Lipid droplets (LD) are organelles present in virtually all cell types, consisting of a hydrophobic core of triacylglycerols and cholesterol. This work shows that LD biogenesis induced by serum, by long-chain fatty acids, or the combination of both in CHO-K1 cells was prevented by phospholipase A2 inhibitors of group IVA cytosolic phospholipase A2 (cPLA2\textsubscript{a}). Knocking down cPLA\textsubscript{a} expression with short interfering RNA was similar to pharmacological inhibition in terms of enzyme activity and LD biogenesis. A Chinese hamster ovary cell clone stably expressing an enhanced green fluorescent protein-cPLA\textsubscript{a} fusion protein (EGFP-cPLA\textsubscript{a}) displayed higher LD occurrence under basal conditions and upon LD induction. Induction of LD took place with concurrent phosphorylation of cPLA\textsubscript{a} at Ser\textsuperscript{505}. Transfection of a S505A mutant cPLA\textsubscript{a} showed that phosphorylation at Ser\textsuperscript{505} is key for enzyme activity and LD formation. cPLA\textsubscript{a} contribution to LD biogenesis was not because of the generation of arachidonic acid, nor was it related to neutral lipid synthesis. cPLA\textsubscript{a} inhibition in cells induced to form LD resulted in the appearance of tubulo-vesicular profiles of the smooth endoplasmic reticulum, compatible with a role of cPLA\textsubscript{a} in the formation of nascent LD from the endoplasmic reticulum.

For the full article, please refer to the original source.
of these is acyl-CoA synthetase (23–25). Inhibition of this enzyme with triacsin C abolishes the formation of LD in cells undergoing apoptosis (15), underlining the need for TAG synthesis in the genesis of LD. Phospholipase D1 has also been found associated with LD (26) and was shown to promote LD budding off from microsomes in a cell-free system, in a manner requiring TAG synthesis and an unidentified cytosolic factor (27). This factor was later identified as ERK2, working apparently downstream of phospholipase D1 to induce dynne association with LD (28). Cytosolic phospholipase A2 (cPLA2), on the other hand, has also been reported associated to LD (29, 30), although its implication in their biogenesis is not clear (31).

Key proteins essential for LD generation do not necessarily have to associate with them, however. In this regard, the TAG-synthesizing diacylglycerol-acyltransferase or the cholesteryl ester synthesizing acyl-CoA:cholesterol acyltransferase (ACAT), whose activities promote LD generation, are known to reside in the ER (32–34). Current models support that nascent LD form in close association with the ER membrane, either between the membrane leaflets (1, 3) or apposed to the bilayer (35). Either way, nascent LD should conceivably have a highly curved geometry, and their formation would involve active reorganization of the ER phospholipid composition to allow the formation of amphiphiles favoring this structure. With this working hypothesis, and taking into account that phospholipases A2 participate in many cellular events involving membrane reorganization and traffic (36), in this study we tested the possible implication of these fatty acid and lysophospholipid-generating enzymes in the formation of LD. Phospholipases A2 are a wide group of enzymes that share the capacity to hydrolyze glycerophospholipids at the sn-2 position to generate the corresponding 2-lysophospholipid and a free fatty acid (36–38). The 15 groups into which PLA2 enzymes have been classified according to nucleotide and amino acid sequence criteria include five distinct types of enzymes, namely the secreted PLA2, the cytosolic PLA2s (cPLA2), the Ca2+-independent PLA2, the platelet-activating factor acylhydrolases, and the lysosomal PLA2s (39).

Using flow cytometric analysis of Nile red-stained cells as a quantitative approach to monitor the occurrence of LD, we present pharmacological and molecular evidence showing the involvement of group IVA phospholipase A2 (cPLA2α) in the biogenesis of this organelle.

**EXPERIMENTAL PROCEDURES**

**Materials**—[56,8,9,11,12,14,15-3H]Arachidonic acid ([3H]AA) (200 Ci/mmol) was purchased from American Radiolabeled Chemicals, and [9,10-3H]palmitic acid (49 Ci/mmol) from Amersham Biosciences. PLA2 inhibitors methylarylidenon fluorophosphonate (MAFP) and bromoeno lactone (BEL) were from Cayman Chemical Co., and arachidonyl trifluoromethyl ketone (AAOCOF2) and pyridoline-2 (Py-2, catalog number 525143) were from Calbiochem. Rabbit anti-cPLA2α, anti-phospho-Ser505 cPLA2α antibodies were from Cell Signaling; chicken anti-ADRP was from BioWay Biotech; rabbit anti-GAPDH was from Ambion; mouse anti-BiP/GRP78 and mouse anti-flotillin-1 were from BD Biosciences, and mouse anti-β-actin was from Sigma. Sodium oleate, sodium arachidonate, palmitic acid, tripalmitin, primuline, triacsin C, and Nile red were from Sigma, and 4,4-difluoro-5-methyl-4-bora-3a,aia diaza-s-indencene-3-dodecanonic acid (C3-BODIPY® 500/510-C12) was from Molecular Probes.

**Cells**—CHO-K1 cells were cultured in Ham’s F-12 medium (Sigma), containing 7.5% fetal bovine serum (FBS, Sigma) or lipoprotein-deficient serum from fetal calf (Sigma), 100 units/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). Cell passages were made once a week by trypsinization (Sigma). When indicated, cells (40–70% confluence) were transfected with 1 μg of plasmid/ml using Lipofectamine Plus™ (Invitrogen), following the manufacturer’s instructions. For stably transfected cells (CHO-cPLA2 and HEK-cPLA2), 1 mg/ml G418 (Invitrogen) was used for selection and subsequent passages. Other cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma), containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. These cell types were human embryo kidney (HEK) cells and HEK cells stably expressing EGFP-cPLA2 (40, 41), primary astrocytes from rat cerebral cortex (42), human neuroblastoma SH-SY5Y (ECACC number 94030304), and rat smooth muscle embryonic aorta A7r5 (ECACC number 86050803) cells, from the European Collection of Cell Cultures, and human chronic B cell leukemia EHEB cells (DSMZ number ACC 67) from the German Collection of Microorganisms and Cell Cultures.

