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Group IVA Phospholipase A_2 Is Necessary for the Biogenesis of Lipid Droplets*[S]

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Lipid droplets (LD) are organelles present in all cell types, consisting of a hydrophobic core of triacylglycerols and cholesteryl esters, surrounded by a monolayer of phospholipids 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 A_2 inhibitors with a pharmacological profile consistent with the implication of group IVA cytosolic phospholipase A_2 ($cPLA_2\alpha$). Knocking down $cPLA_2\alpha$ 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-$cPLA_2\alpha$ fusion protein (EGFP-$cPLA_2$) displayed higher LD occurrence under basal conditions and upon LD induction. Induction of LD took place with concurrent phosphorylation of $cPLA_2\alpha$ at Ser^{505}. Transfection of a S505A mutant $cPLA_2\alpha$ showed that phosphorylation at Ser^{505} is key for enzyme activity and LD formation. $cPLA_2\alpha$ contribution to LD biogenesis was not because of the generation of arachidonic acid, nor was it related to neutral lipid synthesis. $cPLA_2\alpha$ 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 $cPLA_2\alpha$ in the formation of nascent LD from the endoplasmic reticulum.

Lipid droplets (LD)[5] are organelles present in virtually all cell types, are formed by a hydrophobic core of triacylglycerols (TAG) and cholesteryl esters, and are surrounded by a monolayer of phospholipids and cholesterol with which a variety of proteins interact (1–3). LD are considered storage organelles for energy generation and membrane-building blocks, although new roles in protein storage and sorting have been proposed recently (4, 5). LD are small in most cells ($<1\ \mu m$), but a single cell may contain hundreds of them, contrasting with the big droplet of adipocytes, the main TAG-storing cells in animals. LD have received increased interest in the last years, fueled by their involvement in the pathogenesis of diseases related to fat storage like obesity, atherosclerosis, and diabetes (6, 7) and possibly in neurodegenerative disorders like Parkinson (8) and Alzheimer diseases (9).

Most cells in culture form LD whenever there is lipid availability from the medium. When maintained in serum-deprived conditions, cells are practically devoid of LD, which appear upon addition of complete serum, containing lipoproteins. LD induced by serum increase in number and size when free fatty acids are supplemented at nontoxic concentrations (10, 11). Lipid availability is not the only physiological parameter governing the occurrence of LD. Stress has been shown in many instances to induce LD formation; cells reaching confluence (12, 13), undergoing apoptosis (14, 15), exposed to acidic pH (10, 16), or engaged in inflammation (17) are some examples. In fact, cellular stress detected by means of NMR, where mobile lipids in LD generate specific signals (16, 18), is the basis for promising imaging techniques for tumor diagnosis and treatment (18, 19).

Over the past years, a number of proteins associated with LD have been characterized. Among them, the best known is the perilipin-adipophilin-TIP47 family of proteins, termed collectively PAT (3, 20). Adipophilin, also called adipose differentiation-related protein (ADRP), is a constitutive PAT protein, which is degraded in the absence of LD, and therefore expression levels of this protein reflect the mass of stored neutral lipids (21). Unlike perilipin, which is specific for adipocytes and steroidogenic cells, ADRP is expressed ubiquitously, and it is not involved in hormone action (22).

A number of additional proteins have been found associated with LD fractions, including lipid-metabolizing enzymes. One

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[5] The abbreviations used are: LD, lipid droplets; AA, arachidonic acid; $AACOCF_3$, arachidonyl trifluoromethyl ketone; ADRP, adipose differentiation-related protein; BEL, bromoenol lactone; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; MAFP, methylarachidonyl fluorophosphonate; Py-2, pyrrolidine-2; TAG, triacylglycerol; cPLA2, cytosolic phospholipase A_2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, short interfering RNA; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; HEK, human embryo kidney.

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 new LD. Phospholipase D_1 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 D_1 to induce dynein association with LD (28). Cytosolic phospholipase A_2 ($cPLA_2$), 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 A_2 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 A_2 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 PLA_2 enzymes have been classified according to nucleotide and amino acid sequence criteria include five distinct types of enzymes, namely the secreted PLA_2, the cytosolic PLA_2s ($cPLA_2$), the Ca^{2+}-independent PLA_2, the platelet-activating factor acetylhydrolases, and the lysosomal PLA_2s (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 A_2 ($cPLA_2\alpha$) in the biogenesis of this organelle.

EXPERIMENTAL PROCEDURES

Materials—[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid ([^{3}H]AA) (200 Ci/mmol) was purchased from American Radiolabeled Chemicals, and [9,10-^{3}H]palmitic acid (49 Ci/mmol) from Amersham Biosciences. PLA_2 inhibitors methylarachidonyl fluorophosphonate (MAFP) and bromoenol lactone (BEL) were from Cayman Chemical Co., and arachidonyl trifluoromethyl ketone ($AACOCF_3$) and pyrrolidine-2 (Py-2, catalog number 525143) were from Calbiochem. Rabbit anti-$cPLA_2\alpha$ and anti-phospho-Ser^{505} $cPLA_2\alpha$ antibodies were from Cell Signaling; chicken anti-ADRP was from GenWay 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-3*a*,4*a*-diaza-*s*-indacene-3-dodecanoic acid (C_1-BODIPY® 500/510-C_{12}) 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-$cPLA_2$ and HEK-$cPLA_2$), 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-$cPLA_2$ (40, 41), primary astrocytes from rat cerebral cortex (42), human neuroblastoma SH-5YSY (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 DCviewer 3.2.0.0 software.

Confocal Microscopy—Serum-starved CHO-K1 cells were treated for 6 h with 7.5% FBS and 1 μM C_1-BODIPY-C_{12}, 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-$cPLA_2$, serum-deprived CHO-PLA_2 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 where 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 M 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 were 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 (5 mg of primuline in 100 ml of acetone/water (80:20, v/v)). 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 fluorescent protein (EGFP) followed by the entire sequence of the human cPLA$_2$α (EGFP-cPLA$_2$) was described elsewhere (40, 41). To obtain the construct for EGFP-S505A-cPLA$_2$, wild-type cPLA$_2$ was mutagenized by replacing Ser^{505} with Ala, using the QuikChange XL site-directed mutagenesis kit (Stratagene) and the oligonucleotides 5′-CAA TAC ATC TTA TCC ACT G**G**C GCC TTT GAG TGA CTT-3′ (forward) and 5′-GCA AAG TCA CTC AAA GGC G**C**C AGT GGA TAA GAT GTA-3′ (reverse). Mutagenesis was confirmed by sequencing.

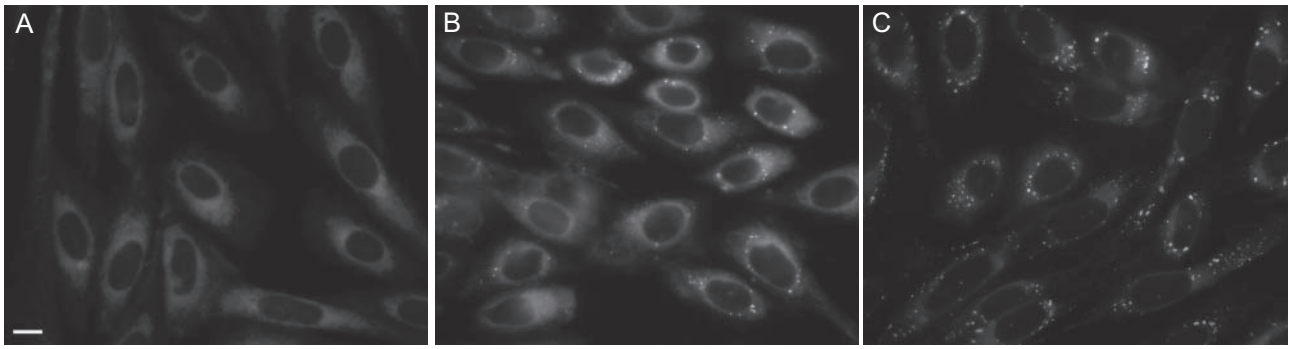
siRNA Transfection—Two pre-designed siRNAs (Gene Link) directed against human cPLA$_2$α were used as follows: sense and antisense PLA2G4A2-(2424) (siRNA1), and sense and antisense PLA2G4A1-(1329) (siRNA2). Cells were transfected at 60% confluence by adding to each 35-mm culture well 1 ml of Opti-MEM (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 cPLA$_2$ 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.

