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Subcellular Localization and Role of Lipin-1 in Human Macrophages

Martín Valdearcos,[1] Esperanza Esquinas,[1] Clara Meana, Luis Gil-de-Gómez, Carlos Guijas, Jesús Balsinde, and María A. Balboa

The lipins have been described as metabolic enzymes that regulate lipid biosynthesis and also signaling processes by controlling the cellular concentration of bioactive lipids, phosphatidic acid, and diacylglycerol. In the present work we have studied the subcellular localization and role of lipin-1 in human monocyte-derived macrophages. Human macrophages express lipin-1 isoforms α and β. A transfected lipin-1α–enhanced GFP construct associates with membranes of cellular organelles that can be stained with Nile Red. Colocalization experiments with lipid droplet (LD)-specific proteins such as adipophilin/adipose differentiation-related protein/perilipin 2 or TIP47/perilipin 3 show that both proteins colocalize with lipin-1α in the same cellular structures. Reduction of the expression levels of lipin-1 by small interfering RNA technology does not impair triacylglycerol biosynthesis but reduces the size of LDs formed in response to oleic acid. In agreement with these data, peritoneal macrophages from animals that carry a mutation in the Lpin-1 gene (*fld* animals) also produce less and smaller LDs in response to oleic acid. Mass spectrometry determinations demonstrate that the fatty acid composition of triacylglycerol in isolated LDs from lipin-1–deficient cells differs from that of control cells. Moreover, activation of cytosolic group IVA phospholipase $A_2\alpha$, a proinflammatory enzyme that is also involved in LD biogenesis, is also compromised in lipin-1–deficient cells. Collectively, these data suggest that lipin-1 associates with LDs and regulates the activation of cytosolic group IVA phospholipase $A_2\alpha$ in human monocyte-derived macrophages. ***The Journal of Immunology*****, 2011, 186: 6004–6013.**

The lipin family of proteins plays multiple roles in cells by regulating lipid biosynthesis and cellular signaling (1–3). Lipins possess phosphatidic acid (PA) phosphatase (PAP) activity that hydrolyzes PA to yield diacylglycerol (DAG). This catalytic activity is highly dependent on Mg^{2+} and specific for PA as a substrate. These enzymes are often referred to as PAP1 to differentiate them from other cellular enzymes whose activity does not depend on Mg^{2+} and are not selective for PA (PAP2 enzymes) (4–6). Both PA and DAG are important signaling lipids, having the capacity to modulate key signaling events (1). In contrast, DAG can be acylated to generate triacylglycerol (TAG), the major energy storage lipid, or bind to choline or ethanolamine, via the Kennedy pathway, to produce phosphatidylcholine or phosphatidylethanolamine, two major membrane phospholipids (6).

Although the lipin coding gene, *Lpin,* and the effects of its mutation and overexpression in whole animals have been known for several years (7, 8), the correlation between lipin and PAP1 was not established until very recently, when Carman and colleagues (9) found that the sequence of a *Saccharomyces cerevisiae* protein possessing PAP1 activity was the ortholog of mammalian lipin-1. Recently, three genes coding for proteins with PAP1 activity have been identified, termed *Lpin1*, *Lpin2*, and *Lpin3*, which exhibit different tissue expression patterns (10, 11). Lipin-1 also has two products due to alternative splicing of the mRNA, lipin-1α and lipin-1β, that are postulated to have different roles during adipogenesis in rodents (12). In humans, another splicing protein from *Lpin1* gene has been recently described, termed lipin-1γ (13). Subcellular localization studies using murine proteins have revealed that lipin-1α and lipin-1β may be cytosolic, nuclear, or associated to the endoplasmic reticulum, whereas lipin-2 is a cytosolic enzyme (12–15). To our knowledge, no studies have been published on the subcellular localization of human lipins.

Most cellular studies on lipin have been conducted in metabolically active tissues such as adipose tissue. As a matter of fact, *Lpin1* is known as an obesity-related gene because its absence promotes the lack of adipose tissue and its overexpression promotes obesity in mice (7, 8). However, little is known on the possible roles of lipin in inflammation and related diseases. Before the nucleotide sequence of the PAP1 enzymes was revealed, our studies with pharmacological inhibitors showed that PAP1 activity is involved in the regulation of the mobilization of free arachidonic acid (AA), the precursor of the inflammatory mediators known as the eicosanoids (16, 17). More recently, these studies were corroborated in immunoinflammatory cells (18, 19). However, identification of the actual PAP1/lipin form involved in AA mobilization has not been elucidated.

Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas, 47003 Valladolid, Spain; and Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, 08036 Barcelona, Spain

[1]M.V. and E.E. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Jesús Balsinde and Dr. María A. Balboa, University of Valladolid School of Medicine, Calle Sanz y Fores 3, 47003 Valladolid, Spain. E-mail addresses: jbalsinde@ibgm.uva.es and mbalboa@ibgm.uva.es

Abbreviations used in this article: AA, arachidonic acid; ADRP, adipose differentiation-related protein; BODIPY493/503, boron dipyrromethene 493/503; $cPLA_2\alpha$, cytosolic phospholipase $A_2\alpha$ (group IVA phospholipase A_2); DAG, diacylglycerol; EGFP, enhanced GFP; HA, hemagglutinin; LD, lipid droplet, PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; siRNA, small interfering RNA; TAG, triacylglycerol.

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Lipid droplets (LDs) are cellular organelles that were regarded as the inert storage places for neutral lipids, and hence for energy generation and membrane building blocks (20). This perception has changed and, at present, LDs are viewed as dynamic structures that regulate a wide variety of cellular processes, and whose production and maintenance are exquisitely regulated (21–23). LDs are formed by a core of TAG and cholesteryl esters with an external monolayer of phospholipids and cholesterol that supports the binding of proteins (20). Among the better known LD-associated proteins are adipophilin, also called adipose differentiation-related protein (ADRP) or perilipin 2 and TIP47/perilipin 3 (24–26).

LDs have been related to prevalent fat storage illnesses such as obesity, atherosclerosis, and diabetes (27, 28), as well as to inflammatory diseases (29). Leukocyte LDs have been shown to contain esterified AA and to associate to proteins that are implicated in the generation of eicosanoids (29–33). In turn, we have recently described that cytosolic phospholipase $A_2\alpha$ (also known as group IVA phospholipase A_2, $cPLA_2\alpha$), that is, the major effector of AA release that leads to PG and leukotriene production, is involved in the biogenesis of LDs (34–36).

In the present work we have studied the subcellular localization and role of human lipin-1 in macrophages. Our findings indicate that lipin-1α is an LD-associated protein that regulates $cPLA_2\alpha$ activation and hence AA mobilization in human macrophages.

Materials and Methods

Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (200 Ci/mmol) and ECL chemiluminiscence substrate were purchased from Amersham Ibérica (Madrid, Spain). [1-^{14}C] Oleic acid (58 mCi/mmol) was from PerkinElmer España (Madrid, Spain). Boron dipyrromethene 493/503 (BODIPY493/503), Nile Red, and goat anti–IgY-Alexa 555 were purchased from Molecular Probes/Invitrogen (Carlsbad, CA). Polyclonal anti-ADRP (IgY) for immunohistochemistry was obtained from GenWay (San Diego, CA). Guinea pig polyclonal anti-ADRP Ab for immunoblot was from Progen Biotechnik (Heidelberg, Germany). Anti–lipin-1 Ab was generated by inoculating rabbits with the peptide CKTDSPSKRDKRS (human sequence) conjugated to keyhole limpet hemocyanin, using standard immunization procedures (Immunostep, Salamanca, Spain). Protein A linked to HRP and Ficoll–Paque Plus were purchased from GE Healthcare (Uppsala, Sweden). Gentamicin was purchased from BioWhittaker (Cambrex, Walkersville, MD). Human macrophage Nucleofection solution was from Amaxa (Gaithersburg, MD). Macrophage serum-free medium and RPMI 1640 were from Life Technologies (Carlsbad, CA). Small interfering RNAs (siRNAs) Silencer Select specific to decrease the expression of human lipin-1 mRNA and negative controls were purchased from Ambion. All other reagents were from Sigma-Aldrich.