**Nile Red Staining and Fluorescence Microscopy**—Cells cultured on glass bottom culture dishes were washed with phosphate-buffered saline (PBS, Sigma), fixed with 3% paraformaldehyde for 10 min, and washed twice with PBS. Cells were overlaid with 0.5 ml of PBS, to which 2.5 μl of a stock solution of Nile red in acetone (0.2 mg/ml) was added, so that the final concentrations of Nile red and acetone were 1 μg/ml and 0.5%, respectively. Samples were kept in the dark until photographed in a Leica Qwin 500 microscope with a Leica DC200 camera, using the Leica Dviewer 3.2.0.0 software.

**Confocal Microscopy**—Serum-starved CHO-K1 cells were treated for 6 h with 7.5% FBS and 1 μM C2-BODIPY-C12, either in the absence or presence of MAFP. After two washes with PBS, cells were fixed as outlined above and photographed in a Leica TCS SP2 AOBs confocal microscope. To monitor re-location of EGFP-cPLA2, serum-deprived CHO-PLA2 cells were treated with 7.5% FBS for 1 h, and then 5 μM ionomycin was added. Images were acquired every 60 s.

**Image Analysis**—Analysis of LD in photomicrographs was performed with ImageJ 1.38x public software (Wayne Rasband, National Institutes of Health; rsb.info.nih.gov), as illustrated in supplemental Fig. S3.

**Electron Microscopy**—Cells were rinsed twice with 0.1 M phosphate-buffered saline (PBS), pH 7.4, and fixed with PBS containing 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde for 2 h at 4°C. After four 10-min washes in PBS, cells were postfixed with 1% osmium tetroxide in PBS for 2 h at 4°C, washed in PBS, dehydrated through an ascending series of acetone concentrations up to 100%, and included in EPON resin. Micrographs were taken with a JEOL JEM-2011 electron microscope equipped with a CCD GATAN 794 MSC 600HP camera.
Flow Cytometry—After each treatment, cells were harvested, washed with PBS, and fixed with 3% paraformaldehyde for 10 min. After two PBS washes, they were resuspended in 1 ml of PBS, to which 5 μl of the stock solution of Nile red was added (final concentration, 1 μg/ml). Samples were kept at least 45 min in the dark to attain equilibrium with the dye. Analysis was carried out with a Cytomics FC 500 (Beckman Coulter) equipped with an argon laser (488 nm), in the FL1 channel (505–545 nm), with the photomultiplier set at 600 V and a gain value of 1. After gating out cellular debris, 30,000 events were taken in all the assays. Given the high avidity of the dye for plastic tubing, and to avoid interference with other flow cytometry applications, a specific pickup tube was used whenever Nile red-stained samples were analyzed.

[^3]H Arachidonic Release—Serum-starved cells, seeded in 24-well plates, were labeled with 0.25 μCi of[^3]H]AA (0.5 μCi/ml) for 24 h, then washed once with PBS, incubated for 5 min with Ham’s F-12 supplemented with 0.5 mg/ml albumin, and washed twice more with PBS (41). Radioactivity in the last wash was subtracted from released[^3]H]AA over the stimulation period. Cells were then treated as described in each experiment. At the end of the treatments, culture media were taken, centrifuged, and counted. Cell monolayers were detached with ice-cold PBS containing 1% Triton X-100 and also counted for radioactivity. Stimulated[^3]H]AA release represents a balance between what has been liberated to the medium and what has been incorporated into the cells.

Cellular Fractionation—Harvested cells were washed with PBS and homogenized with a 10-s sonication in 0.6 ml of 10 mM Tris-HCl, containing 0.25 mM sucrose, 1 mM EDTA, and protease inhibitors. The homogenate was centrifuged 1 h at 20,000 × g. The supernatant was kept aside, and the pellet was resuspended in 0.6 ml of homogenization buffer. 0.2 ml of each fraction was used for Western blot of marker proteins, and lipids were extracted from the remaining volume.

Thin Layer Chromatography—Cells were harvested on ice, washed with 1 ml of PBS, and pelleted for extraction of lipids (43). To separate the major lipid species, 0.2-ml aliquots of the chloroform phases were evaporated under vacuum, resuspended in 15 μl of chloroform/methanol (3:1, v/v), and spotted onto Silica Gel G thin layer chromatography plates (Merck), which were developed in hexane/diethyl ether/acetic acid (70:30:1, v/v), and stained with iodine vapor or with primuline spray which were developed in hexane/diethyl ether/acetic acid (70:30:1, v/v), and stained with iodine vapor or with primuline spray. Identification of phospholipids, diacylglycerol, cholesterol, free fatty acids, TAG, and cholesteryl esters was made by co-migration with authentic standards. Quantification of radioactive lipids was done by scraping into vials the silica gel from regions corresponding to migration of the standards. Primuline-stained TAG was quantified by densitometry after acquiring images under UV (340 nm) light.

Immunoblots—Cells were lysed with 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% bromphenol blue, and around 20 μg of protein were separated by standard 10% SDS-PAGE and transferred to nitrocellulose membranes. Primary (1:1,000) and secondary antibodies (1:5,000) were diluted in 25 mM Tris-HCl buffer, pH 7.4, containing 140 mM NaCl, 0.5% defatted dry milk, and 0.1% Tween 20, with the exception of ADRP antibody, which was blocked with 0.5% bovine serum albumin. Membranes were developed using ECL detection reagents from Amersham Biosciences and visualized using a GeneGenome HR chemiluminescence detection system coupled to a CCD camera.