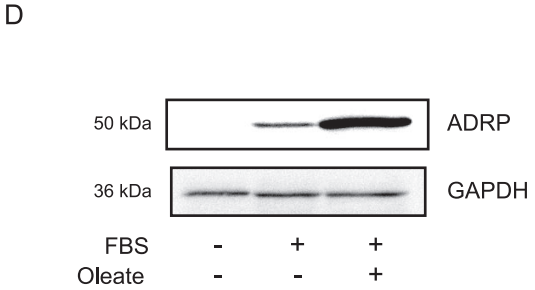
Calcium Imaging—Cells grown onto polylysine-coated coverslips were incubated with the calcium indicator Fura-2/AM at 4 μM in Krebs buffer of the following composition (in mM): 119 NaCl, 4.75 KCl, 5 $NaHCO_3$, 1.2 $MgSO_4$, 1.18 KH_2PO_4, 1.3 $CaCl_2$, 20 Hepes, and 5 glucose, pH 7.4. After 1 h, cells 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.

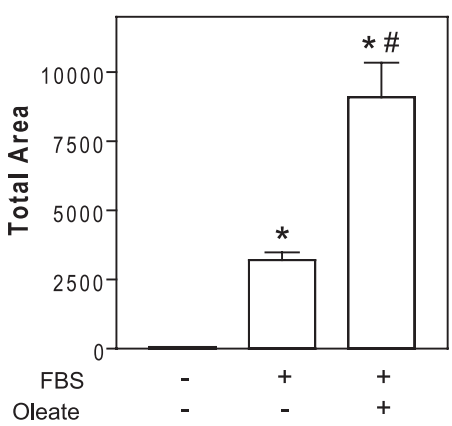
Statistical Analysis—Data analysis was carried out with Prism software (GraphPad). Responses among different treatments were analyzed with one-way analysis of variance followed by Bonferroni's multiple comparison test.

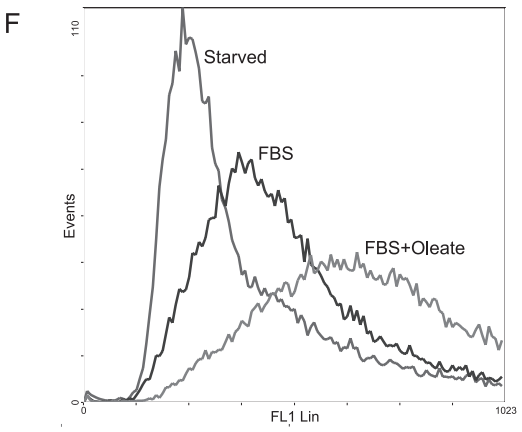
RESULTS

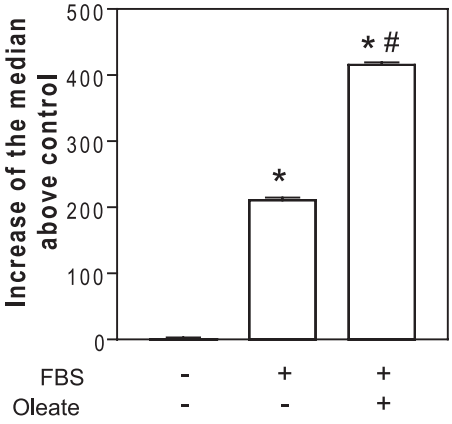
Flow Cytometry as a Tool to Quantify LD—Our aim was to study LD dynamics under regular culture conditions, avoiding whenever possible the induction of cellular stress, which has been shown to induce the formation of LD. It has long been known that cells accumulate LD from serum lipoproteins (10), and 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

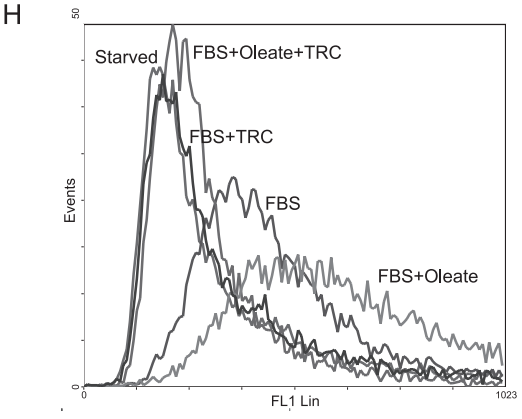


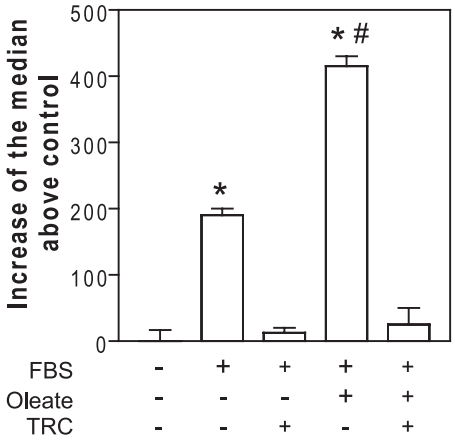












this, we monitored initially the percentage of cells containing two or more LD (13) at various days after plating in serum-containing medium and after serum withdrawal (supplemental Fig. S1). Using this criterion, 100% CHO-K1 cells cultured in FBS-containing medium were LD-positive from day 1 onward. 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. 1*A*), whereas a 6-h treatment with 7.5% FBS induced LD in most cells (Fig. 1*B*). The same is true when LD formation was induced with FBS together with an overload of exogenous sodium oleate (Fig. 1*C*). 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. 1*D*). These two conditions were discriminated after image analysis of the photomicrographs (Fig. 1*E*; 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. 1*F*, 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. 1*G*). 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-associated fluorescence (supplemental Fig. S3, *B* and *D*). As expected, treatment with lipoprotein-deficient serum did not induce LD, quantified either by microscopic examination (not shown) or by flow cytometry (supplemental Fig. S3, *C* and *D*). Overall, the remarkable reproducibility of the data and the high number of cells that can be analyzed in a single fluorescence profile (30,000 events were acquired for each sample) show that flow cytometric analysis of Nile red-stained cells is a very accurate method for the indirect quantification of LD.

Inhibition of $cPLA_2$ Abolishes the Release of Arachidonate but Not Palmitate from Cells Treated with FBS—To determine the possible implication of PLA_2 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 [^{3}H]AA and [^{3}H]palmitic acid, but only release of [^{3}H]AA was inhibited by a 10 μM concentration of the $cPLA_2$ inhibitor methyl arachidonyl fluorophosphonate (MAFP) (supplemental Fig. S4, *A* and *B*). 10 μM arachidonyl trifluoromethyl ketone ($AACOCF_3$) and 1 μM pyrrolidine-2 (Py-2), but not 10 μM BEL, also inhibited [^{3}H]AA release (supplemental Fig. S4*C*). Unlike complete FBS, lipoprotein-deficient serum did not stimulate the release of [^{3}H]AA (not shown). Although $AACOCF_3$ and MAFP inhibit $cPLA_2$ and $iPLA_2$ (groups IVA and VI, respectively) (44), Py-2 is relatively specific for group IVA PLA_2 ($cPLA_2\alpha$) (45), although it has been shown that it also inhibits group IVF ($cPLA_2\zeta$) (46). In contrast, BEL is an inhibitor specific for $iPLA_2$ (group VI) (44). These results suggest that FBS stimulates $cPLA_2\alpha$.