Animals

BALB/cByJ-Lpin1$^{fld/J}$ carrying a spontaneous mutation in the Lpin1 gene were purchased from The Jackson Laboratory and bred to generate fld/fld and +/fld animals (7). Animals were fed a standard chow diet (Purina 5001). Only male mice were used for experimentation. All animal experimentation was conducted in accordance with accepted standards of animal care.

PCR

RNA from macrophages was obtained using the TRIzol method (Invitrogen), following the manufacturer's instructions. First-strand cDNA was then obtained by using the Moloney murine leukemia virus reverse transcriptase from 1 μg RNA. PCR was then performed using the specific primers for human lipin-1α and -1β described by Lindegaard et al. (37).

Plasmids

The construct lipin-1α–enhanced GFP (EGFP) was obtained as follows. The human lipin-1α cDNA was purchased from Origene (TC125492; Rockville, MD) and was subcloned into the pEGFP-N3 vector (Clontech, Palo Alto, CA) using XhoI and PstI cloning sites. To generate a mutant lacking enzymatic activity, Asp at position 678 was substituted to Glu (D678E) by using the QuickChange XL site-directed mutagenesis kit from Stratagene and the oligonucleotides 5′-GGGATGATAAAGTCATCATTTCTGAGATTGATGGGACAATTACCAG-3′ (forward) and 5′-CTGGTAATTGTCCCATCAATCTCAGAAATGATGACTTTATCATCCC-3′ (reverse) (38). Mutagenesis was confirmed by sequencing. The construct lipin-1β was obtained by inserting the 108 nucleotides that make human lipin-1β different from lipin-1α (13). The insert was obtained by PCR from a human macrophage cDNA using the primers 5′-GGATAGAGAGTGGTCACCCAC-3′ (forward) and 5′-AAGACTTAGCAGCCTGCGGCA-3′ (reverse). Afterwards, the sequence was introduced into the lipin-1α–EGFP using the restriction enzymes BstEII and HindIII. The inset was then sequenced and confirmed to be the same as previously described for human lipin-1β (13). The construct hemagglutinin (HA)–lipin-1α was generated by cutting the lipin-1α sequence from the lipin-1α–EGFP construct using the restriction enzymes EcoR1 and Xba1 and introducing it into the plasmid pEF-HA.

Cells

Human macrophages were obtained from buffy coats of healthy volunteer donors obtained from the Centro de Hemoterapia y Hemodonación de Castilla y León (Valladolid, Spain) as described previously (39, 40). Briefly, blood cells were diluted 1:1 with PBS, layered over a cushion of Ficoll–Paque, and centrifuged at 750 × *g* for 30 min. Macrophage differentiation was achieved by incubating plastic-adhered monocytes in RPMI 1640 with heat-inactivated 5% human serum for 2 wk, in the absence of exogenous cytokine mixtures. Transfection of human macrophages was achieved by the Nucleofection method, following the kit specifications for human macrophages. Briefly, the cells were resuspended in 100 μl Human Macrophage Nucleofector solution plus 5 μg plasmid or siRNAs. Nucleofection was carried out using the program Y-010, and the cells were resuspended in 400 μl macrophage serum-free medium (Life Technologies) plus 5% heat-inactivated human serum. When oleic acid was used, the cells were treated with 100 μM fatty acid complexed to BSA in a ratio 5:1 for 18 h.

Resident peritoneal macrophages were obtained by peritoneal lavage using 5 ml cold PBS as described (41, 42). The cells were plated at 2×10^6 per well (6-well plates) in 2 ml RPMI 1640 with 10% heat-inactivated serum and allowed to adhere for 2 h in a humidified atmosphere of 5% CO_2 at 37°C. Wells were then extensively washed with PBS to remove nonadherent cells. Adherent macrophages were then used for experimentation.

Cellular stainings

Transfected cells were seeded in coverslips and allowed to adhere for 24 h in RPMI 1640 supplemented with 2 mM L-glutamine, 40 mg/ml gentamicin, and 5% heat-inactivated human serum. After treatments, cells were fixed with 4% paraformaldehyde in PBS containing 2% sucrose for 20 min. Cells were then permeabilized with 0.1% Triton X-100 for 2 min, washed with PBS, and blocked with 0.2% BSA, 100 mM glycine, and 10% goat serum in PBS for 30 min (43). ADRP immunostaining was performed by incubating fixed cells with an IgY polyclonal Ab at 1:200 dilution for 1 h followed by a goat anti–IgY-Alexa 555 at 1:500. TIP47 immunostainings were performed after cellular treatment with 200 μM oleic acid for 18 h by using 1:100 anti-TIP47 rabbit antiserum (AnaSpec, San Jose, CA) and then a goat anti–rabbit-Alexa 555 at 1:500. Nile Red, BODIPY493/503, and DAPI stainings were carried out by treating with the cells with these dyes at 100 ng/ml, 2.5 μg/ml, and 1 mg/ml, respectively, in PBS for 5 min. Cells were then mounted with an antifade solution.

Confocal microscopy

Fluorescence was monitored by confocal microscopy using a Bio-Rad Radiance 2100 laser-scanning system coupled to a Nikon TE-2000U. The objective was CFI Plan Apo ×60, 1.4 numerical aperture, oil immersion. The fluorescence from EGFP and BODIPY493/503 was monitored at 488 nm argon excitation using the combination of a long-pass filter HQ500LP and a short-pass filter HQ560SP. Nile Red and Alexa 555 fluorescences were analyzed at 543 nm HeNe excitation using a long-pass filter HQ570LP.

AA release assay

This assay was performed as previously described (44, 45). In brief, the cells were labeled with 0.5 μCi/ml [^{3}H]AA overnight, washed, stimulated for the indicated time, and supernatants were removed. Cell monolayers were overlaid with ice-cold PBS containing 0.05% Triton X-100 and scraped. Radioactivity was quantified by liquid scintillation counting in supernatants and homogenates, and AA release was referred to total radioactivity for each condition.

PGE_2 production

Supernatants from stimulated cells (10^6 cells) were collected and assayed for PGE_2 using an ELISA kit from Cayman Chemical, following the manufacturer's instructions.

Preparation of zymosan

Zymosan was prepared as described elsewhere (41, 46). Briefly, zymosan particles were suspended in PBS, boiled for 60 min, and washed three times. The final pellet was resuspended in PBS at 20 mg/ml and stored frozen. Zymosan aliquots were diluted in serum-free medium and sonicated before addition to the cells. No phospholipase A_2 activity was detected in the zymosan batches used in this study, as assessed by in vitro activity assay (47–49).

Cell fractionation

Cells from 15-cm culture dishes were washed with PBS, scraped in 200 μl homogenization buffer (10 mM HEPES [pH 7.4] and 5 mM EDTA plus Sigma-Aldrich protease inhibitor mixture), and homogenized by 35 passages through a 25-gauge needle. The homogenate was then centrifuged at 50,000 × g for 30 min in an ultracentrifuge (Optima TL; Beckman Coulter). The top 200 μl, containing the LDs, were harvested, and LD proteins were collected by acetone precipitation (50). Protein from total homogenates, cytosol, membranes, and LDs was then analyzed by immunoblot.