Constructs—The construct codifying for the expression of a fusion protein containing N-terminal enhanced green fluorescence protein (EGFP) followed by the entire sequence of the human cPLA2α (EGFP-cPLA2) was described elsewhere (40, 41). To obtain the construct for EGFP-S505A-cPLA2, wild-type cPLA2 was mutagenized by replacing Ser505 with Ala, using the QuikChange XL site-directed mutagenesis kit (Stratagene) and the oligonucleotides 5’-CAA TAC ATC TTC ACC ATT GCC GCC TTC TTT GAG TGA CTT-3’ (forward) and 5’-GCA AAG TCA CTC AAA GGC AGT GGA TAA GAT GTA-3’ (reverse). Mutagenesis was confirmed by sequencing.

siRNA Transfection—Two pre-designed siRNAs (Gene Link) directed against human cPLA2α were used as follows: sense and antisense PLA2G4A2-(2424) (siRNA1), and sense and antisense PLA2G4A1-(1329) (siRNA2). Cells were transfected at 60% confluence by adding to each 35-mm culture well 1 ml of Opti-MEM (Invitrogen) containing 1.5 μl of the stock siRNA solution (20 μM) and 5 μl of Lipofectamine Plus™ (1 mg/ml). After 5 h, 1 ml of Ham’s F-12 medium containing 7.5% FBS was added, and the cells were incubated for 48 h and then changed to serum-free medium during 24 h prior to stimulation with FBS. For the assays of cPLA2 activity, prelabeling with[^3]H]AA was done during these last 24 h. In some experiments, a siRNA directed against human GAPDH (Ambion) was used as control.

Calcium Imaging—Cells grown onto polylysine-coated coverslips were incubated with the calcium indicator Fura-2/AM at 4 μM in Krebs buffer of the following composition (in mM): 119 NaCl, 4.75 KCl, 5 NaHCO3, 1.2 MgSO4, 1.18 KH2PO4, 1.3 CaCl2, 20 Heps, and 5 glucose, pH 7.4. After 1 h, cells were washed and coverslips mounted in a static chamber on an inverted Nikon TE2000U microscope of a conventional epifluorescence system. Cells were excited alternatively at 340 and 380 nm, and emission light was collected at 510 nm every 10 s using a 12-bit-CCD ERG ORCA Hamamatsu camera. Ratio image of cells was analyzed using the Metafluor software (Universal Imaging). 14–20 cells were analyzed in each experiment.

Statistical Analysis—Data analysis was carried out with Prism software (GraphPad). Responses among different treatments were analyzed with one-way analysis of variance followed by Bonferroni’s multiple comparison test.

RESULTS

Flow Cytometry as a Tool to Quantify LD—Our aim was to study LD dynamics under regular culture conditions, avoiding whenever possible the induction of cellular stress, which has been shown to induce the formation of LD. It has long been known that cells accumulate LD from serum lipoproteins (10), and therefore our first goal was to set an experimental system with the highest variation in LD content among quiescent, serum-starved cells and cells treated with FBS. For this purpose, we examined various cell models for the occurrence of LD, which we labeled with the lipophilic dye Nile red. To quantify
this, we monitored initially the percentage of cells containing two or more LD (13) at various days after plating in serum-containing medium and after serum withdrawal (supplemental Fig. S1). Using this criterion, 100% CHO-K1 cells cultured in FBS-containing medium were LD-positive from day 1 onward. Serum deprivation at day 2 reduced LD-containing cells after 24 h to only 10–15%. HeLa cells and primary astrocytes also reduced LD content after serum deprivation but to a lesser extent. We therefore decided to explore deeper the mechanisms of LD formation when serum-deprived CHO-K1 cells were challenged with 7.5% FBS.

The criterion to consider LD-positive every cell containing two or more LD may be convenient as a first approach, but it does not discriminate cells containing more than two droplets or cells containing LD of different sizes. This is the case when comparing LD content in cells treated with FBS in the presence or absence of 100 μM sodium oleate, as shown in Fig. 1. Serum-deprived cells were virtually devoid of LD (Fig. 1A), whereas a 6-h treatment with 7.5% FBS induced LD in most cells (Fig. 1B). The same is true when LD formation was induced with FBS together with an overload of exogenous sodium oleate (Fig. 1C). However, the overall occurrence of LD was higher with the latter treatment, as evidenced by simple visual inspection or by the level of expression of ADRP (Fig. 1D). These two conditions were discriminated after image analysis of the photomicrographs (Fig. 1E; see also supplement Fig. S2) or after indirect quantification with cell cytometry (Fig. 1, F and G). Compared with serum-starved cells, the right shifts of the fluorescence profiles, shown in Fig. 1F, indicate a stronger signal in the presence of oleate than in its absence, and this can be quantified after the median value of each distribution of events (Fig. 1G). LD induction by both treatments was abolished in the presence of the acyl-CoA synthetase inhibitor triacsin C, as evidenced by microscopic examination (not shown) and the left shift of the fluorescence distributions (Fig. 1, H and I), which were similar to those from serum-starved cells. Furthermore, the time course of LD formation after addition of FBS was easily monitored by flow cytometry, with an increase in the fluorescent signal up to 16 h (supplemental Fig. S3, A and D). Oleate in the presence of FBS induced a faster increase of the Nile red-stained cells is a very accurate method for the indirect quantification of LD.