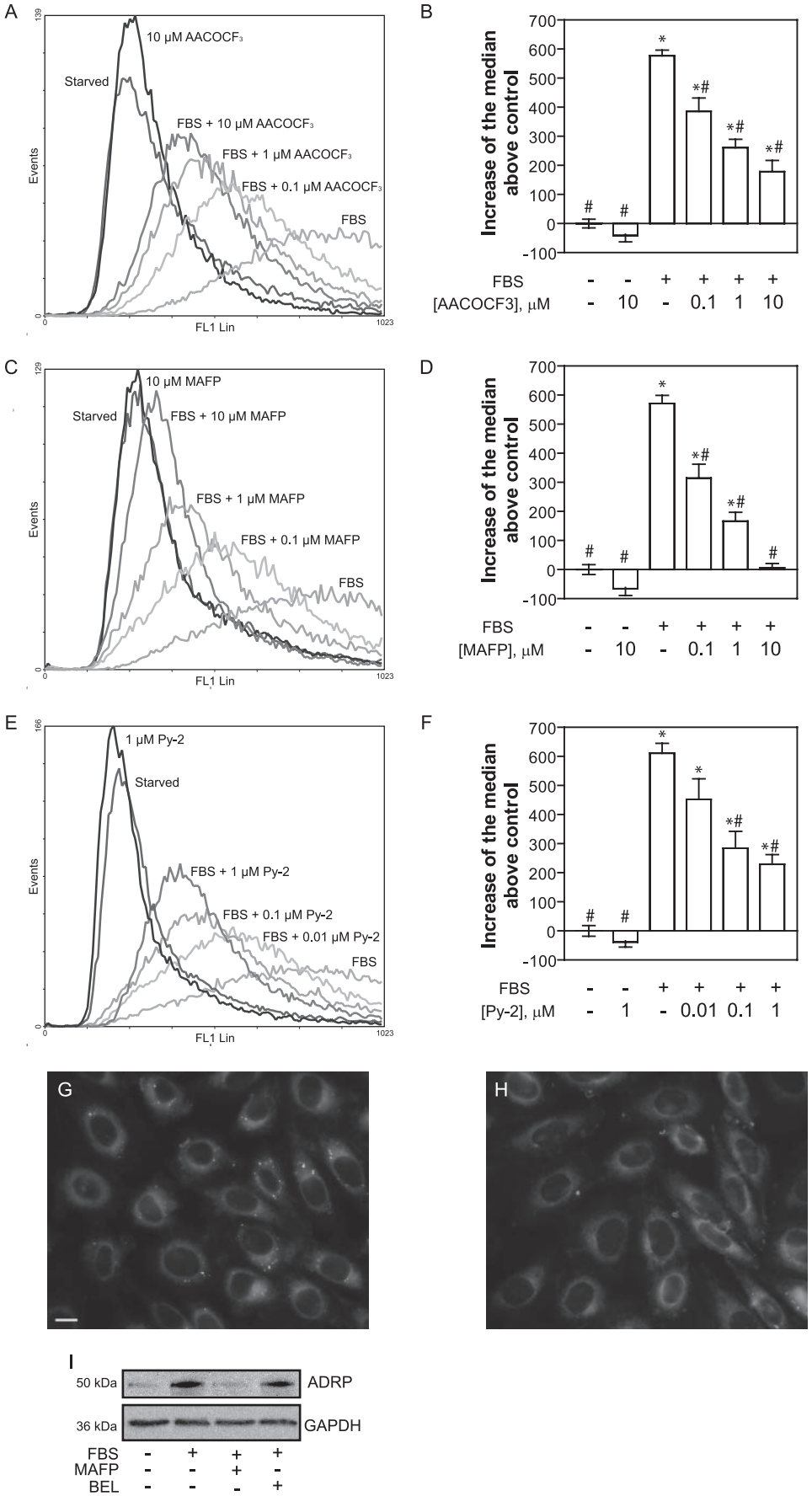
Inhibition of PLA_2 Precludes FBS-stimulated Formation of LD—To test pharmacologically the implication of $cPLA_2\alpha$ in the appearance of LD, we designed experiments to show inhibitor concentration-effect relationships for the reversal of LD induction by FBS (Fig. 2). For this purpose, we treated serum-starved cells (see Fig. 1*A*) with FBS in the presence of inhibitors and measured LD by flow cytometry. $AACOCF_3$ (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 IC_{50} 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. 2*I*). We found no evidence of cytotoxicity because of 24-h treatments with 10 μM concentrations of $AACOCF_3$ 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 $cPLA_2$ Inhibits the Formation of LD—Nonpharmacological inhibition $cPLA_2\alpha$ was undertaken by silenced expression of the enzyme. For this purpose, we transfected two different siRNAs and, after serum deprivation, looked for $cPLA_2\alpha$ protein and activity and LD formation in response to FBS (Fig. 3). Of the two siRNAs, only one (siRNA1) reduced the expression of $cPLA_2\alpha$ and also of ADRP (Fig. 3*A*). Silenced expression paralleled the reduction in [^{3}H]AA release (Fig. 3*B*) and LD occurrence (Fig. 3, *C* and *D*) to levels similar to

FIGURE 1. **Indirect LD quantification by flow cytometry.** Serum-starved CHO-K1 cells were kept untreated (*A*) or treated for 6 h with medium containing 7.5% FBS alone (*B*) or in combination with 100 μM sodium oleate (*C*). *Scale bar* represents 10 μm. A Western blot of ADRP in total cellular extracts under these three conditions is shown in *D*. After fixation, cells were stained with Nile red, and the presence of LD was quantified either by ImageJ analysis of total LD area in 10 micrographs from each condition (*E*) or by flow cytometry (*F* and *G*). *F* shows the distributions of 30,000 events for each condition in the FL1 channel, in linear scale, obtained in a representative experiment. Fluorescence intensities, quantified as the median value of each event distribution, are presented in *G*, which shows means ± S.E. of the median obtained in three independent experiments. *H* and *I*, cells were treated as in *F* and *G*, but in the absence or presence of 5 μM triacsin C (*TRC*). *H* shows the distribution profiles of 10,000 events; and *I* shows the means ± S.E. of the median values in three experiments. *, $p < 0.001$ compared with control in the absence of FBS and oleate; #, $p < 0.001$ compared with FBS alone.

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$_2\alpha$ in LD biogenesis.

Overexpression of cPLA$_2\alpha$ Enhances the Occurrence of LD—Additional evidence, summarized in Fig. 4, came from a CHO-K1-derived cell clone (CHO-cPLA$_2$) stably expressing an EGFP-cPLA$_2\alpha$ fusion protein (40, 41). As shown in Fig. 4*A* (*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. 4*A*, *right*). In addition to calcium at the micromolar level, cPLA$_2\alpha$ is regulated positively by phosphorylation at Ser^{505} (38). In agreement with this, phospho-Ser^{505}-cPLA$_2$ increased as starved CHO-K1 cells were exposed to FBS (Fig. 4*B*). Unlike the phosphorylated endogenous enzyme, unambiguous detection of EGFP-cPLA$_2$ phosphorylated at Ser^{505} in CHO-cPLA$_2$ 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$_2$ cells maintained a higher level of phosphorylated enzyme than CHO-K1 cells, and also that phosphorylation of both endogenous cPLA$_2$ and EGFP-cPLA$_2$ increased in CHO-cPLA$_2$ 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 [^{3}H]AA-prelabeled CHO-cPLA$_2$ 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. 4*C*). Regarding LD occurrence, CHO-cPLA$_2$ cells closely paralleled data obtained in [^{3}H]AA release