Immunoblot

Cells were lysed (20 mM Tris [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, plus Sigma-Aldrich protease inhibitor mixture), centrifuged at 12,000 rpm for 10 min to eliminate debris, and 50–100 μg total protein was separated by standard 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Primary Abs were used at 1:1000 in PBS [pH 7.4] with 0.5% defatted dry milk and 0.1% Tween 20. After extensive washes, HRP linked to anti-rabbit donkey IgG or anti-IgY goat IgG was used as secondary Abs (1:5000). Specific proteins were visualized using ECL chemiluminiscent substrate (Amersham Biosciences).

Fluorescence protease protection assay

The fluorescence protease protection assay was performed as described (51) with minor modifications. Briefly, transfected human macrophages were seeded on glass-bottom culture dishes (MatTek, Ashland, MA), allowed to adhere overnight in RPMI 1640 supplemented with 2 mM L-glutamine, 40 mg/ml gentamicin, and heat-inactivated 5% human serum. Cells were then washed with a buffer consisting of 110 mM potassium acetate, 20 mM HEPES, and 2 mM $MgCl_2$ and placed on the confocal microscope stage at 37˚C. EGFP fluorescence from live cells was recorded. Images were taken before and after digitonin permeabilization (20 μM) and after trypsin (4 μM) treatment.

Cellular TAG synthesis assay

This assay was performed as previously described (52, 53). Briefly, human macrophages or peritoneal macrophages were treated with 1 μM [1-^{14}C] oleate (0.5 μCi/ml) in serum and albumin-free medium for different periods of time. Cells were washed, the lipids were extracted according to Bligh and Dyer (54), and the lipids in the chloroform phase were separated by thin-layer chromatography with *n*-hexane/diethyl ether/acetic acid (70:30:1) as a mobile phase. The spots corresponding to TAG were scraped into scintillation vials, and the amount of radioactivity was estimated by liquid scintillation counting.

Flow cytometry

Macrophages were separated from the plastic by treatment with trypsin/EDTA, washed with PBS, and labeled with 0.2 μg/ml BODIPY493/503 for 5 min. Cells were washed and fluorescence was analyzed by flow cytometry in a FACSAria using the FL1 detector. Fluorescence data were measured in linear scale.

Gas chromatography/mass spectrometry analysis of fatty acid methyl esters

Lipid extracts from purified LD fractions or from whole-cell homogenates were transmethylated with 500 μl 0.5 M KOH in MeOH for 30 min at 37˚C (55). One volume of 0.5 M HCl was added to neutralize, and fatty acid methyl esters were extracted twice with 2 vol *n*-hexane. Analysis of fatty acid methyl esters was carried out in an Agilent 6890N gas chromatograph coupled to an Agilent 5975 mass-selective detector operated in electron impact mode (EI, 70 eV), equipped with an Agilent DB23 column (60 m × 0.25 mm interior diameter × 0.15 μm film thickness). One microliter sample was injected in splitless mode. Inlet temperature was mantained at 250˚C. Oven temperature was held at 50˚C for 1 min, then increased to 175˚C at intervals of 25˚C per minute, and to 230˚C at intervals of 2.75˚C per minute. The final temperature was maintained for 5 min, and the run time was 33 min. The mass spectrometry transfer line was maintained at 250˚C and the mass spectrometer quadrupole and source at 150˚C and 230˚C, respectively. Helium was used as a carrier gas at a constant pressure of 180 kPa. Data acquisition was carried out both in scan and selected ion monitoring mode. Scan mode was used for compound identification, comparing with authentic fatty acid methyl ester standards and the National Institute of Standards and Technology mass spectroscopy library spectra. Selected ion monitoring mode was used for quantitation, using 74 and 87 fragments for saturated, 83 for monounsaturated, 67 and 81 for diunsaturated, and 79 and 91 for polyunsaturated fatty acid methyl esters. A 37-component mixture from Supelco was used for calibration curves, and nonadecanoic acid was used as an internal standard. Data analysis was carried out with the Agilent G1701EA MSD Productivity ChemStation software, revision E.02.00 (55).

Analysis of AA-containing phospholipids by liquid chromatography/mass spectrometry

For lipid separation by HPLC, a binary pump Hitachi LaChrom Elite L-2130 was used, together with a Hitachi Autosampler L-2200 (Merck). The liquid chromatography system was coupled online to a Bruker esquire6000 ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). In all cases the effluent was split and 0.2 ml/min entered the electrospray interface of the mass spectrometer. Nebulizer was set to 30 ψ, dry gas to 8 l/min, and dry temperature to 350˚C. Analysis of AA-containing phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine species was carried out exactly as described elsewhere (56, 57).

Results

Subcellular localization of lipin-1α

Human monocyte-derived macrophages were transfected with a fusion protein of human lipin-1α and EGFP (lipin-1α–EGFP) and analyzed under the confocal microscope. A clear association of lipin-1α–EGFP with the periphery of cytoplasmic vesicles was appreciated (Fig. 1*A*). To rule out a possible localization artifact due to the EGFP tail, comparative immunolocalization studies were carried out utilizing an HA-tagged lipin-1α construct (Fig. 1*B*). In agreement with the lipin-1α–EGFP results, the HA–lipin-1α also localized in the periphery of intracellular vesicles, thus suggesting that the presence of lipin-1α at this location does reflect the physiological behavior of the enzyme. Mutation of the first Asp present in the catalytic motif DXDXT of lipin-1α to Glu (D678E) has recently been shown to abolish the enzymatic activity of the enzyme (38). When this mutant was transfected into the macrophage cells, the same pattern of intracellular distribution as that of the wild-type enzyme was observed, that is, in the periphery of cytoplasmic vesicles (Fig. 1*C*). These data suggest that the enzymatic activity of lipin-1α has no influence on its subcellular localization.

Because lipin-1α has been found to possess a nuclear location signal at its N terminus, experiments were conducted in the presence of leptomycin B, a potent and specific inhibitor of nuclear export (58), to assess whether there was a shuttling of lipin-1α between the nucleus and the cytosol. Fig. 1*D* shows that leptomycin B did not alter the lipin-1α localization pattern in human macrophages, suggesting that lipin-1α is a cytoplasmic protein in these cells.