**Inhibition of cPLA2 Abolishes the Release of Arachidonate but Not Palmitate from Cells Treated with FBS**—To determine the possible implication of cPLA2 in the mechanisms of LD formation, we first looked for fatty acid-releasing activities induced by FBS. Exposure of serum-starved CHO-K1 cells to FBS induced the release of [3H]AA and [3H]palmitic acid, but only release of [3H]AA was inhibited by a 10 μM concentration of the cPLA2 inhibitor methyl arachidonyl fluorophosphonate (MAFP) (supplemental Fig. S4, A and B). 10 μM arachidonyl trifluoromethyl ketone (AACOCF3) and 1 μM pyrrolidine-2 (Py-2), but not 10 μM BEL, also inhibited [3H]AA release (supplemental Fig. S4C). Unlike complete FBS, lipoprotein-deficient serum did not stimulate the release of [3H]AA (not shown). Although AACOCF3 and MAFP inhibit cPLA2 and iPLA2 (groups IVA and VI, respectively) (44), Py-2 is relatively specific for group IVA PLAs (cPLA2α) (45), although it has been shown that it also inhibits group IVF (cPLA2γ) (46). In contrast, BEL is an inhibitor specific for iPLA2 (group VI) (44). These results suggest that FBS stimulates cPLA2α.

**Inhibition of PLA2 Precludes FBS-stimulated Formation of LD**—To test pharmacologically the implication of cPLA2α in the appearance of LD, we designed experiments to show inhibitor concentration-effect relationships for the reversal of LD induction by FBS (Fig. 2). For this purpose, we treated serum-starved cells (see Fig. 1A) with FBS in the presence of inhibitors and measured LD by flow cytometry. AACOCF3 (Fig. 2, A and B), MAFP (Fig. 2, C and D), and Py-2 (Fig. 2, E and F) inhibited the formation of LD in a concentration-dependent fashion that allowed the calculation of IC50 values (0.98, 0.29, and 0.11 μM, respectively). 10 μM BEL had no effect in the formation of LD as assessed by flow cytometry (not shown) or microscopic inspection (compare micrographs G and H in Fig. 2 for the effects of BEL and MAFP, respectively). Furthermore, MAFP but not BEL inhibited the increase of ADRP induced by FBS (Fig. 2I). We found no evidence of cytotoxicity because of 24-h treatments with 10 μM concentrations of AACOCF3 and MAFP or 1 μM Py-2 either in serum-starved cells, in the presence of 7.5% FBS, or with 7.5% FBS plus 100 μM oleate, as assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction assay (not shown).

**Silenced Expression of cPLA2 Inhibits the Formation of LD**—Nonpharmacological inhibition cPLA2α was undertaken by silenced expression of the enzyme. For this purpose, we transfected two different siRNAs and, after serum deprivation, looked for cPLA2α protein and activity and LD formation in response to FBS (Fig. 3). Of the two siRNAs, only one (siRNA1) reduced the expression of cPLA2α and also of ADRP (Fig. 3A). Silenced expression paralleled the reduction in [3H]AA release (Fig. 3B) and LD occurrence (Fig. 3, C and D) to levels similar to
those in serum-starved cells or in FBS-stimulated cells in the presence of MAFP. These results, together with the pharmacological approach, are consistent with the implication of cPLA<sub>2</sub>/H9251 in LD biogenesis.

Overexpression of cPLA<sub>2</sub>/α Enhances the Occurrence of LD—Additional evidence, summarized in Fig. 4, came from a CHO-K1-derived cell clone (CHO-cPLA<sub>2</sub>) stably expressing an EGFP-cPLA<sub>2</sub>/α fusion protein (40, 41). As shown in Fig. 4A (left), the bigger size of the transfected protein, with an apparent molecular mass of 135 kDa, made it easily discriminated from the endogenous enzyme, with an apparent molecular mass of 105 kDa. When maintained in medium containing 7.5% FBS, this clone expressed a higher level of ADRP than the parental line (Fig. 4A, right). In addition to calcium at the micromolar level, cPLA<sub>2</sub>/H9251 is regulated positively by phosphorylation at Ser<sup>505</sup> (38). In agreement with this, phospho-Ser<sup>505</sup>-cPLA<sub>2</sub> increased as starved CHO-K1 cells were exposed to FBS (Fig. 4B). Unlike the phosphorylated endogenous enzyme, unambiguous detection of EGFP-cPLA<sub>2</sub> phosphorylated at Ser<sup>505</sup> in CHO-cPLA<sub>2</sub> cells was somewhat hampered by a nonspecific band of the same size also appearing in CHO-K1 cells. Despite this, it is apparent that serum-starved CHO-cPLA<sub>2</sub> cells maintained a higher level of phosphorylated enzyme than CHO-K1 cells, and also that phosphorylation of both endogenous cPLA<sub>2</sub> and EGFP-cPLA<sub>2</sub> increased in CHO-cPLA<sub>2</sub> cells in response to FBS. Importantly, FBS did not alter the nonspecific band present in CHO-K1 lysates. In close agreement with phosphorylation results, release of radioactivity from [³H]AA-prelabeled CHO-cPLA<sub>2</sub> cells was 2.5-fold that of CHO-K1 cells under serum-starved conditions and 2.2-fold after stimulation with FBS, and this effect was sensitive to MAFP inhibition (Fig. 4C). Regarding LD occurrence, CHO-cPLA<sub>2</sub> cells closely paralleled data obtained in [³H]AA release.
found that phosphorylation at Ser505 is relevant for enzyme activity and LD biogenesis, we silenced expression of cPLA2 with siRNA(1) but not with siRNA(2), (A). Silenced expression with siRNA(1) was in line with decreased cPLA2 activity, similar to the inhibition of control cells with MAFP, and decreased capacity of FBS to induce LD in CHO-K1 cells and 1.7-fold after stimulation with FBS (Fig. 5C and D). B represents means ± S.E. of three independent experiments with triplicate determinations. Data in D are means ± S.E. of the median values of the event distributions obtained in independent experiments. *, significantly different (p < 0.05) from serum-starved conditions.