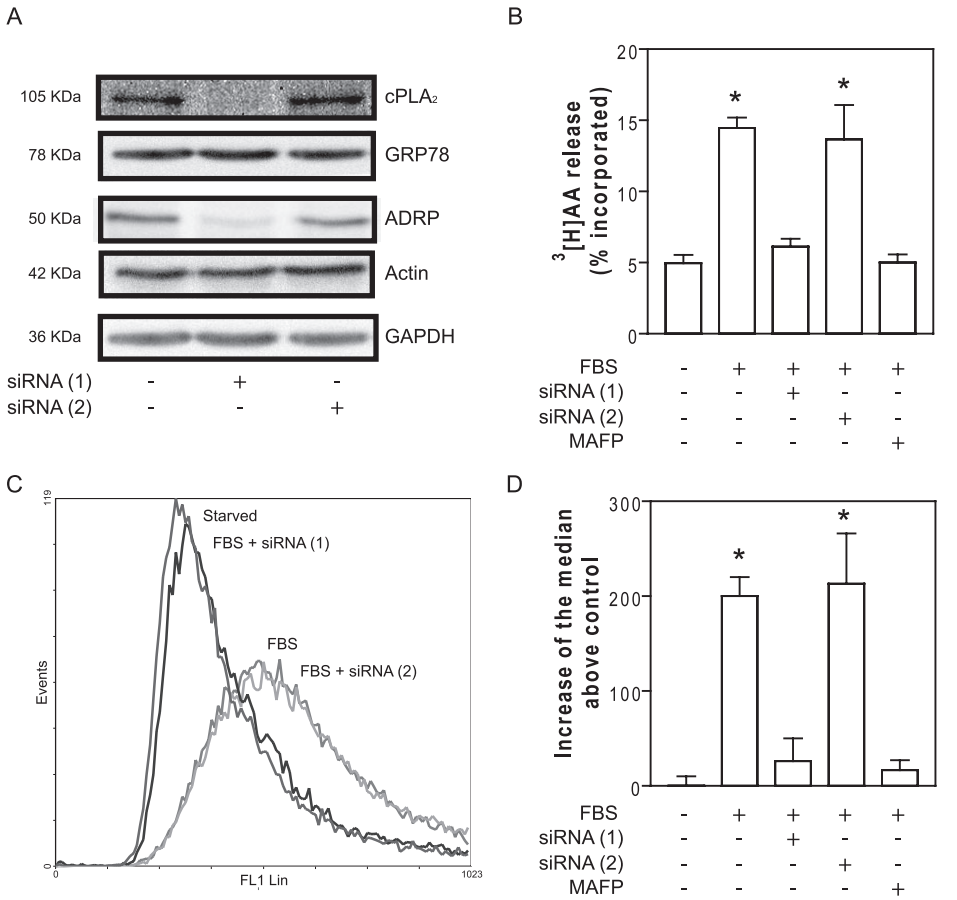


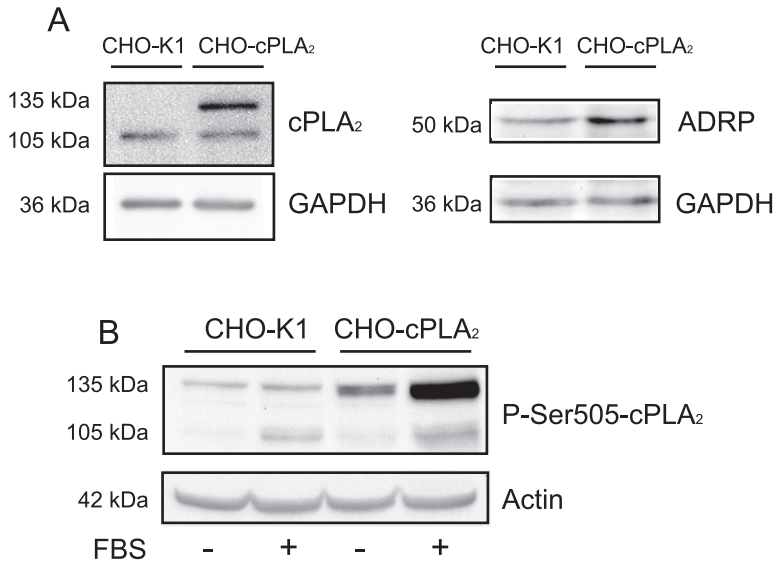
FIGURE 3. **Silencing the expression of cPLA$_2\alpha$ also prevents the appearance of LD.** CHO-K1 cells were transfected with two cPLA$_2\alpha$-siRNA sequences as described under "Experimental Procedures," resulting in silenced expression of cPLA$_2\alpha$ with siRNA(1) but not with siRNA(2), (*A*). Silenced expression with siRNA(1) was in line with decreased cPLA$_2$ activity, similar to the inhibition of control cells with MAFP (*B*), and decreased capacity of FBS to induce LD (*C* 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 three event distributions obtained in independent experiments. *, significantly different ($p < 0.05$) from serum-starved conditions.

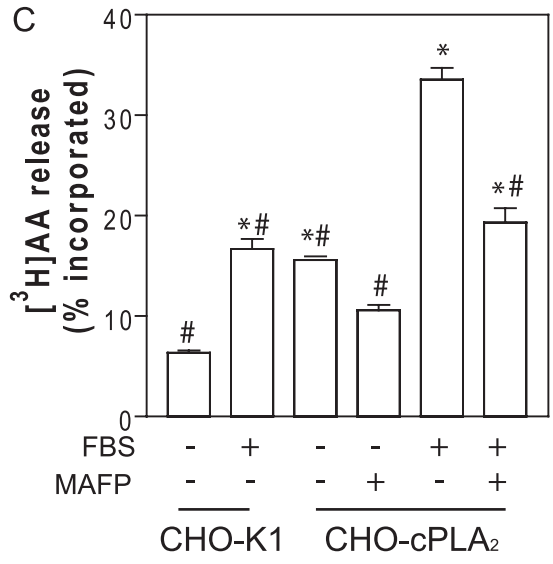
experiments; under serum-starved conditions, the median of the fluorescence distribution of CHO-cPLA$_2$ cells was 1.8-fold that of CHO-K1 cells and 1.7-fold after stimulation with FBS (Fig. 4, *D* and *E*). Again, the increased occurrence of LD in CHO-cPLA$_2$ cells, both under serum-starved and FBS-stimulated conditions, which is illustrated in Fig. 4, *F* and *G*, was inhibited by MAFP (Fig. 4*E*). Taken together, these results show that overexpression of cPLA$_2\alpha$ enhances the occurrence of LD.

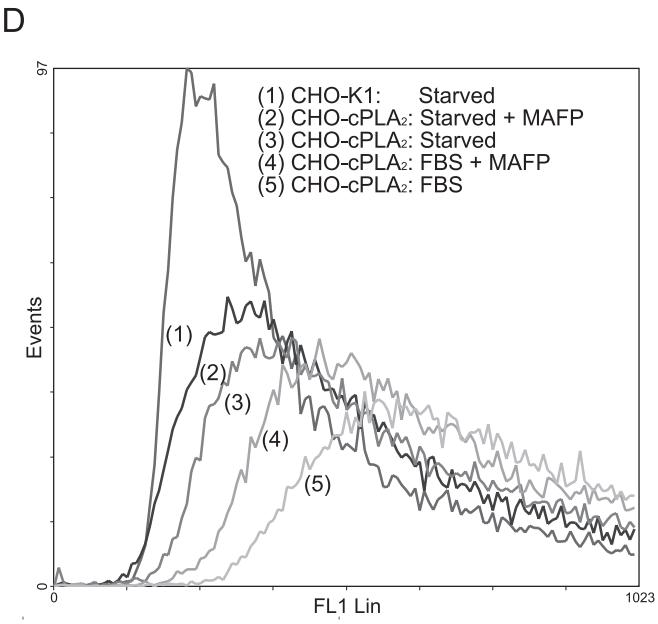
cPLA$_2\alpha$ Phosphorylation at Ser^{505} Is Required for the Formation of LD—To determine whether phosphorylation of cPLA$_2\alpha$ at Ser^{505} is relevant for enzyme activity and LD biogenesis, we transiently transfected Chinese hamster ovary K1 cells with an EGFP-cPLA$_2\alpha$ fusion protein with a S505A mutation, with EGFP-cPLA$_2\alpha$, or with EGFP alone (Fig. 5). Transfection was monitored by fluorescence microscopy (not shown) and by Western blot (Fig. 5*A*). After a 6-h stimulation with FBS, cPLA$_2\alpha$ was phosphorylated at Ser^{505} but S505A-cPLA$_2\alpha$ was not. Furthermore, AA release as stimulated by FBS in cells transfected with S505A-cPLA$_2\alpha$ was similar to that in cells transfected with EGFP alone, and significantly lower than in EGFP-cPLA$_2\alpha$-transfected cells (Fig. 5*B*). Likewise, LD occurrence in cells transfected with EGFP-S505A-cPLA$_2$ was the same as that in cells transfected with EGFP alone and significantly lower than in EGFP-cPLA$_2\alpha$-transfected cells (Fig. 5, *C* and *D*). These results show a key role of Ser^{505} phosphorylation of cPLA$_2\alpha$ for enzyme activation and LD biogenesis. This phosphorylation site has been shown to play an important role in regulating enzyme activity under conditions of transient increase of the intracellular calcium concentrations, enhancing the membrane affinity of cPLA$_2\alpha$ (46, 47). Consistent with this, FBS induced a relatively small and transient (5–7 min) increase in cytosolic calcium, clearly different from the robust signal elicited by ionomycin (supplemental Fig. S5). In agreement with these different calcium responses, FBS did not induce any apparent change in the cellular distribution of EGFP-cPLA$_2$, in contrast with the translocation to nuclear and perinuclear membranes induced by ionomycin (supplemental Fig. S5).