Experiments were conducted next to identify the intracellular structures to which lipin-1α is associated in human macrophages. Nile Red is an oxazone analog that is widely used to stain LDs (59). Fig. 2*A* shows that the green fluorescence arising from lipin-1α–EGFP-transfected cells surrounded the Nile Red-stained

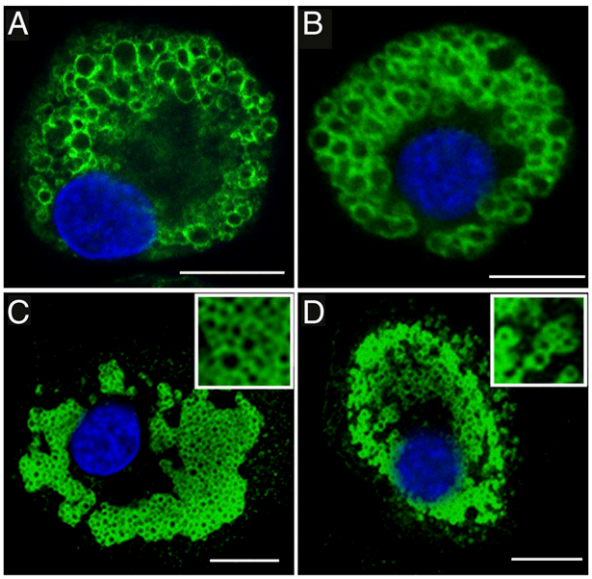

FIGURE 1. Human lipin-1α localizes to intracellular membranes. Human macrophages transfected with lipin-1α–EGFP (*A*), HA–lipin-1α (*B*), lipin-1α–D678E–EGFP (*C*), or with lipin-1α–EGFP and afterwards treated with 10 ng/ml leptomycin B for 24 h (*D*) were stained with DAPI (blue) and analyzed by confocal microscopy as described in *Materials and Methods*. *Inserts* in *C* and *D* are magnifications of selected regions of the cells. The cells shown in the pictures are representative of many analyzed. Scale bars, 10 μm.

structures, suggesting that lipin-1α is associated with the surface of LDs. This finding can be appreciated with detail in a tridimensional reconstruction of a macrophage cell fluorescence from a series of Z-stacks (Fig. 2*B*). In contrast, we failed to detect significant colocalization between lipin-1α–EGFP and markers of endoplasmic reticulum, Golgi, mitochondria, or lysosomes, indicating that the enzyme does not significantly interact with any of these structures (results not shown).

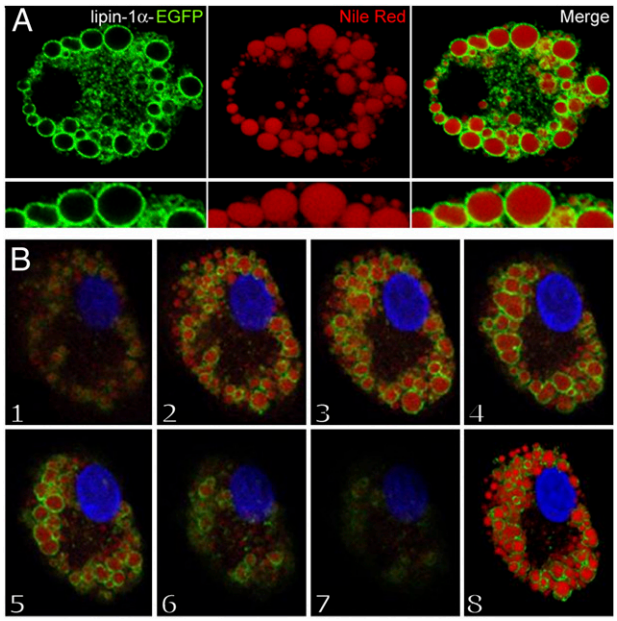

FIGURE 2. Human lipin-1α associates with LDs. *A*, Human macrophages transfected with lipin-1α–EGFP (green) were stained with 100 ng/ml Nile Red (red) and analyzed by confocal microscopy as described in *Materials and Methods*. *B*, Z-stack fluorescence series from the bottom to the upper part of a cell (1–7). Nuclei are stained with DAPI (blue). The image in the *lower right panel* is a tridimensional image generated from 15 Z-stacks using the confocal software (8). Cells shown are representative of many cells analyzed. Original magnification ×600.

In our laboratory we have generated a rabbit polyclonal Ab against lipin-1α. This Ab recognizes by immunoblot a 130-kDa protein present in homogenates of human macrophages and human U937 cells, which is the expected molecular mass of lipin-1α. Additionally, the Ab recognizes a 160-kDa protein in homogenates from lipin-1α–EGFP–expressing HEK cells, a band that is also recognized by anti-GFP Abs (Fig. 3*A*). Unfortunately, the anti–lipin-1α Ab generated by us also recognizes many other bands, making it unsuitable for immunomicroscopy experiments. Because commercial Abs against lipin-1α from different sources were found to be unsuitable as well, the association of endogenous lipin-1α with LDs was studied by immunoblot analyses of subcellular fractions obtained by ultracentrifugation. As shown in Fig. 3*B*, the LD marker ADRP only appears in the fraction containing LDs and in the total homogenate. The anti–lipin-1α Ab recognized a wide band in the total homogenate fraction and also reacted with material from the LD-enriched and cytosolic fractions, but not from the total membrane fraction (Fig. 3*B*). Note that the molecular mass of the cytosolic band is slightly higher than that of the LD-enriched fraction (Fig. 3*B*). Because the peptide used to generate the antiserum is present in both lipin-1α and lipin-1β, and lipin-1β has a slightly higher molecular mass than lipin-1α (12), we speculate that the protein that appears in the cytosolic fraction is lipin-1β, and that the protein appearing in the LD fraction is lipin-1α. mRNA for both lipin forms was readily detectable by RT-PCR (Fig. 3*C*)

To confirm the presence of lipin-1β in the cytosol, a lipin-1β–EGFP construct was transfected into the macrophages, and its subcellular localization was examined by confocal microscopy. Fig. 4 clearly suggests that lipin-1β localizes in the cytosol and does not colocalize with the structures stained with Nile Red or ADRP, implying that lipin-1β is not associated with LDs.

In the next series of studies we examined the orientation of lipin-1α with respect to the LD phospholipid monolayer, that is,

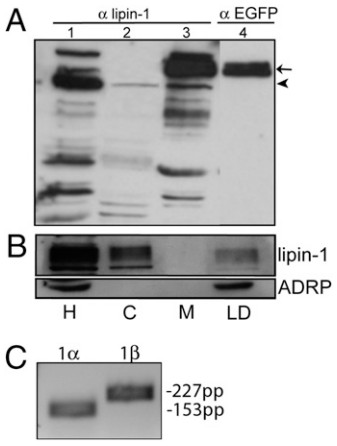

FIGURE 3. Analysis of the association of endogenous lipin-1α expression with LDs. *A*, Cell homogenates from U937 cells (*lane 1*) or HEK-293 untransfected (*lane 2*) or transfected (*lanes 3* and *4*) with lipin-1α–EGFP were analyzed by immunoblot using an Ab against lipin-1 (*lanes 1–3*) or an Ab against EGFP (*lane 4*). The arrowhead points to the endogenous protein and the arrow points to the construct lipin-1α–EGFP. *B*, Equivalent amounts of total cellular proteins (H) or from the cytosol (C), membrane (M), or LD-enriched fraction were analyzed by immunoblot using the antiserum against lipin-1 or against ADRP, as indicated. *C*, PCR analysis of mRNA expression of lipin-1α and lipin-1β in human macrophages.

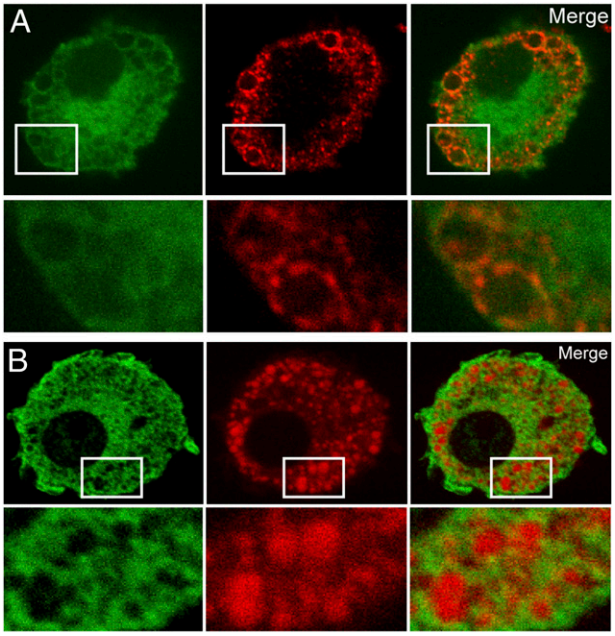

FIGURE 4. Human lipin-1β is not associated with LDs. Human macrophages transfected with lipin-1β–EGFP (green) were immunostained with Abs against ADRP (red) (*A*) or stained with Nile Red (red) (*B*) and analyzed by confocal microscopy as described in *Materials and Methods*. Lower images are magnifications of selected areas. Cells shown are representative of many cells analyzed. Original magnification ×600.