In agreement with these different calcium responses, FBS did not induce any apparent change in the cellular distribution of EGFP-cPLA2, in contrast with the translocation to nuclear and perinuclear membranes induced by ionomycin (supplemental Fig. S5).

cPLA2 Is Not Involved in the Synthesis of Neutral Lipids during LD Biogenesis—To test whether the role of cPLA2 in the biogenesis of LD is to provide AA for neutral lipid synthesis (TAG and cholesteryl esters), we assayed the ability of exogenous AA to induce LD in serum-starved cells and also to stimulate cPLA2 (Fig. 6). AA at a 10 μM concentration induced the increase of ADRP, and also the phosphorylation of cPLA2 at Ser505 (Fig. 6A). Higher concentrations (100 μM) of the fatty acid were toxic in the absence of FBS, resulting in 60% reduction in cell viability over 6 h (data not shown). Exogenous AA (10 μM) also stimulated the release of [3H]AA (Fig. 6B), in an MAFP-sensitive manner, to a level similar to that attained with 7.5% FBS, and this response was stronger at 100 μM AA in the presence of 7.5% FBS. Results on cPLA2 activity were mirrored by

cPLA2α was phosphorylated at Ser505 but S505A-cPLA2α was not. Furthermore, AA release as stimulated by FBS in cells transfected with S505A-cPLA2α was similar to that in cells transfected with EGFP alone, and significantly lower than in EGFP-cPLA2α-transfected cells (Fig. 5B). Likewise, LD occurrence in cells transfected with EGFP-S505A-cPLA2α was the same as that in cells transfected with EGFP alone and significantly lower than in EGFP-cPLA2α-transfected cells (Fig. 5, C and D). These results show a key role of Ser505 phosphorylation of cPLA2α for enzyme activation and LD biogenesis.
**Group IVA PLA₂ and Lipid Droplet Biogenesis**

**A**

<table>
<thead>
<tr>
<th>CHO-K1</th>
<th>CHO-cPLA₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>135 kDa</td>
<td>cPLA₂</td>
</tr>
<tr>
<td>105 kDa</td>
<td></td>
</tr>
<tr>
<td>36 kDa</td>
<td>GAPDH</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>CHO-K1</th>
<th>CHO-cPLA₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>135 kDa</td>
<td></td>
</tr>
<tr>
<td>105 kDa</td>
<td>P-Ser505-cPLA₂</td>
</tr>
<tr>
<td>42 kDa</td>
<td>Actin</td>
</tr>
</tbody>
</table>

**C**

![](chart.png)

**D**

(1) CHO-K1: Starved
(2) CHO-cPLA₂: Starved + MAFP
(3) CHO-cPLA₂: Starved
(4) CHO-cPLA₂: FBS + MAFP
(5) CHO-cPLA₂: FBS

**E**

![](chart.png)

**F**

![](image.png)

**G**

![](image.png)
Group IVA PLA₂ and Lipid Droplet Biogenesis

FIGURE 5. Phosphorylation of cPLA₂α in Ser⁵⁰⁵ is required for the formation of LD. CHO-K1 cells were transiently transfected with plasmids encoding EGFP alone, EGFP-cPLA₂, or EGFP-S505A-cPLA₂, and maintained in FBS (A) or deprived from serum for 24 h prior to stimulation with FBS for 6 h (B–D). A, Western blot of total cPLA₂, phospho-Ser⁵⁰⁵-cPLA₂, and GAPDH as a loading control. B, AA release from cells containing EGFP alone, EGFP-cPLA₂, or EGFP-S505A-cPLA₂ over a 6-h stimulation with FBS. C and D, indirect quantification of lipid droplets after 6 h with FBS. Results are representative of three independent experiments (A and C) or means ± S.E. of three independent experiments (B and D). *, significantly different (p < 0.01) from EGFP-transfected cells.

FIGURE 4. Overexpression of cPLA₂α enhances the occurrence of LD. A CHO-K1-derived clone stably expressing EGFP-cPLA₂α (A and B) displayed enhanced basal (under serum-starved conditions) and FBS-induced cPLA₂ activity (C) and enhanced LD occurrence under serum-starved or FBS-stimulated conditions (D–G). A and B show Western blots of cell lysates from CHO-K1 and CHO-cPLA₂ cells against cPLA₂ (A) or phospho-Ser⁵⁰⁵-cPLA₂ (B), the former from cells in medium containing 7.5% FBS, and the latter from serum-starved or FBS-treated cells. GAPDH (A) or actin (B) was used as loading controls. C shows means ± S.E. of three independent experiments with triplicate determinations and represents radioactivity released into the medium from [³H]AA-prelabeled CHO-K1 or CHO-cPLA₂ cells during a 6-h stimulation with 7.5% FBS, and with or without 20 μM MAFP. D and E show LD indirect quantification in CHO-K1 or CHO-cPLA₂ cells treated for 6 h with 7.5% FBS and with or without 20 μM MAFP and represent event distributions in a representative experiment (D) or means ± S.E. of the median values of the event distributions (increase above control) in three independent experiments, respectively. F and G show LD in CHO-cPLA₂ cells maintained in serum-free medium or in medium containing 7.5% FBS, respectively. Note the high occurrence of LD in serum-starved cells. Scale bar, 10 μm. *, significantly different (p < 0.01) from serum-starved conditions; #, significantly different (p < 0.01) from serum-induced LD in CHO-cPLA₂; $, significantly different (p < 0.05) from serum-starved CHO-cPLA₂.

Fatty Acids into Neutral Lipids—A different possibility we considered is that cPLA₂α could be required at LD in the channeling of fatty acids from the medium into the synthesis of TAG and cholesteryl esters from the medium into the synthesis of TAG and cholesteryl esters. This was confirmed by following the incorporation of [³H]AA among the main neutral lipids after a 24-h prelabeling period, followed by a 6-h stimulation with FBS to induce LD (Fig. 6E); about 17,000 dpm were released to the medium in an MAFP-sensitive manner, indicative of cPLA₂α stimulation. However, barely 70 dpm were incorporated into TAG and even less into cholesteryl esters under these LD-inducing conditions. The results show that forming LD content is not a major fate of AA released by cPLA₂α.