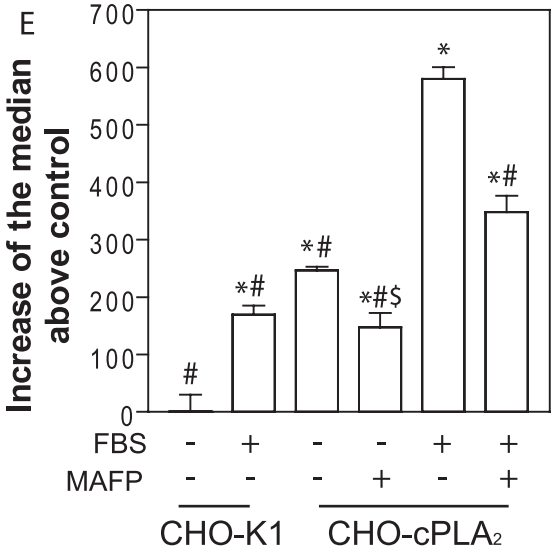
cPLA$_2$ Is Not Involved in the Synthesis of Neutral Lipids during LD Biogenesis—To test whether the role of cPLA$_2$ in the biogenesis of LD is to provide AA for neutral lipid synthesis (TAG and cholesteryl esters), we assayed the ability of exogenous AA to induce LD in serum-starved cells and also to stimulate cPLA$_2\alpha$ (Fig. 6). AA at a 10 μM concentration induced the increase of ADRP, and also the phosphorylation of cPLA$_2\alpha$ at Ser^{505} (Fig. 6*A*). 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 [^{3}H]AA (Fig. 6*B*), 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 cPLA$_2\alpha$ activity were mir-

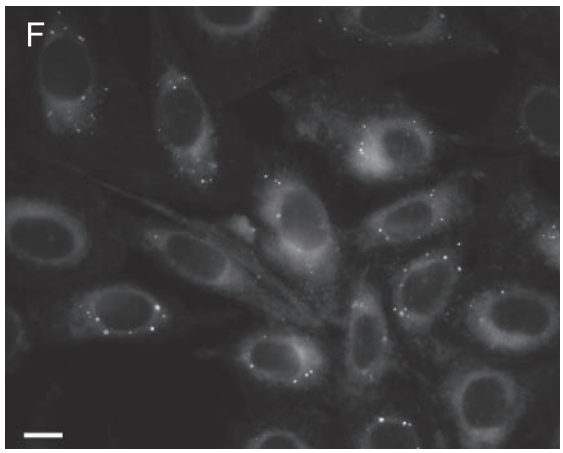
FIGURE 2. **cPLA$_2$ inhibitors prevent the appearance of FBS-induced LD.** Serum-deprived CHO-K1 cells were treated for 16 h with 7.5% FBS in the presence of different concentrations of the indicated cPLA$_2$ inhibitors, and LD were quantified by flow cytometry. *A, C,* and *E* show event distributions in representative experiments. *B, D,* and *F* present means ± S.E., obtained in three independent experiments, of the median values of the event distributions for each condition, expressed as the increase above the control in the absence of FBS, which averaged 405 ± 13 ($n = 9$). *G* and *H* show Nile red-stained cells treated for 6 h with 7.5% FBS in the presence of 10 μM BEL or MAFP, respectively. *Scale bar*, 10 μm. *I* shows a Western blot of ADRP from serum-starved cells or cells treated with FBS and in the absence or presence of BEL or MAFP. *, significantly different ($p < 0.01$) from serum-starved conditions; #, significantly different ($p < 0.01$) from serum-induced LD.

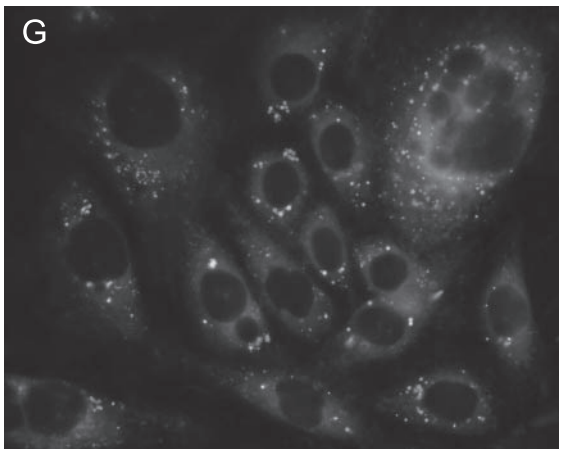












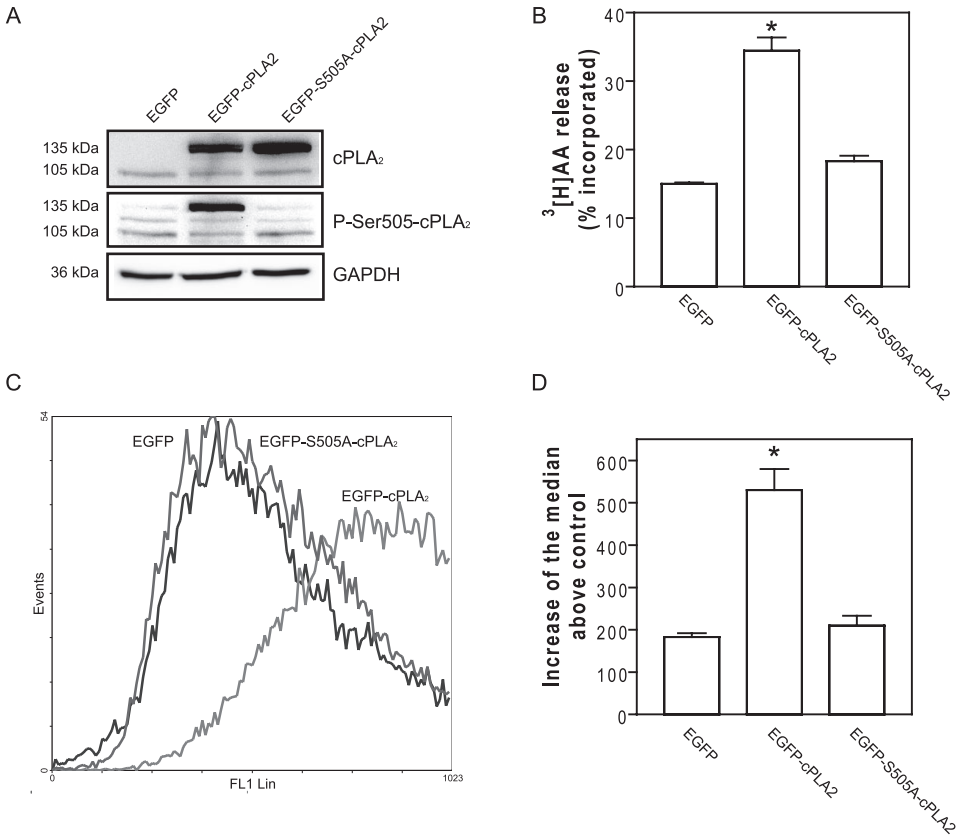


FIGURE 5. **Phosphorylation of cPLA$_2\alpha$ in Ser^{505} is required for the formation of LD.** CHO-K1 cells were transiently transfected with plasmids encoding EGFP alone, EGFP-cPLA$_2$, or EGFP-S505A-cPLA$_2$, and maintained in FBS (*A*) or deprived from serum for 24 h prior to stimulation with FBS for 6 h (*B–D*). *A,* Western blot of total cPLA$_2$, phospho-Ser^{505}-cPLA$_2$, and GAPDH as a loading control. *B,* AA release from cells containing EGFP alone, EGFP-cPLA$_2$, or EGFP-S505A-PLA$_2$ 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 experiments (*A* and *C*) or means ± S.E. of three independent experiments (*B* and *D*). *, significantly different ($p < 0.01$) from EGFP-transfected cells.

