM) might reflect a
re-localization and sequestration of calcium in unvisualized nuclear foci.

Direct stimulation of nuclei with oLPA (as well as sLPA) also provoked dose-dependent, calcium- and PTX-sensitive iNOS gene induction (Fig. 7), consistent with results from calcium mobilization assays (see Fig. 6A). Along with the presence of nuclear Akt (Fig. 7A, inset), LPA effects were also associated with phosphorylation (and resultant activation) of this kinase (Fig. 7B), which in turn participated in iNOS gene induction. Accordingly, this latter effect was abolished by PI 3-kinase-activated protein kinase Akt inhibitor wortmannin (Fig. 7, A and B). In similar experimental conditions, LPA failed to induce phosphorylation of nuclear Erk1/2 kinases (not shown).

**DISCUSSION**

The generally accepted view of signaling to immediate-early growth-related genes by cell surface GPCRs for lysosphospholipid growth factors centers on ras- and rho-dependent activation of transcription through the multicomponent serum-response element (1). The results of our present study support an alternative signaling pathway by which LPA-LPA1R complexes in the nucleus more directly evoke transcriptional events. In this context, we showed that in pCMVEC membrane, LPA1Rs are partitioned in both clathrin and caveolar endocytic microdomains (Fig. 4); the former domain usually being ascribed for receptor recycling or degradation (57), whereas the latter is thought to serve as a protein carrier responsible for intracellular redistribution of various acid sequences implying that other nuclear import mechanisms may prevail, possibly brought into play following receptor bio-

**FIG. 6. Nuclear calcium signaling induced by LPA and closely related lysophospholipids.** A, calcium signals were obtained from fura-2-AM-loaded nuclei of rat liver and LPA1-R expressing HTC4 cell transfectants and detected by spectrofluorometry. Non-transfected HTC4 cells were unresponsive to LPA (not shown), as reported (24). B, intranuclear calcium mobilization induced by oLPA in a single nucleus of rat hepatocyte measured by confocal microscopy. Upper panel (left to right): three-dimensional reconstruction of a series of transverse sections taken from an isolated fluo-4-AM-loaded nucleus. Note the increase in fluorescence from baseline when nucleus is challenged with a mixed solution of ionomycin and calcium; this increase disappears after adding excess calcium chelator EGTA. The fluorescent DNA probe Syto-11 confirmed the from an isolated fluo-4-AM-loaded nucleus. Note the increase in fluorescence from baseline when nucleus is challenged with a mixed solution of ionomycin and calcium; this increase disappears after adding excess calcium chelator EGTA. The fluorescent DNA probe Syto-11 confirmed the nuclear entity. Middle panel: continuous recording of calcium oscillations from a single transverse section (1 μm). Note the fluorescence signal stability in the absence of stimulant (Vehicle). Bottom panel: rapid, massive, and transitory nuclear free calcium oscillations induced by oLPA (1 nM). The pseudo-color ladder indicates relative intensity. C, concentration-dependent effect of oLPA on calcium transients measured as in B; concentrations are presented in log molar. D, effect of repeated stimulation of a nucleus with oLPA measured as in B; concentrations are presented in log molar. Note: live calcium fluorescent intensity obtained by confocal microscopy was automatically converted to numerical data using the Molecular Dynamics software. Images are representative of three and five separate experiments.
signaling components (18). Notably, other transmembrane receptors such as β2 adrenergic (58), endothelin ETA (59), and interferon γ receptors (60), have been reported to be dually compartmentalized into caveolae and clathrin-coated pits in cell membranes. On the other hand, our results do not support a nuclear translocation of cell surface LPA₁ receptor-evoked inflammatory (COX-2) gene expression mediated through endocytic caveolar pathway (Fig. 5B), despite LPA₁-R and caveolin-1 co-localization at the nucleus (Fig. 4, A–C). Rather, our data suggest a major role for released PLÅ₂ products in LPA-induced-COX-2 expression (Fig. 5B); these PLÅ₂ products are unlikely to derive from cyclooxygenase, lipoxigenase, or cytochrome P450 enzymatic systems, but possibly LPA. Interest-ingly, a similar PLÅ₂-dependent mechanism for αₐ-adrenergic receptor-mediated mitogen-activated protein kinases (MAPKs) activation has been recently documented (36).

A novel pathway has recently been reported to explain LPA-evoked signaling of cellular proliferation, which involves MAPK activation through RTK autophosphorylation (e.g. EGF and PDGF receptors) (19, 61). The presumption of auto-induced generation of LPA in extracellular LPA-evoked COX-2 expression is strengthened from experiments describing the limited effectiveness of the RTK-specific inhibitor tyrphostin and MEK kinase inhibitor PD 90859 (Fig. 5B) compared with PLÅ₂ inhibitors. This may suggest that LPA-induced COX-2 up-regu-

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