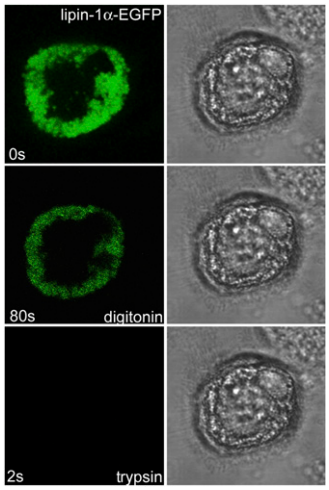

FIGURE 5. Human lipin-1α associates with the cytoplasmic side of LDs membranes. Live human macrophages transfected with lipin-1α–EGFP were analyzed under the confocal microscope. Fluorescence was recorder before (0 s) and after permeabilization with 20 μM digitonin (80 s) and after 4 μM trypsin treatment for 2 s, as indicated. Images in the *left column* show green fluorescence while images in the *right column* show light transmission. Original magnification ×600.

whether the enzyme is facing the cytosol or the lumen of the organelle. For this purpose we carried out a fluorescence protease protection assay (51). This technique is performed in live, digitonin-permeabilized cells and measures, in real time, the proteolysis of a GFP-tagged protein after trypsin incubation (51). Only those proteins that are in the luminal face of organelles would be protected from proteolysis. Also, fluorescent signals that resist cell permeabilization indicate that the protein of interest is actually bound to the organelle under study (51). As shown in Fig. 5, fluorescence from lipin-1α–EGFP resisted digitonin permeabilization for 80 s. In some occasions we observed lipin-1α–EGFP fluorescence signals even after 6 min of permeabilization. After trypsin was added to the cell cultures, the fluorescence disappeared in <2 s, implying that lipin-1α interacts with the cytosolic face of LDs.

Lipin-1α colocalizes with ADRP and TIP47 in the membranes of LDs and does not change its localization after macrophage stimulation

To characterize in depth the lipin-1α–containing LDs, a comparative study of colocalization with the LD markers ADRP and perilipin 3/TIP47 were carried out. Fig. 6 confirms that both lipin-1α and ADRP were present in LDs. The colocalization index of lipin-1α and ADRP, calculated with the LaserPix confocal image software (Bio-Rad), was 0.5 for both fluorescences. Perilipin 3/TIP47 is well described to be recruited to nascent LDs after stimulation of the cells (26, 60). Interestingly, lipin-1α–EGFP colocalized with perilipin 2/TIP47 on the surface of the LDs after a long-term treatment of the cells with oleic acid (Fig. 6*B*).

Previous results have demonstrated that in cells of murine origin, the subcellular localization of lipin-1 can be modified upon stimulation by insulin or oleic acid (38). To assess whether in human macrophages the localization of lipin-1α also changes upon cellular stimulation, the lipin-1α–EGFP–transfected cells were treated with the calcium ionophore ionomycin and PMA, two stimuli that induce strong macrophage activation (61). We also tested bacterial LPS, a well-known inducer of innate immune responses, and oleic acid complexed to albumin to induce LD formation. As shown in Fig. 6*C*, lipin-1α remained associated with the surface of LDs under all of these situations. Thus, in human macrophages, the subcellular localization of lipin-1α does not appear to depend on the activation state of the cell.

Lipin-1 does not participate in TAG synthesis in human macrophages

The Mg^{2+}-dependent PAP activity of lipins is known to participate in the de novo pathway of TAG biosynthesis by providing the DAG moiety that is acylated with a free fatty acid (3, 6). Because lipin-1α is present in LDs and these organelles are the main cellular storage sites of TAG, it is logical to assume a role for this particular form in regulating cellular TAG synthesis. To address this possibility, fatty acid incorporation experiments into TAG were performed in cells made deficient in lipin-1 by an siRNA approach (Fig. 7). The silencing efficiency of the siRNA against human lipin-1 used in these studies was very high, yielding the quantitative disappearance of lipin-1 expression after a 48-h treatment (Fig. 7*A*). Lipin-1–deficient macrophages were exposed to concentrations of [^{3}H]oleic acid in the low micromolar range (1–10 μM) so as to minimally perturb cellular lipid metabolism, and the rate of incorporation into TAG was measured and compared with that of control untreated cells. As shown in Fig. 8, no differences were detected in the incorporation of [^{3}H]oleic acid into TAG up to 90 min. The same experiment was repeated using another fatty acid, namely arachidonic acid, but again no differences were detected between control and lipin-deficient cells under any condition tested (not shown). To further substantiate this unexpected finding, we repeated the experiments utilizing peritoneal macrophages from *fld* mice, which constitutively lack lipin-1 due to a spontaneous mutation (7). Cells from *fld* mice not only did not show decreased incorporation of [^{3}H]oleic acid into TAG, but the response was actually slightly increased (Fig. 7*C*), raising the possibility of the existence of compensatory mechanisms for the lack of lipin-1 in these cells, as discussed elsewhere by others (14).

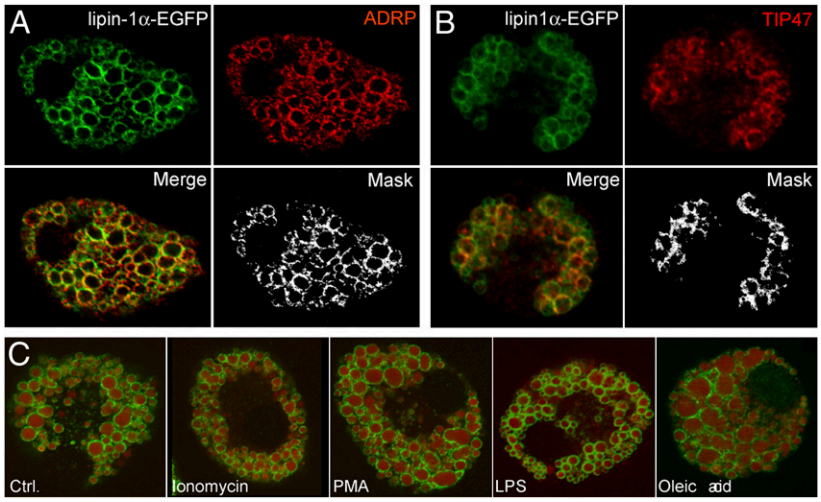

FIGURE 6. Human lipin-1α colocalizes with ADRP, and TIP47 and does not change its localization after cellular activation in LDs. Human macrophages transfected with lipin-1α–EGFP (green) were immunostained with Abs against ADRP (red) (*A*), or treated for 18 h with 200 μM oleic acid and immunostained with Abs against TIP47 (Alexa 555, red) (*B*), or treated with 1 μM ionomycin, 80 nM PMA, 100 ng/ml LPS, 200 μM oleic acid, or vehicle for 1 h as indicated and stained with with 100 ng/ml Nile Red (red) (*C*). Cells were then analyzed by confocal microscopy. A colocalization mask was generated by the confocal software (Bio-Rad) to show colocalization pixels (*A*, *B*). The cells shown are representative of many analyzed. Original magnification ×600.