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Group IVA PLA₂ and Lipid Droplet Biogenesis

A

105 kDa
50 kDa
36 kDa

P-Ser505-cPLA₂
ADRP
GAPDH

B

\[ \frac{3}{H} \text{AA release} \]

(CAR

\% incorporated)

\[
\begin{array}{cccc}
\text{FBS} & \text{AA,} \mu \text{M} & \text{MAFP} & \\
- & 100 & - & + \\
+ & 10 & - & + \\
+ & 10 & + & + \\
\end{array}
\]

C

Control
AA+MAFP
AA+MAFP+
FBS+AA+MAFP
FBS+AA
FBS

D

Increase of the median
above control

\[
\begin{array}{cccc}
\text{FBS} & \text{AA,} \mu \text{M} & \text{MAFP} & \\
- & 100 & - & + \\
+ & 10 & - & + \\
+ & 10 & + & + \\
\end{array}
\]

E

TAG

FBS
MAFP

Released

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\begin{array}{cccc}
\text{FBS} & \text{MAFP} & \\
- & - & \\
+ & + & \\
\end{array}
\]

FIGURE 6. cPLA₂α is not involved in the synthesis of neutral lipids during LD biogenesis. A, serum-starved CHO-K1 cells were exposed for 6 h to 10 \( \mu \)M AA or to 100 \( \mu \)M AA in combination with 7.5% FBS, and phospho-Ser505-cPLA₂ and ADRP were detected by Western blot. B, serum-starved CHO-K1 cells were labeled 24 h with \({^3}H\)AA and then treated for 6 h with 10 \( \mu \)M AA or with 100 \( \mu \)M AA in combination with 7.5% FBS and in the presence or absence of 10 \( \mu \)M MAFP. These same treatments were used to quantify the occurrence of LD by flow cytometry (C and D). C shows the event distribution profiles in FL1 of a representative experiment, whereas the means \( \pm \) S.E. of the median values of three event distributions are shown in D. E shows the event distribution profiles in FL1 of a representative experiment, whereas the means \( \pm \) S.E. of the median values of three event distributions are shown in D. E, serum-starved CHO-K1 cells were labeled 24 h with \({^3}H\)AA and treated for 6 h with 7.5% FBS in the absence or presence of 10 \( \mu \)M MAFP. Radioactivity in TAG (left) and cholesteryl esters (center) was compared with radioactivity released to the medium, indicative of cPLA₂ activity (right). *, significantly different \((p < 0.01)\) from serum-starved conditions; #, significantly different \((p < 0.01)\) from serum plus AA conditions.

mainly into phospholipids, and this was inhibited by MAFP to some extent, probably because of the housekeeping activity of MAFP-sensitive iPLA₂, which is involved in an ongoing decylation-reacylation cycle for phospholipid remodeling (49). It is noticeable, however, that at all three concentrations of the fatty acid, regardless of whether they were enough to induce LD formation (Fig. 7C) or not (Fig. 7, A and B), the inhibition of cPLA₂α did not decrease \({^3}H\)AA incorporation into TAG or cholesteryl esters. Rather, there was a tendency, although it did not reach significance, of increased TAG labeling in the presence of MAFP. These results suggest that, during LD biogenesis, cPLA₂α is necessary at a step beyond the synthesis of neutral lipids. This was confirmed after measuring total TAG content in serum-starved cells and in cells treated for 6 h with FBS plus 100 \( \mu \)M AA, either in the absence or presence of MAFP (Fig. 7, D and E). Induction of LD with FBS plus oleate took place with an increase in TAG from 5.6 ± 0.6 to 9.3 ± 0.3 \( \mu \)g/2 \( \times \) 10⁵ cells, and it was not altered by inhibition of LD biogenesis by MAFP (9.5 ± 1.1 \( \mu \)g/2 \( \times \) 10⁵ cells). These results show that cPLA₂α is not involved in the channelling of extracellular fatty acids into neutral lipids during LD biogenesis.

Inhibition of cPLA₂α under LD-forming Conditions Alters ER Structure—As cPLA₂α did not affect neutral lipid synthesis, it could be required at a later step to allow LD formation from the ER. To test this, we monitored the distribution of the fluorescent fatty acid C₁₂-BODIPY-C₁₂ inside the cells during a 6-h stimulation with FBS. As shown in Fig. 8, A–D, the tracer incorporated mainly into LD-like structures but was retained in intracellular membranes in the presence of MAFP. An ultrastructural study revealed that treatment with FBS together with 100 \( \mu \)M oleate during 10 h induced massive appearance of LD and the development of smooth ER, often in close apposition to LD (Fig. 8, E and F; see also supplemental Fig. S6). When LD formation was partially inhibited by MAFP (see supplemental Fig. S6, C and D, showing that LD are not totally abolished after this overload of fatty acid), there was a marked development of tubulovesicular structures, probably related to the smooth ER, which filled practically all the cell (Fig. 8, G and H; see also supplemental Fig. S7G), eventually forming aberrant membrane stacks (supplemental Fig. S7H). In contrast, serum-starved cells contained no LD, a well defined rough ER, and a poorly developed smooth ER (supplemental Fig. S7, A and B). Treatment of serum-starved cells with MAFP revealed no alterations in the intracellular membrane compartments (supplemental Fig. S7, C and D). Consistent with the ultrastructural study, a simple cellular fractionation of FBS and oleate-treated cells showed that inhibition of cPLA₂α promoted re-distribution of TAG from a cytosol-enriched to a membrane-enriched fraction (Fig. 8, I–L).
MAFP Inhibits LD Biogenesis in all Cell Types Tested—Finally, we tested in other cell types the protocol of LD induction by FBS during 6 h and the reversion of this response by MAFP (Table 1). LD content decreased in all cells upon serum withdrawal for 24 h (not shown). Addition of FBS increased LD-associated fluorescence of Nile red in all cells, and this was sensitive to inhibition by MAFP. Of particular interest is the comparison among the LD occurrence in HEK cells and HEK stably transfected with EGFP-cPLA2 (HEK-cPLA2), where the latter showed a general increase in LD occurrence either in serum-starved or FBS-stimulated conditions, consistent with data on CHO-cPLA2 cells (Fig. 4, D and E). Also, transfection of siRNA(1) and also of siRNA(2), the latter without effect in Chinese hamster ovary cells, decreased cPLA2α expression (not shown) and LD in human SHSY5Y cells (Table 1). Taken together, and considering the ubiquitous expression of cPLA2α in mammalian tissues (47), the results suggest a general implication of the enzyme in LD biogenesis.

