

Lipid Droplet Biogenesis Induced by Stress Involves Triacylglycerol Synthesis That Depends on Group VIA Phospholipase A₂*

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This work investigates the metabolic origin of triacylglycerol (TAG) formed during lipid droplet (LD) biogenesis induced by stress. Cytotoxic inhibitors of fatty acid synthase induced TAG synthesis and LD biogenesis in CHO-K1 cells, in the absence of external sources of fatty acids. TAG synthesis was required for LD biogenesis and was sensitive to inhibition and down-regulation of the expression of group VIA phospholipase A₂ (iPLA₂-VIA). Induction of stress with acidic pH, C₂-ceramide, tunicamycin, or deprivation of glucose also stimulated TAG synthesis and LD formation in a manner dependent on iPLA₂-VIA. Overexpression of the enzyme enhanced TAG synthesis from endogenous fatty acids and LD occurrence. During stress, LD biogenesis but not TAG synthesis required phosphorylation and activation of group IVA PLA₂ (cPLA₂α). The results demonstrate that iPLA₂-VIA provides fatty acids for TAG synthesis while cPLA₂α allows LD biogenesis. LD biogenesis during stress may be a survival strategy, recycling structural phospholipids into energy-generating substrates.

Intracellular lipid droplets (LDs)⁵ are cytosolic inclusions present in most eukaryotic cells, containing a core of triacylglycerol (TAG) and cholesteryl esters, surrounded by a phospho-

lipid monolayer and by specific proteins, among which the best characterized belong to the perilipin family (1–3). The biology of LD has received increasing interest, due to the link between excess lipid storage in certain tissues and pathologies such as obesity, diabetes, or atherosclerosis (3, 4). LDs have been shown to interfere with membrane translocation of the insulin-sensitive glucose transporter, an observation that might account for insulin resistance in type 2 diabetes (5). The recent identification of 132 genes controlling LD number, size, and distribution in *Drosophila* (6) illustrates the complexity of this organelle, whose dynamic nature is far from being understood fully.

LDs are formed in two very different environmental conditions and, presumably, the physiological significance in each case is different. First, cells accumulate LD in response to exogenous lipid availability (4), present in serum lipoproteins or as free fatty acids. There is general agreement that LD content arising from the medium has a storing purpose for energy generation and membrane building. Using this experimental paradigm of exogenous lipid loading, we have shown the implication of group IVA PLA₂ (cPLA₂α) in LD biogenesis at a step beyond the synthesis of TAG (7). Second, many kinds of cellular stress, including inflammation, apoptosis induced by different insults or contact inhibition, also induce LD biogenesis. An account of this situation is the human T lymphoblastoid cell line (HuT 78) undergoing apoptosis triggered by Fas antibody (8). In these cases, the metabolic origins of LD-associated neutral lipids and the physiological function they subserve are not known.

Altering phospholipid metabolism has been shown to cause programmed cell death in some instances (9–12), and, conversely, programmed cell death often alters phospholipid metabolism, resulting in the accumulation of lysophospholipids and fatty acids (13). Current evidence suggests that group VIA PLA₂ (iPLA₂-VIA) is involved in the generation of lysophosphatidylcholine during programmed cell death, which may mediate attraction and recognition/engulfment signals for apoptotic cell clearance by phagocytes (13–17). Unlike cPLA₂α, which shows a marked preference for arachidonic acid-containing phospholipids, iPLA₂-VIA has no substrate specificity regarding the fatty acid residue at the *sn*-2 position, and its implication in eicosanoid synthesis may be minor. Instead, the enzyme has a housekeeping role mediating phospholipid

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⁵ The abbreviations used are: LD, lipid droplets; AA, arachidonic acid; ADRP, adipophilin (adipose differentiation-related protein); BEL, bromoenol lactone; C₂-ceramide, *N*-acetyl-*D*-sphingosine; CCT, CTP:phosphocholine cytidyltransferase; DAG, diacylglycerol; EYFP, enhanced yellow fluorescent protein; FAS, fatty acid synthase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; PAP, phosphatidate phosphohydrolase; PBS, phosphate-buffered saline; PLA₂, phospholipase A₂; py-2, pyrrolidine-2; siRNA, short interfering RNA; TAG, triacylglycerol; CHO, Chinese hamster ovary; C₁-BODIPY, 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid.