Lipin-1 regulates the number and size of LDs in macrophages

Given the above results indicating that the blockade of lipin-1 expression does not affect the rate of incorporation of fatty acids into TAG, we speculated that lipin-1 could be implicated instead in regulating the physical properties of the LDs. To study this possibility, the lipin-1–deficient human macrophages were acutely exposed to a high concentration of oleic acid (100 μM), which induces rapid LD formation (34–36). Inspection of the LDs formed under these conditions revealed notable differences between lipin-1–depleted and control cells (Fig. 8). LDs in the lipin-1–deficient cells were smaller and localized more dispersedly throughout the cytoplasm than those produced by control cells (Fig. 8*A*). Quantitative measurements by flow cytometry confirmed a decreased production of LDs in lipin-1–deficient cells versus control cells in response to oleic acid treatment (Fig. 8*B*). Mean fluorescence from BODIPY493/503 doubled in control cells after oleic acid treatment (from 241 ± 10 to 454 ± 41), whereas the mean fluorescence from lipin-1–deficient cells increased modestly (from 374 ± 18 to 384 ± 15) (Fig. 8*B*). In agreement with all these findings, confocal microscopy analyses of LD in peritoneal macrophages from heterozygous and *fld* mice upon exposure to oleic acid revealed again that lipin-1 deficiency results both in decreased size, number, and in atypical distribution of cytoplasmic LDs (Fig. 8*C–E*).

In the next series of experiments, the effect of depleting lipin-1 on the fatty acid composition of TAG in LDs was studied. For these experiments, the various lipid classes were separated by thin-layer chromatography, and their fatty acid composition and mass were analyzed by gas chromatography/mass spectrometry after conversion to methyl esters. Fig. 9*A* shows the fatty acid distribution within TAG in LDs in untreated versus lipin-1–deficient cells. A small but significant increase ($p < 0.05$) in the levels of myristic acid (14:0) and palmitic acid (16:0) was appreciated in the LDs from lipin-1–deficient cells, which was counteracted by a decrease in linoleic acid (18:2 *n*-6). Of note, the fatty acid distribution of TAG in whole cells was essentially identical whether the cells were deficient in lipin-1 or not (Fig. 9*B*). The precise biological significance of these changes in the fatty acid composition of TAG in LDs remain to be elucidated, but they clearly emphasize that lipin-1 plays a role in regulating the TAG composition of LDs.

Given the above findings indicating that the silencing of lipin-1 alters the number, size, and TAG composition of LDs, but cellular TAG synthesis is not impaired, the possibility arises that lipin-1α–deficient cells may have a reduced capacity to incorporate TAG into LDs. To test this possibility, the accumulation of [^{3}H]oleic acid in isolated LDs was measured. In the otherwise untreated cells, incubation with 0.5 μCi/ml [^{3}H]oleic acid resulted in the accumulation of 27 ± 1% of the total radiolabel in the LD fraction after 24 h (mean ± SEM, $n = 3$). In contrast, in the lipin-1–deficient cells, the amount of [^{3}H]oleic acid incorporated into LDs under identical conditions as those described above was 19 ± 2% (mean ± SEM, $n = 3$). These data suggest that lipin-1 may be necessary for the proper incorporation of TAG into LDs.

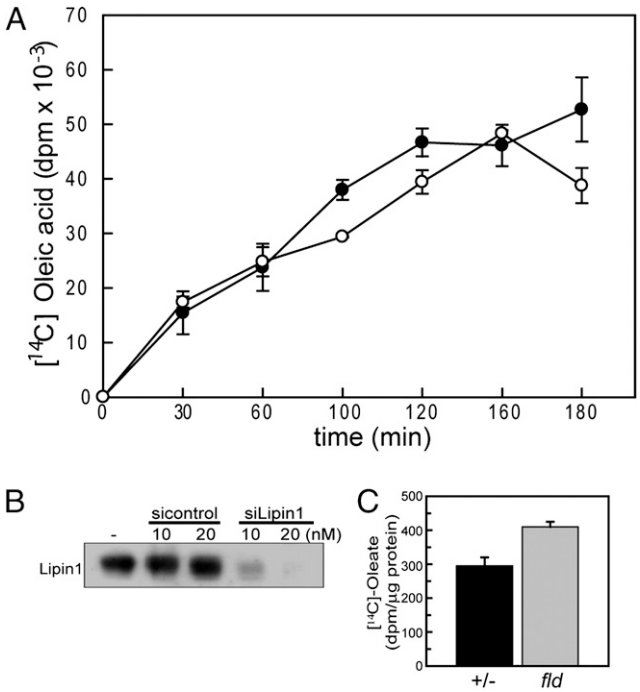

FIGURE 7. Depletion of lipin-1 does not decrease TAG synthesis in human macrophages. *A*, Human macrophages were treated with a siRNA negative control (20 nM, filled symbols) or with a siRNA against lipin-1 (20 nM, open symbols). After 48 h, the cells were treated with [^{14}C]oleate (1 μM) for the indicated times, and radioactivity incorporated in TAG was measured as described in *Materials and Methods*. *B*, Disappearance of lipin-1 from cell homogenates after cell treatment with siRNA against lipin. *C*, Peritoneal macrophages obtained from heterozygous (+/−, black bar) or *fld* animals (gray bar) were treated with [^{14}C]oleate (1 μM) for 1 h, and radioactivity incorporated in TAG was determined. The results are shown as means ± SEM of three independent determinations. Experiments are representative of at least three different ones.

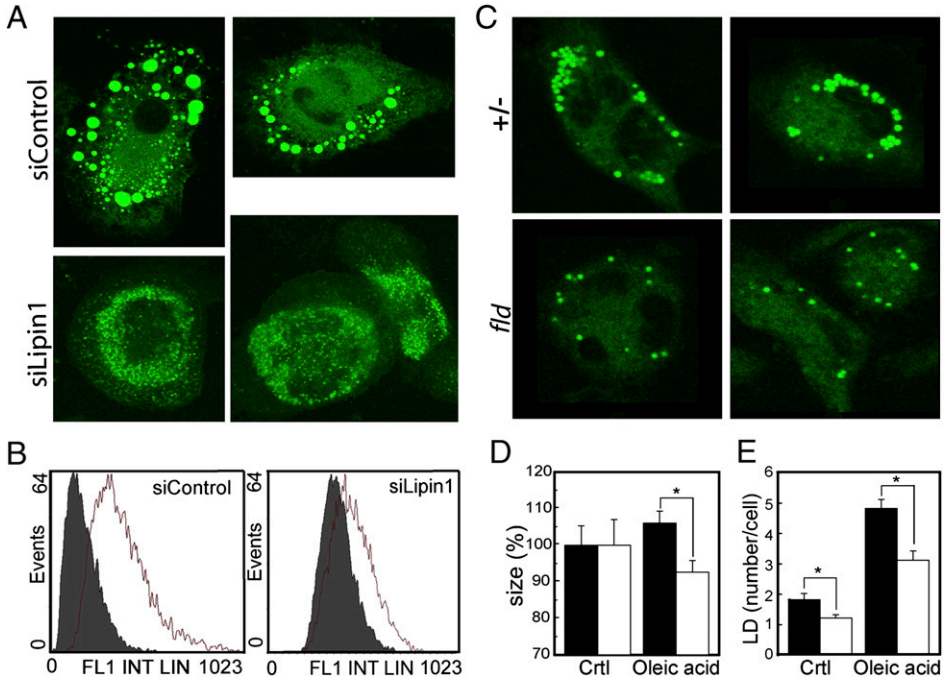

FIGURE 8. Lipin-1 expression levels influence LD size and number in macrophages. Human macrophages (*A*, *B*) treated with a negative control siRNA or with a siRNA against lipin-1 for 48 h, or peritoneal macrophages from heterozygous (+/−) or *fld* animals (*C*–*E*) were treated with 100 μM oleic acid for 18 h. Cells were then stained with BODIPY493/503 and analyzed by confocal microscopy (*A*, *C*). Fluorescence from human macrophages was also analyzed by flow cytometry. Original magnification ×600. Gray plots are from cells without oleic acid treatment and the open plots represent the oleic acid-treated cells (*B*). The size (*D*) and number (*E*) of LDs from peritoneal macrophages from heterozygous (filled bars) or *fld* animals (open bars) in untreated cells or treated with 100 μM oleic acid (as indicated) were evaluated under the microscope from >600 cells are shown as means ± SEM. *$p < 0.05$.