**DISCUSSION**

An important drawback in the study of LD is the limited choice of quantitative methods. Reliable quantification of LD visualized in cells stained with lipophilic dyes has been reported after thorough analysis of droplet and cell dimensions (13) or a careful image treatment (28). There has been a limited use of flow cytometry, however, for quantification purposes. Nile red is a lipophilic dye widely used in the study of LD, with an emission spectrum that shifts to shorter wavelengths in hydrophobic environments. When Nile red-stained cells are examined at wavelengths of 580 nm or less, the fluorescence of the probe interacting with the extremely hydrophobic environment of LD is maximized, whereas that of cellular membranes is minimized (50, 51). This makes Nile red a suitable probe for indirect quantification of LD by flow cytometry (32). In fact, fluorescence intensities of the event distributions have shown a close agreement with LD under microscopic examination and with NMR signals (52). A similar indirect quantification of LD by flow cytometry in BODIPY-stained D3922 cells undergoing apoptosis has been reported (53). This technique offers the advantage of a rapid analysis of multiple samples each consisting of thousands of cells. Also, Nile red staining of the cells does not require treatment with organic solvents that could extract LD or simply provide fatty acids for the generation of TAG or cholesteryl esters, the main lipids contained in LD. Induction of LD with lipoproteins present in FBS or with exogenous fatty acids...
Group IVA PLA₂ and Lipid Droplet Biogenesis

FIGURE 8. Precluding LD formation by the inhibition of cPLA₂ alters the structure of the endoplasmic reticulum and re-distributes TAG from cytosolic to membrane compartments. A–D, serum-starved Chinese hamster ovary K1 cells were treated for 6 h with 7.5% FBS and 1 μM C₇-BODIPY 500/510 C₁₂, in the absence (A and B) or presence of 10 μM MAFP (C and D), fixed in paraformaldehyde, and visualized in the confocal microscope. Scale bars are as follows: A and C, 47.62 μm; B, 12.42 μm; D, 27.31 μm. E–H, cells were treated 10 h with 7.5% FBS plus 100 μM oleate in the absence (E and F) or presence of 10 μM MAFP (G and H), fixed and processed for electron microscopy. N and LD denote nuclei and lipid droplets, respectively; arrows, smooth ER; arrowheads, abnormal tubulovesicular structures. Magnification: E, ×20,000; F, ×80,000; G, ×12,000; H, ×40,000. I–L, serum-starved cells were left untreated or treated 10 h with 7.5% FBS plus 100 μM oleate, and with or without 10 μM MAFP, then homogenized and centrifuged 1 h at 20,000 × g to obtain pellet and supernatant fractions, denoted as P and S, respectively. I shows a representative Western blot of 15 μg of protein from P and S fractions, which were enriched in flotillin-1 and GAPDH, respectively. Ctrl, control. TAG from both fractions were separated by TLC (I) and quantified (J). K, n.s., not significant. MAFP did not affect total TAG content (K), but induced a re-distribution of TAG from supernatant (L, open bars) to the pellet fraction (L, solid bars). Lanes 1–3 in I correspond to 1, 5, and 10 μg of tripalmitine standard. Asterisks in L denote the significant (p < 0.001) effect of MAFP on TAG distribution among pellet and supernatant fractions.

(arachidonate, olate, and palmitate) can be regarded as a simple channeling of material into neutral lipids that are stored in LD, and therefore the role of cPLA₂α in LD biogenesis is less obvious than merely providing AA for neutral lipid synthesis. In contrast to fatty acids, lipoproteins are internalized by receptor-mediated endocytosis, and in this regard a BEL-sensitive PLA₂ has been found required for vesicle fusion (55) and sorting (56) along the endocytic pathway, an effect that may be mimicked by exogenous AA supplementation. Clearly this is not the present case, because (a) we show that BEL is not effective to block LD biogenesis; and (b) more importantly, fatty acids in the absence of lipoproteins also promote LD appearance. These observations strongly argue against a link between cPLA₂α and LD biogenesis involving lipoprotein metabolism.

Group VI PLA₂ (iPLA₂) was our best candidate in initial experiments, because it is involved in membrane traffic events other than the endocytic pathway, including retrograde membrane movement from the Golgi apparatus to the ER (56) or phagosome formation (57). However, although AACOCF₃ and MAFP inhibit cPLA₂α and iPLA₂, BEL is selective for iPLA₂ and had no effect on LD biogenesis. In contrast to iPLA₂, cPLA₂α is considered the key enzyme mediating AA release for the production of eicosanoids (38). As mentioned earlier, LD develop in cells associated with inflammation, and it has been suggested that LD may be a source for inflammatory precursors (17). In this regard, we have found that cyclooxygenase inhibitors (20 μM indomethacin or 500 μM ibuprofen) are unable to mimic the effect of cPLA₂α inhibitors in blocking the biogenesis of LD induced by FBS,₆ suggesting that, although LD may serve as an AA-rich reservoir for the initiation of inflammatory cascades, eicosanoid production is not involved in their biogenesis. This is in keeping with Bozza et al. (17), who showed that, although aspirin inhibited fatty acid-induced LD formation, this effect was independent of COX inhibition. We also took into account the possibility that AA generated by cPLA₂α could act as a ligand for peroxisome proliferator-activated receptor-γ and mediate lipogenesis and LD formation (58). Again, we found that treatment with the peroxisome proliferator-activated receptor-γ agonist pioglitazone at 50 μM did not induce LD over a 6-h treatment nor did it potentiate the effect of FBS; also the antagonist GW9662 at 10 μM had no effect.₆ Long-chain polyunsaturated fatty acids have been shown to regulate ADRP expression (59), and therefore the role of cPLA₂α could be to promote ADRP expression after the generation of AA. We have shown, however, that addition of exogenous AA, which by itself induced LD, did not restore LD biogenesis either in MAFP-treated cells or after knocking down cPLA₂α expression. There-