rored by the occurrence of LD (Fig. 6, *C* and *D*); AA alone at subtoxic concentrations (10 μM) induced the appearance of LD, and this was prevented by MAFP. These results show that exogenous AA is sufficient to induce the formation of LD, again with a mechanism involving cPLA$_2\alpha$ activity and, importantly, that effects of cPLA$_2\alpha$ inhibition on LD biogenesis cannot be overcome by supplementation of its product. Identical experiments were conducted using sodium oleate (supplemental Fig. S6) or palmitic acid (not shown) as exogenous fatty acids; in the absence of FBS, both fatty acids at 10 μM were not toxic, induced ADRP expression, cPLA$_2\alpha$ phosphorylation and activity, and LD biogenesis. As with AA, 100 μM oleate or palmitic were toxic in the absence of FBS, but in its presence they induced cPLA$_2\alpha$ activity and LD appearance in an MAFP-sensitive manner. Furthermore, enzyme activity and LD occurrence after treatment with 10 μM oleate decreased to basal levels after silencing enzyme expression with siRNA(1) (supplemental Fig. S6, *E* and *F*), and the same results were obtained with 10 μM AA or palmitate (data not shown). Essentially identical results regarding LD occurrence (supplemental Fig. S6*G*) were obtained when 10 μM oleate was supplied in a more physiological way, after complexion with bovine serum albumin at a ratio of 6:1 (48). Taken together, these results show that fatty acid-induced formation of LD is a cPLA$_2\alpha$-dependent process and suggest that the enzyme may be necessary for roles other than generating fatty acids for TAG and cholesteryl ester synthesis. This was confirmed by following the distribution of [^{3}H]AA among the main neutral lipids after a 24-h prelabeling period, followed by a 6-h stimulation with FBS to induce LD (Fig. 6*E*); about 17,000 dpm were released to the medium in an MAFP-sensitive manner, indicative of cPLA$_2\alpha$ 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 cPLA$_2\alpha$.

cPLA$_2\alpha$ Is Not Involved in the Channeling of Extracellular Fatty Acids into Neutral Lipids—A different possibility we considered is that cPLA$_2\alpha$ could be required for the channeling of fatty acids from the medium into the synthesis of TAG and cholesteryl esters. To address this, we pulsed cells with [^{3}H]AA in the absence of FBS during 6 h and measured its incorporation into the major cellular lipid species, using three different AA concentrations as follows: 10 nM (Fig. 7*A*), which is a concentration of the fatty acid far below that required to induce LD; 1 μM (Fig. 7*B*), which still is not enough for LD induction (not shown); and 10 μM (Fig. 7*C*), which stimulates cPLA$_2\alpha$ phosphorylation and activity, ADRP expression, and LD occurrence, as shown in Fig. 6. In all situations, [^{3}H]AA was incorporated

FIGURE 4. **Overexpression of cPLA$_2\alpha$ enhances the occurrence of LD.** A CHO-K1-derived clone stably expressing EGFP-cPLA$_2\alpha$ (*A* and *B*) displayed enhanced basal (under serum-starved conditions) and FBS-induced cPLA$_2$ 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-cPLA$_2$ cells against cPLA$_2$ (*A*) or phospho-Ser^{505}-cPLA$_2$ (*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 [^{3}H]AA-prelabeled CHO-K1 or CHO-cPLA$_2$ 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-cPLA$_2$ 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-cPLA$_2$ 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-cPLA$_2$; $, significantly different ($p < 0.05$) from serum-starved CHO-cPLA$_2$.