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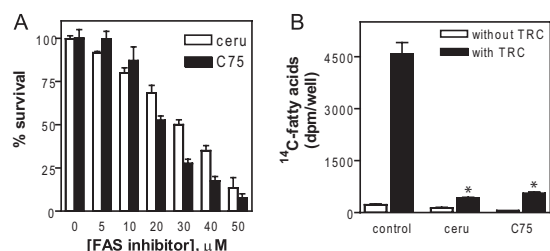


FIGURE 1. Cerulenin and C75 inhibit fatty acid synthesis. *A*, cells were treated for 24 h with cerulenin (open bars) or C75 (filled bars), and viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay. *B*, serum-starved cells were incubated with [¹⁴C]acetate (2 μCi/ml, 34.4 μM) for 6 h in the presence (filled bars) or absence (open bars) of 5 μM triacsin C (TRC) and with or without 30 μM cerulenin or 20 μM C75. Lipids were separated by TLC, and areas of the silica co-migrating with palmitic acid were counted. Results are means ± S.E. of three independent experiments each carried out with triplicate determinations. *, significantly different ($p < 0.001$) from controls treated only with TRC.

remodeling through deacylation/reacylation reactions (18). Bearing this in mind, we hypothesized that during cellular stress part of the fatty acids released by phospholipid degradation could be incorporated into TAG and stored in LD. In essence, our results show that TAG synthesis associated with the formation of LD during stress depends on iPLA₂-VIA. Further, we show that the implication of cPLA₂α in LD formation still holds in this new paradigm.

EXPERIMENTAL PROCEDURES

Materials—[5,6,8,9,11,12,14,15-³H]Arachidonic acid ([³H]-AA, 200 Ci/mmol) was from American Radiolabeled Chemicals, [9,10(*n*)-³H]palmitic acid (49 Ci/mmol) was from Amersham Biosciences, and [1-¹⁴C]acetate (58 mCi/mmol) was from PerkinElmer Life Sciences. Bromoenol lactone (BEL), (S)-BEL, (R)-BEL, and cerulenin were from Cayman Chemical Co., and pyrrolidine-2 (py-2, cat. no. 525143) and *N*-acetyl-D-sphingosine (C₂-ceramide) were from Calbiochem. Rabbit anti-cPLA₂α and anti-phospho Ser⁵⁰⁵ cPLA₂α antibodies were from Cell Signaling, chicken anti-ADRP from GenWay Biotech, rabbit anti-GAPDH from Ambion, mouse anti-BiP/GRP78 from BD Bioscience, rabbit anti-iPLA₂-VIA from Cayman, and rabbit anti-GFP from Abcam. Tripalmitin, primuline, Nile Red, triacsin C, C75, tunicamycin, phorbol 12-myristate 13-acetate, and U73122 were from Sigma-Aldrich, and 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid (C₁-BODIPY® 500/510-C₁₂) was from Molecular Probes.

Constructs—The sequence of human iPLA₂-VIA2 was cloned in the pEYFP-N3 vector (Clontech), using the HindIII and SalI restriction sites. This construct encodes the expression of a fluorescent fusion protein containing N-terminal iPLA₂-VIA2 followed by enhanced yellow fluorescent protein (EYFP).

Cells—CHO-K1 cells were cultured as described (7). For the experiments, cells were seeded at a density of 30,000 cells/ml in 24- (0.5 ml) or 6-well (2 ml) plates and maintained in FBS-containing medium for 24 h. Before LD induction, cells were switched to serum-free medium for 24 h to set control conditions with minimal occurrence of LD (7). When indicated, cells (40–70% confluence) were transfected