₆ A. Gubern and E. Clar, unpublished observations.
fore, the role of cPLA2α in the biogenesis of LD induced either by lipoproteins present in serum, fatty acids at subtoxic concentrations, or the combination of serum and higher fatty acid concentrations does not seem related to the generation of AA or its metabolites.

Our results reveal that TAG and cholesteryl ester synthesis can be dissociated from LD occurrence, because we found no MAFP-sensitive difference in [3H]AA incorporation into these lipid species under LD-forming and nonforming conditions. The ability to synthesize TAG, together with the incapacity to form LD, is most probably the basis for the altered smooth ER structure. This situation is clearly different from the inhibition of acyl-CoA synthetase with triacsin C, which abolishes LD formation together with that of TAG and cholesteryl esters (15). Interestingly, Nile red fluorescence was able to discriminate ER- from LD-associated TAG. We found that triacsin C precluded LD formation in FBS- and FBS plus oleate-treated cells, and this effect was similar to that of MAFP in terms of Nile red fluorescence profiles. As cPLA2α inhibition does not affect TAG synthesis, this indicates that the hydrophobicity of excess TAG accumulating in the ER is closer to that of membranes than that of LD. Keeping this in mind, our finding that inhibition of cPLA2α decreases ADRP content fits with that of MAFP in terms of Nile red fluorescence. However, because of the higher LD content, the photomultiplier voltage was set at 550 V instead of 600 V.

Another question arising is how cPLA2α is activated by serum lipoproteins or free fatty acids, the two LD-forming conditions used in this study. Regulation of cPLA2α (see Ref. 38 and references therein) is because of increases in cytosolic calcium concentrations, which interact with a C2 domain of the protein and promote its membrane association to access the phospholipid substrate. Besides, phosphorylation on Ser505 plays a relevant role under transient, physiological submicromolar [Ca2+]i, increasing the phospholipid binding affinity of the enzyme (47), but it appears less important in response to higher sustained [Ca2+]i. Our results show that addition of FBS to serum-starved cells stimulates cPLA2α in a manner that requires phosphorylation on Ser505, and we have obtained pharmacological and molecular evidence showing that this event involves c-Jun kinase.7 This aspect may have been overlooked before, as most studies are done in cells maintained in complete medium, and conceivably phosphorylation is already present in control conditions. Future efforts to address what role [Ca2+]i and perhaps signaling lipids like phosphatidylinositol 4,5-bisphosphate (40, 64) and ceramide 1-phosphate (65) play in cPLA2α activation, and also what are the upstream events leading to cPLA2α phosphorylation, will contribute to clarifying the mechanisms of LD biogenesis.

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REFERENCES


Either way, the elucidation of the precise role of cPLA2α in these processes awaits further investigation. Both mechanisms, droplet formation from the ER and fusion of already formed ones, would be favored by PL2α-generated lyso phospholipids, because of their inverted cone shape that may drive the formation of positive membrane curvature (36, 62). A similar mechanism has been proposed for the calcium-dependent, MAFP-sensitive PL2α implicated in Golgi vesiculation induced by cholesterol (63).

**TABLE 1**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>Control</th>
<th>10% FBS</th>
<th>10% FBS, 10 μM MAFP</th>
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<tr>
<td>Primary astrocytes</td>
<td></td>
<td>519 ± 29</td>
<td>719 ± 42</td>
<td>594 ± 70</td>
</tr>
<tr>
<td>HEK</td>
<td></td>
<td>243 ± 2</td>
<td>303 ± 5</td>
<td>255 ± 5</td>
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<td>HEK-cPLA2</td>
<td></td>
<td>408 ± 2</td>
<td>488 ± 6</td>
<td>366 ± 8</td>
</tr>
<tr>
<td>B cell leukemia EHEB</td>
<td></td>
<td>317 ± 1</td>
<td>477 ± 5</td>
<td>418 ± 8</td>
</tr>
<tr>
<td>Smooth muscle A7r5</td>
<td></td>
<td>227 ± 8</td>
<td>295 ± 5</td>
<td>260 ± 4</td>
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<tr>
<td>Hepatoma HepG2</td>
<td></td>
<td>387 ± 18</td>
<td>618 ± 13</td>
<td>430 ± 23</td>
</tr>
<tr>
<td>Neuroblastoma H11006</td>
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<td>209 ± 9</td>
<td>330 ± 13</td>
<td>226 ± 28</td>
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<tr>
<td>Neuroblastoma H11006-siRNA(1)</td>
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<td>236 ± 13</td>
<td>254 ± 17</td>
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<tr>
<td>Neuroblastoma H11006-siRNA(2)</td>
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<td>212 ± 8</td>
<td>230 ± 9</td>
<td></td>
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<tr>
<td>Neuroblastoma H11006-siRNA(3)</td>
<td></td>
<td>198 ± 12</td>
<td>350 ± 20</td>
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</table>

* Different from control (p < 0.05).
* Different from 10% FBS (p < 0.05).
* Because of the higher LD content, the photomultiplier voltage was set at 550 V instead of 600 V.