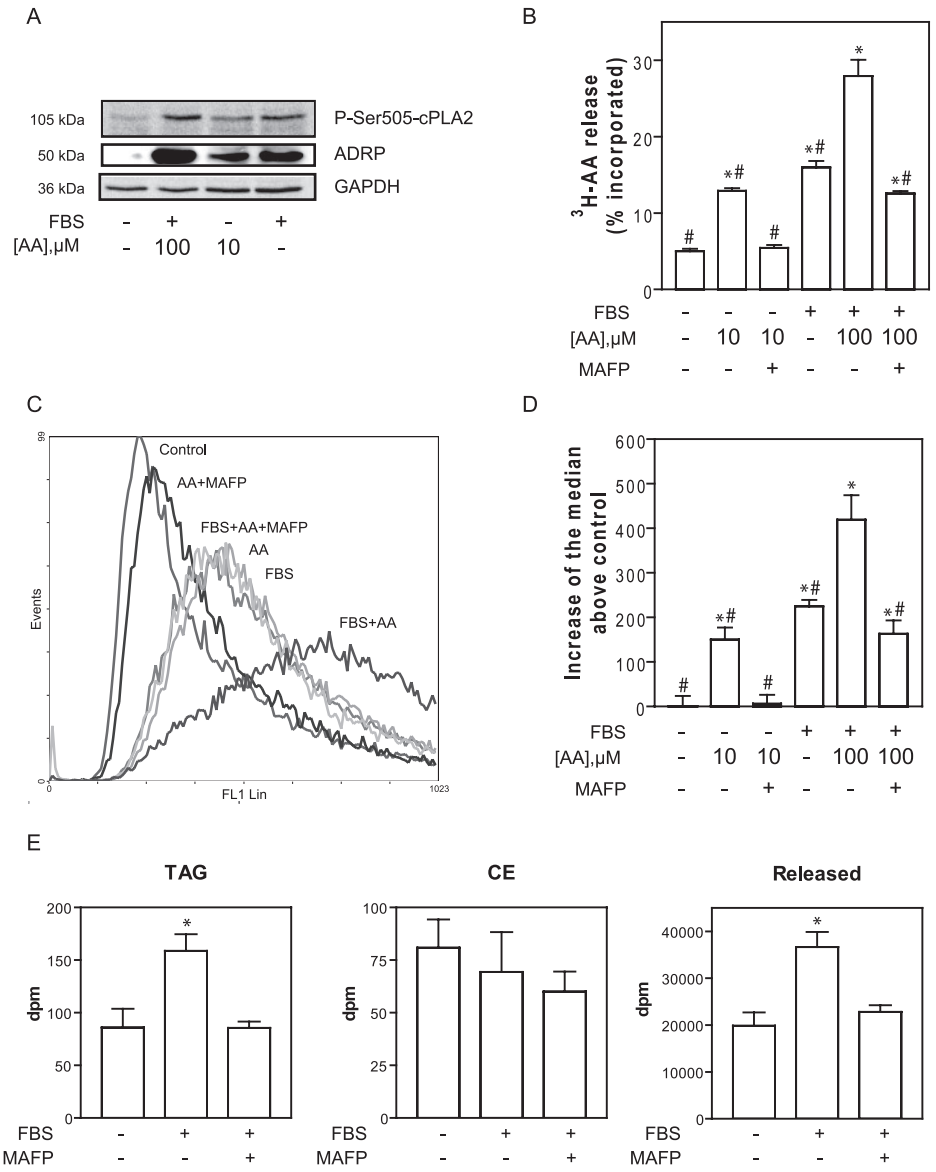


FIGURE 6. **cPLA$_2\alpha$ is not involved in the synthesis of neutral lipids during LD biogenesis.** *A,* serum-starved CHO-K1 cells were exposed for 6 h to 10 μM AA or to 100 μM AA in combination with 7.5% FBS, and phospho-Ser^{505}-cPLA$_2$ and ADRP were detected by Western blot. *B,* serum-starved CHO-K1 cells were labeled 24 h with [^{3}H]AA and then treated for 6 h with 10 μM AA or with 100 μM AA in combination with 7.5% FBS and in the presence or absence of 10 μM 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 ± S.E. of the median values of three event distributions are shown in *D*. *E,* serum-starved CHO-K1 cells were prelabeled 24 h with [^{3}H]AA and treated for 6 h with 7.5% FBS in the absence or presence 10 μM MAFP. Radioactivity in TAG (*left*) and cholesteryl esters (*center*) was compared with radioactivity released to the medium, indicative of cPLA$_2$ 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 iPLA$_2$, which is involved in an ongoing deacylation-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. 7*C*) or not (Fig. 7, *A* and *B*), the inhibition of cPLA$_2\alpha$ 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, cPLA$_2\alpha$ 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 μM oleate, 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 μg/2 × 10^5 cells, and it was not altered by inhibition of LD biogenesis by MAFP (9.5 ± 1.1 μg/2 × 10^5 cells). These results show that cPLA$_2$ is not involved in the channeling of extracellular fatty acids into neutral lipids during LD biogenesis.

Inhibition of cPLA$_2\alpha$ under LD-forming Conditions Alters ER Structure—As cPLA$_2\alpha$ 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 C_1-BODIPY-C_{12} 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 μM 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. S7, *E* and *F*). 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. S7*G*), eventually forming aberrant membrane stacks (supplemental Fig. S7*H*). 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 cPLA$_2\alpha$ promoted re-distribution of TAG from a cytosol-enriched to a membrane-enriched fraction (Fig. 8, *I–L*).

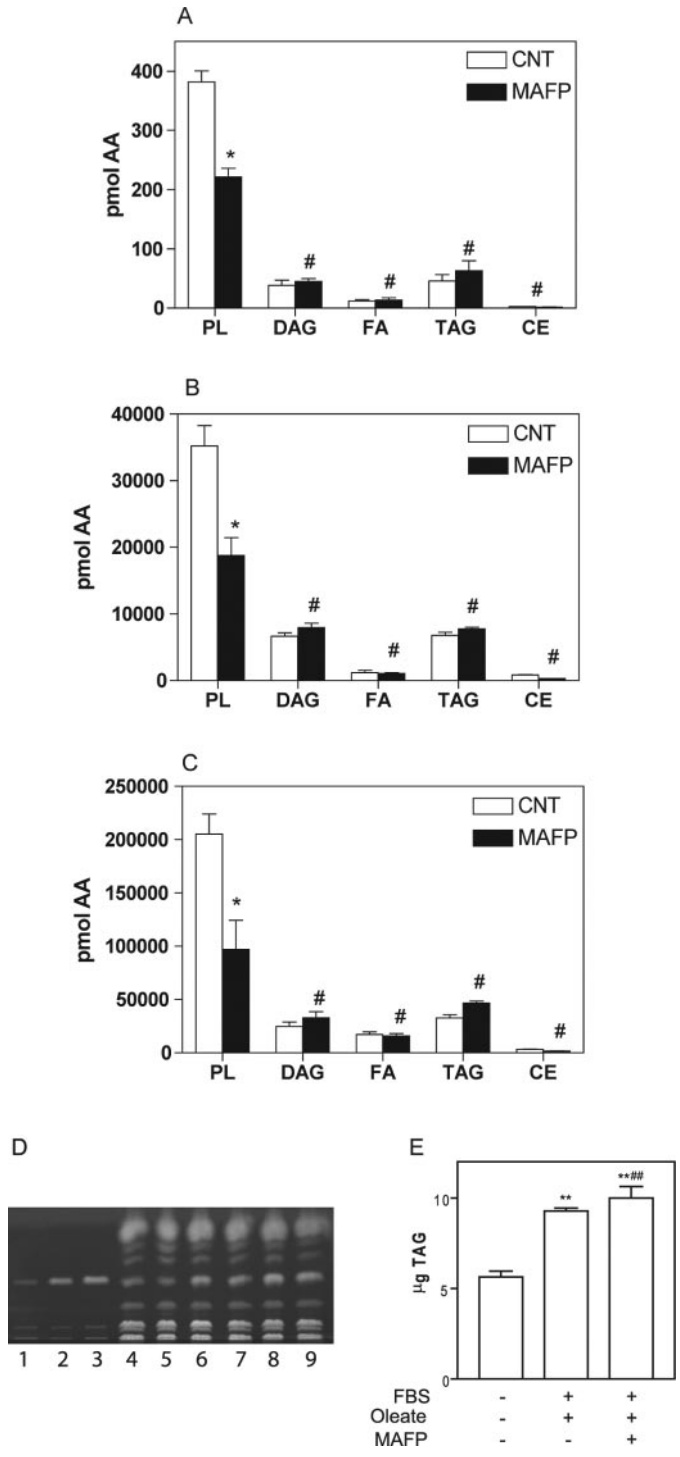


FIGURE 7. **cPLA$_2\alpha$ is not required for the channeling of fatty acids from the medium into LD.** *A–C,* serum-starved CHO-K1 cells were incubated for 6 h with 2 μCi/ml [^{3}H]AA alone (*A*), which yielded a final AA concentration of 10 nM, or together with 1 μM (*B*) or 10 μM unlabeled AA (*C*), in the absence (*open bars*) or presence of 10 μM MAFP (*filled bars*). 1 or 10 μM unlabeled AA reduced the specific activity of the tracer to 2 or 0.2 Ci/mmol, respectively. Lipids were extracted and separated by TLC. Results are expressed in pmol of AA incorporated into the major lipid species: *PL,* phospholipids; *DAG,* diacylglycerol; *FA,* fatty acids; *TAG,* triglycerides; *CE,* cholesteryl esters. *, different from control ($p < 0.05$); #, not different from control ($p > 0.05$). *D* and *E,* Chinese hamster ovary K1 cells were left untreated (*lanes 4* and *5*) or treated 10 h with 7.5% FBS plus 100 μM oleate in the absence (*lanes 6* and *7*) or presence of 10 μM MAFP (*lanes 8* and *9*). Lipids were extracted, separated in TLC, and stained with primuline spray (*D*). *Lanes 1–3* contain 1, 5, and 10 μg tripalmitine, respectively. *E* represents densitometry quantification of primuline-stained TAG (μg of TAG in 2×10^5 cells), and are means ± range of two independent experiments with duplicate determinations. **, different from serum-starved conditions ($p < 0.05$); ##, not different from FBS + oleate ($p > 0.05$).

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-cPLA$_2$ (HEK-cPLA$_2$), where the latter showed a general increase in LD occurrence either in serum-starved or FBS-stimulated conditions, consistent with data on CHO-cPLA$_2$ 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 cPLA$_2\alpha$ expression (not shown) and LD in human SH5YSY cells (Table 1). Taken together, and considering the ubiquitous expression of cPLA$_2\alpha$ 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 content and alter their size and shape (2, 54). Furthermore, in contrast with microscopy, flow cytometry does not involve the need of washing out excess dye that could result in its re-distribution under nonequilibrium conditions.