Lipin-1 regulates AA mobilization in human macrophages

We have recently shown that cPLA$_2$α regulates the biogenesis of LDs in a model cell system (34–36). Interestingly, previous work has demonstrated that, under certain conditions, cPLA$_2$α activation may occur downstream of a Mg^{2+}-dependent PAP enzyme via DAG generation (16, 18). Putting these observations together with the data presented in this study, we hypothesized that lipin-1 may act to modulate the cPLA$_2$α-mediated AA mobilization process. In keeping with this possibility, Fig. 10*A* shows that macrophage cells made deficient in lipin-1 by siRNA mobilized significantly less AA than did control cells upon treatment with 100 μM oleic acid, an inducer of LD formation, and with yeast-derived zymosan, an innate immunity stimulus. AA mobilization under these conditions was abolished by treating the cells with the selective inhibitor pyrrophenone (1 μM), confirming that cPLA$_2$α is the effector of the response (data not shown). Furthermore, cPLA$_2$α phosphorylation was found to be significantly reduced ($p < 0.05$) in homogenates from cells deficient in lipin-1, as measured by immunoblot using an Ab specific for the phosphorylated protein (Fig. 10*B*). In keeping with these observations, measurements of PGE_2 formation demonstrated that cells deficient in lipin-1 manifest a marked defect in eicosanoid generation (Fig. 10*C*). Altogether, these findings support the existence of a link between lipin-1 and inflammation.

The distribution of AA among glycerophospholipids in untreated versus lipin-1–deficient cells was studied by mass spectrometry (Fig. 11). The results demonstrated that lipin-1 deficiency did not significantly alter the distribution of AA among phospholipids, thus suggesting that diminished cPLA$_2$ activation in the lipin-1–deficient cells is unlikely due to altered availability of substrate.

Discussion

Lipins are well established to play important metabolic roles (3, 7). Since the discovery that these proteins possess intrinsic enzymatic activity as Mg^{2+}-dependent PAP enzymes (9), much interest has grown as to their participation in signal transduction events, particularly in those pathways where DAG and PA play defined roles. In this regard, this work describes lipin-1 as a key regulator of LD formation in human monocyte-derived macrophages, likely through the regulation of cPLA$_2$α-derived AA mobilization. We have also identified LDs as the cellular organelles where human lipin-1α resides. These findings provide new insights into the cell biology of lipin-1, particularly from an immunoinflammatory viewpoint.

The idea that Mg^{2+}-dependent PAP enzymes may participate in the regulation of cellular free AA availability has been the subject of active research over the years. More than a decade ago, we

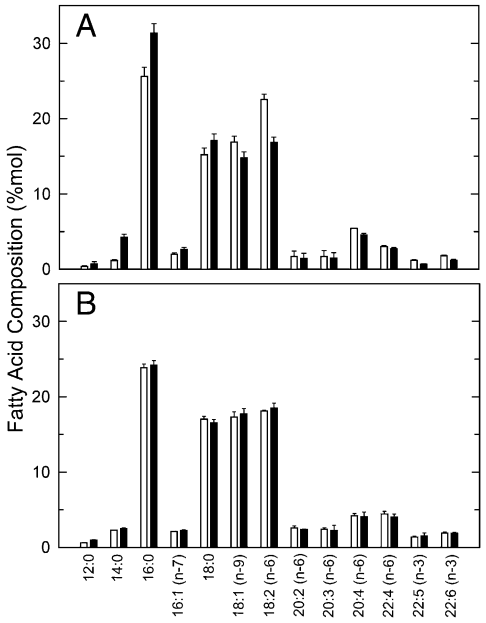

FIGURE 9. Fatty acid distribution of TAG in LDs (*A*) and whole macrophages (*B*). Human macrophages were transfected with 20 nM siRNA negative control (open bars) or 20 nM siRNA against lipin-1 (filled bars) for 48 h. Afterwards, the analysis of TAG fatty acids from LDs (*A*) or whole cells (*B*) was carried out by gas chromatography/mass spectrometry after converting the fatty acid glyceryl esters into fatty acid methyl esters. Data are expressed as means ± range of a representative experiment with duplicate determinations.