When considering the putative role of cPLA$_2\alpha$, it is important to bear in mind that the enzyme could work after the generation of eicosanoids that in turn might modify signal transduction pathways, or directly affect membrane function after the generation of lysophospholipids or free fatty acids (36), 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

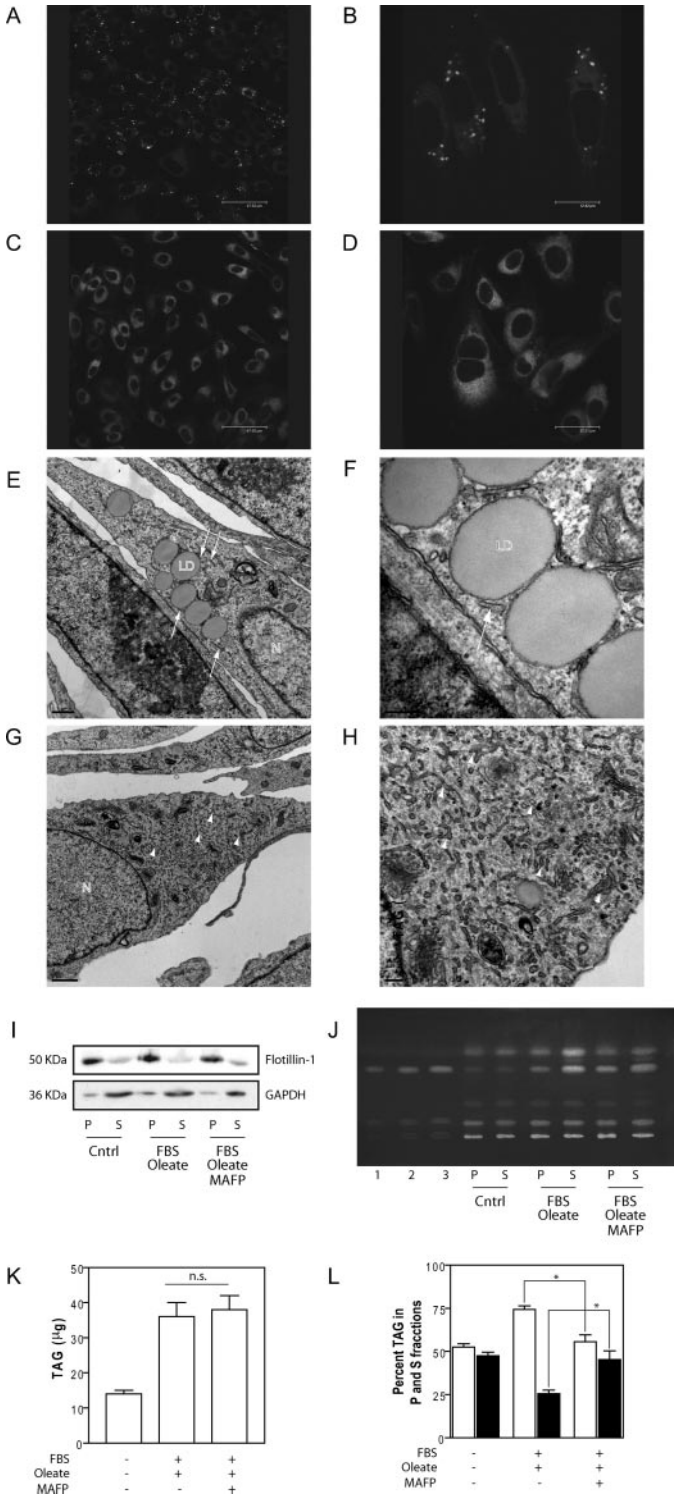


FIGURE 8. **Precluding LD formation by the inhibition of $cPLA_2$ alters the structure of the endoplasmic reticulum and re-distributes TAG from cytosolic to membrane compartments.** *A–D,* serum-starved Chinese hamster ovary K1 cells were treated for 6 h with 7.5% FBS and 1 μM C_1-BODIPY 500/510 C_{12}, 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. *Cntrl*, control. TAG from both fractions were separated by TLC (*J*) and quantified (*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 *J* 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, oleate, and palmitate) can be regarded as a simple channeling of material into neutral lipids that are stored in LD, and therefore the role of $cPLA_2\alpha$ 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 PLA_2 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 $cPLA_2\alpha$ and LD biogenesis involving lipoprotein metabolism.

Group VI PLA_2 ($iPLA_2$) 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 $AACOCF_3$ and MAFP inhibit $cPLA_2\alpha$ and $iPLA_2$, BEL is selective for $iPLA_2$ and had no effect on LD biogenesis. In contrast to $iPLA_2$, $cPLA_2\alpha$ 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 $cPLA_2\alpha$ 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 $cPLA_2\alpha$ 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 $cPLA_2\alpha$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 $cPLA_2\alpha$ expression. There-

[6] A. Gubern and E. Claro, unpublished observations.

TABLE 1

MAFP inhibits LD formation in all cell types tested

Different cellular models were deprived of serum during 24 h and then treated for 6 h as indicated. The occurrence of LD was monitored by flow cytometry of Nile red-stained cells. In some experiments, SH5YSY cells were transfected with the indicated siRNA 72 h before the treatment with FBS. Results are expressed as means ± range ($n = 2$) of the median values of the event distributions in FL1 (30,000 events).

Cell type	Treatment		
	Control	10% FBS	10% FBS, 10 μM MAFP
Primary astrocytes	519 ± 29	719 ± 42[a]	594 ± 7[b]
HEK	243 ± 2	303 ± 5[a]	255 ± 5[b]
HEK-cPLA$_2$	408 ± 2	488 ± 6[a]	366 ± 8[b]
B cell leukemia EHEB	317 ± 1	477 ± 5[a]	414 ± 8[b]
Smooth muscle A7r5	227 ± 8	295 ± 5[a]	260 ± 4[b]
Hepatoma HepG2[c]	387 ± 18	618 ± 13[a]	430 ± 23[b]
Neuroblastoma SH5YSY	209 ± 9	330 ± 13[a]	226 ± 28[b]
Neuroblastoma SH5YSY-siRNA(1)	226 ± 13	254 ± 17	
Neuroblastoma SH5YSY-siRNA(2)	212 ± 8	230 ± 9	
Neuroblastoma SH5YSY-GAPDH siRNA	198 ± 12	350 ± 20[a]	

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

fore, the role of cPLA$_2\alpha$ 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 [^{3}H]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 cPLA$_2\alpha$ 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 cPLA$_2\alpha$ decreases ADRP content fits with the observation of Wolins *et al.* (60), who found that in oleate-loaded adipocytes ADRP moves to already formed nascent LD coated with S3-12. Therefore, the role of cPLA$_2\alpha$ in LD biogenesis would fit somewhere between the synthesis of TAG and cholesteryl esters and the generation of nascent LD. It could be required to allow the formation of primordial, nascent LD from the ER. Alternatively, it might favor fusion events between newly formed LD, which have been shown to increase in size independently of TAG synthesis (61), and may not be detectable either by epifluorescence microscopy, by flow cytometry, or by NMR, as proposed recently (13). The latter possibility, however, does not quite fit our results, as it is difficult to envisage how inhibiting fusion of micro-LD would induce the marked alterations in ER structure revealed by electron microscopy. Either way, the elucidation of the precise role of cPLA$_2\alpha$ in these processes awaits further investigation. Both mechanisms, droplet formation from the ER and fusion of already formed ones, would be favored by PLA$_2$-generated lysophospholipids, 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 PLA$_2$ implicated in Golgi vesiculation induced by cholesterol (63).

Another question arising is how cPLA$_2\alpha$ is activated by serum lipoproteins or free fatty acids, the two LD-forming conditions used in this study. Regulation of cPLA$_2\alpha$ (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 Ser^{505} plays a relevant role under transient, physiological submicromolar [Ca^{2+}], increasing the phospholipid binding affinity of the enzyme (47), but it appears less important in response to higher sustained [Ca^{2+}]. Our results show that addition of FBS to serum-starved cells stimulates cPLA$_2\alpha$ in a manner that requires phosphorylation on Ser^{505}, 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 [Ca^{2+}] and perhaps signaling lipids like phosphatidylinositol 4,5-bisphosphate (40, 64) and ceramide 1-phosphate (65) play in cPLA$_2\alpha$ activation, and also what are the upstream events leading to cPLA$_2\alpha$ phosphorylation, will contribute to clarifying the mechanisms of LD biogenesis.

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