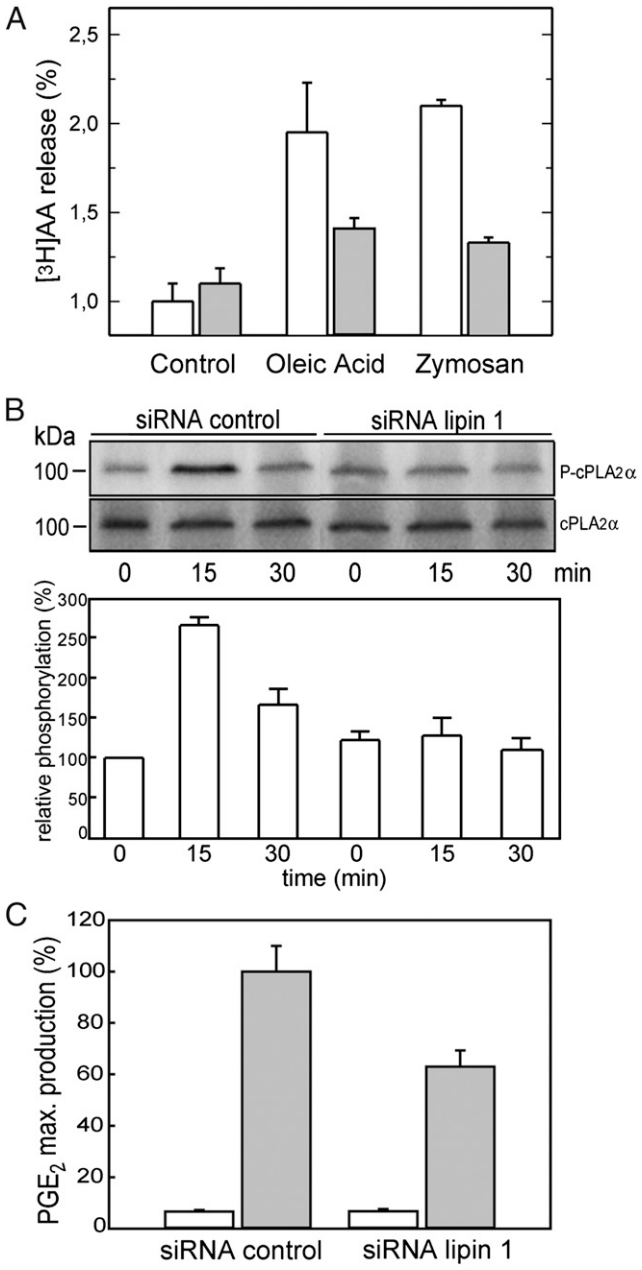

FIGURE 10. Lipin-1 regulates AA mobilization in human macrophages. *A*, Human macrophages transfected with 20 nM siRNA negative control (open bars) or 20 nM siRNA against lipin-1 (gray bars) for 48 h were labeled with [^{3}H]AA and treated with 100 μM oleic acid, 1 mg/ml opsonized zymosan, or vehicle (control) for 6 h, as indicated. Release of [^{3}H]AA was assayed as described in *Materials and Methods*. *B*, Transfected macrophages were stimulated with 1 mg/ml opsonized zymosan for the indicated periods of time, and cellular protein was analyzed by immunoblot using specific Abs for total cPLA$_2$α or the Ser^{505} phosphorylated form, as indicated. Relative quantification of the phosphorylated cPLA$_2$α bands is shown in the *lower panel*, normalized with respect to the total cPLA$_2$α content. *C*, Transfected macrophages were stimulated with 1 mg/ml opsonized zymosan for 18 h and cellular supernatants were assayed for PGE$_2$ content by ELISA. Data are expressed relative to the response observed in control untreated cells and are shown as means ± SEM of three independent determinations.

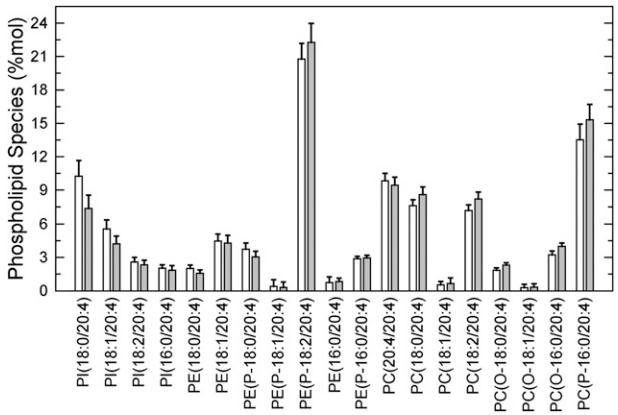

FIGURE 11. Mass spectrometry analysis of AA-containing glycerophospholipids in human macrophages. Human macrophages were transfected with 20 nM siRNA negative control (open bars) or 20 nM siRNA against lipin-1 (gray bars) for 48 h. Afterwards, the analysis of AA-containing phospholipids was carried out by ion-trap mass spectrometry. Data are expressed as means ± range of a representative experiment with duplicate determinations. Shorthand notation of glycerophospholipids (abscissa) follows the guidelines proposed by Fahy et al. (62).

demonstrated that pharmacological inhibition of PAP1 with propranolol or bromoenol lactone reduced AA mobilization in stimulated WISH cells (16). Similar results were reported more recently in U937 promonocytic cells exposed to bacterial LPS (18). At the time our experiments were performed, the PAP1 sequence was not known and, thus, no genetic experimental approaches could be undertaken. In the present work we have used human macrophages treated with oleic acid to induce LD formation, and we found that depleting the cells of lipin-1 by siRNA not only affects LD size, number, and TAG composition, but it also reduces the cPLA$_2$α-dependent AA release, thus providing genetic evidence for a regulatoy role of lipin-1 in these processes.

Lipin-1 could regulate cPLA$_2$α activation by two separate mechanisms. The first one is by generating DAG that could favor penetration of cPLA$_2$α into biological membranes, as discussed elsewhere (63, 64). cPLA$_2$α activity is known to be enhanced by at least 5-fold in in vitro assays when DAG is incorporated into the substrate (58), and the accumulation of DAG in cell membranes has been demonstrated to enable cPLA$_2$α to penetrate into densely packed membranes (64). Because cPLA$_2$α lacks a DAG docking domain, the suggestion is that DAG acts as a spacer to spread their polar headgroups apart, thus allowing cPLA$_2$α to penetrate into the membrane (64).

Another possibility is that lipin-1–derived DAG regulates cPLA$_2$α activity by activating kinases that influence, either directly or indirectly, the phosphorylation state of the enzyme, and hence its activity. Our results suggesting that depletion of lipin-1 results in reduced phosphorylation of cPLA$_2$α would be consistent with this possibility. Additionally, other results by others support this scenario. For example, during IgG-mediated phagocytosis by human monocytes, activation of DAG-dependent forms of protein kinase C precedes AA release (65). If protein kinase C or related kinases mediate the DAG effect on cPLA$_2$α, this would probably be an indirect effect, for example, via the MAPK pathway (66–68). In this regard, we have recently described the JNK-dependent phosphorylation activation of cPLA$_2$α in human monocyte-derived macrophages (39). Importantly, in other series of experiments we also found that JNK activation is required for LD production in serum-activated cells (36). Whether JNK activation provides a molecular link between cPLA$_2$α activation and proper LD formation under lipin-1 modulation is currently under active investigation in our laboratory.

Increasing evidence suggests that LDs may constitute intracellular sites for inflammatory eicosanoid biosynthesis (29). LDs from leukocytes have been found to contain significant amounts of

AA esterified in phospholipids, and some AA-metabolizing enzymes have been localized in part to LDs, including cyclooxygenase-2, 5- and 15-lipoxygenases, and PGE_2 synthase (29). Very recently, translocation of cPLA$_2$α to LDs has also been shown (35, 69). This work describes lipin-1α as another lipid signaling enzyme associated to LD membranes. Given the regulatory link between lipin-1α, LDs, and AA mobilization, it appears that the latter enzyme may exert key, previously unrecognized roles in regulating the inflammatory process.

With the exception of lipin-1α, all of the enzymes potentially implicated in the biosynthesis of TAG have been localized in the endoplasmic reticulum (70). Such a finding makes it difficult to envision a role for lipin-1α in homeostatic TAG biosynthesis, at least in the endoplasmic reticulum. Moreover, cells made deficient in lipin-1 by siRNA exhibit an incorporation of fatty acids into TAG that is indistinguishable from that occurring in cells displaying normal lipin-1 levels. Hence, another lipin may be involved in TAG synthesis in human macrophages, or compensatory mechanisms may occur to make up for the lack of lipin-1, that is, increases in either lipin-2 or lipin-3, as recently shown to occur in liver cells (14, 71). Collectively, our results would be compatible with a scenario where the homeostatic levels of TAGs would be governed by lipin-2/lipin-3, whereas the proper assembly of LDs would be a much more complex process that requires a signaling network where lipin-1, probably lipin-1α, has a unique role. The latter would be reminiscent of the situation described in 3T3 adipocytes undergoing differentiation (12, 14). In the preadipocyte stage, lipin-2 is expressed and maintains PAP cellular basal levels. However, when cells are induced to differentiate, lipin-2 disappears and lipin-1 expression is induced, controlling an intricate network of adipocyte differentiation together with TAG accumulation (12, 14).

Certain metabolic conditions such as obesity, metabolic syndrome, and insulin resistance have an important immune component. A good example of it is the recruitment of macrophages to the adipose tissue that takes place during obesity and that, by generating proinflammatory lipids and cytokines, is responsible for a low-grade adipose inflammation (72). In this study, we have shown that an obesity-related protein, lipin-1, also has important repercussions in the biology of the macrophages. These findings should add new information for the development of novel and effective drugs in the treatment of the aforementioned conditions.

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Disclosures

The authors have no financial conflicts of interest.

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