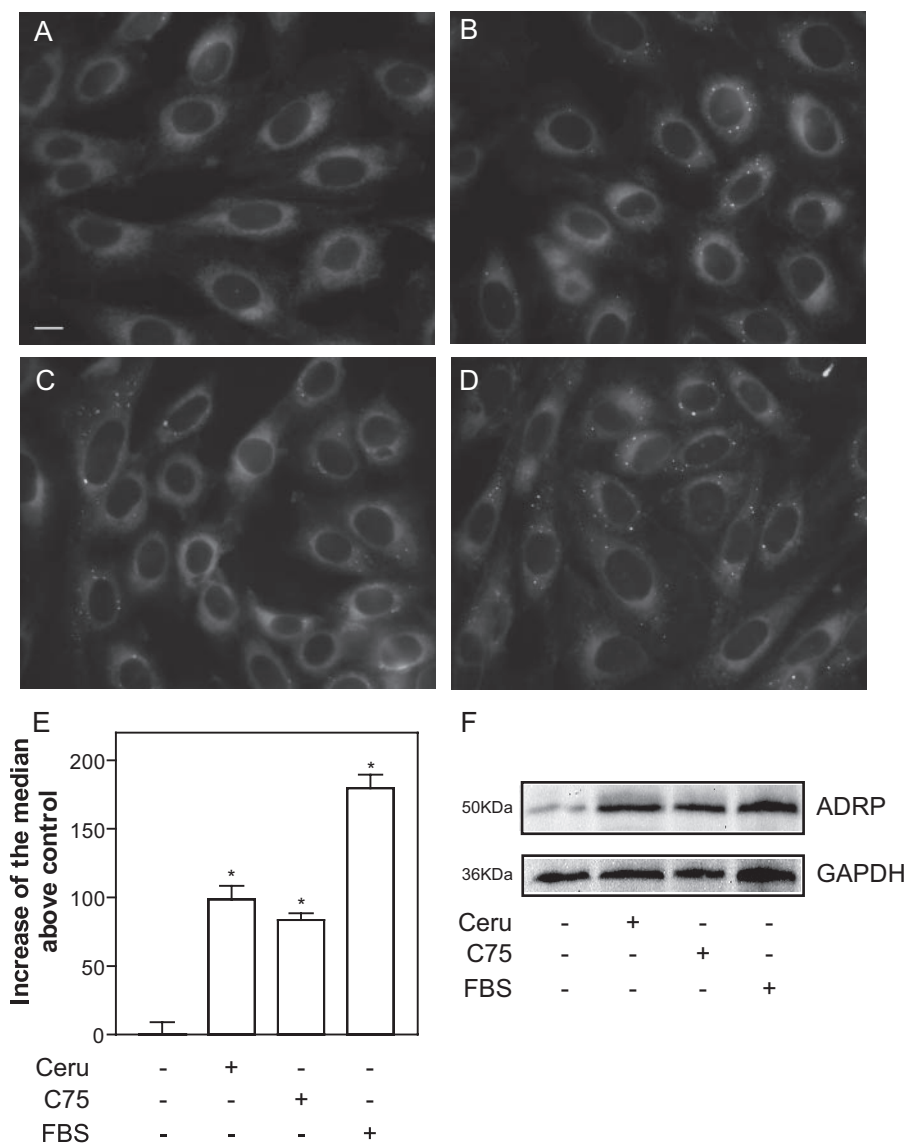


FIGURE 2. Inhibition of fatty acid synthesis induces LD biogenesis. Cells were left untreated (*A*) or treated for 6 h with 30 μM cerulenin (*B*), 20 μM C75 (*C*), or 7.5% FBS (*D*). LD occurrence was indirectly quantified by flow cytometry (*E*). In this case, fluorescence intensities in FL1 were quantified as the median values of the event distributions for each condition, expressed as the increase above the control, which averaged 434 ± 9 ($n = 6$). Results are means ± S.E. obtained in six independent experiments. *Panel F* shows Western blots of ADRP and GAPDH. Scale bar represents 10 μm. *, significantly different ($p < 0.01$) from controls.

with 1 μg of plasmid/ml (pGFP-C3 from Clontech or iPLA₂-VIA2-EYFP) using Lipofectamine PlusTM (Invitrogen), following the manufacturer's instructions.

Nile Red Staining and Fluorescence Microscopy—Cells cultured on glass coverslips were washed with phosphate-buffered saline (PBS, Sigma-Aldrich), 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 solution of Nile Red in acetone (0.2 mg/ml) was added, so that the final concentrations of Nile Red and acetone were 1 $\mu\text{g}/\text{ml}$ 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.

Flow Cytometry—Indirect quantification of LD by flow cytometry in Nile Red-stained cells was performed exactly as described (7). Briefly, paraformaldehyde-fixed cells were stained with 1 $\mu\text{g}/\text{ml}$ Nile Red during 45 min and analyzed with a Cytomics FC 500 (Beckman Coulter) equipped with an argon laser (488 nm), in the FL1 channel (505–545 nm). After gating out cellular debris, 30,000 events were acquired in all the assays, in linear scale. Fluorescence intensities in the different treatments were quantified as the median value of each distribution of events, and expressed as increase above the median of the control (serum-starved cells), which do not contain LD (7).

[³H]AA Release—Serum-starved cells, seeded in 24-well plates, were labeled with 0.25 μCi of [³H]AA (0.5 $\mu\text{Ci}/\text{ml}$) for 24 h, then washed once with PBS, incubated 5 min with Ham's F-12 supplemented with 0.5 mg/ml albumin, and washed twice more with PBS (19). Radioactivity in the last wash was subtracted from released [³H]AA over the stimulation period. At the end of the treatments, culture media containing released [³H]AA were taken, centrifuged, and counted. Cell monolayers were detached with ice-cold PBS containing 1% Triton X-100, and

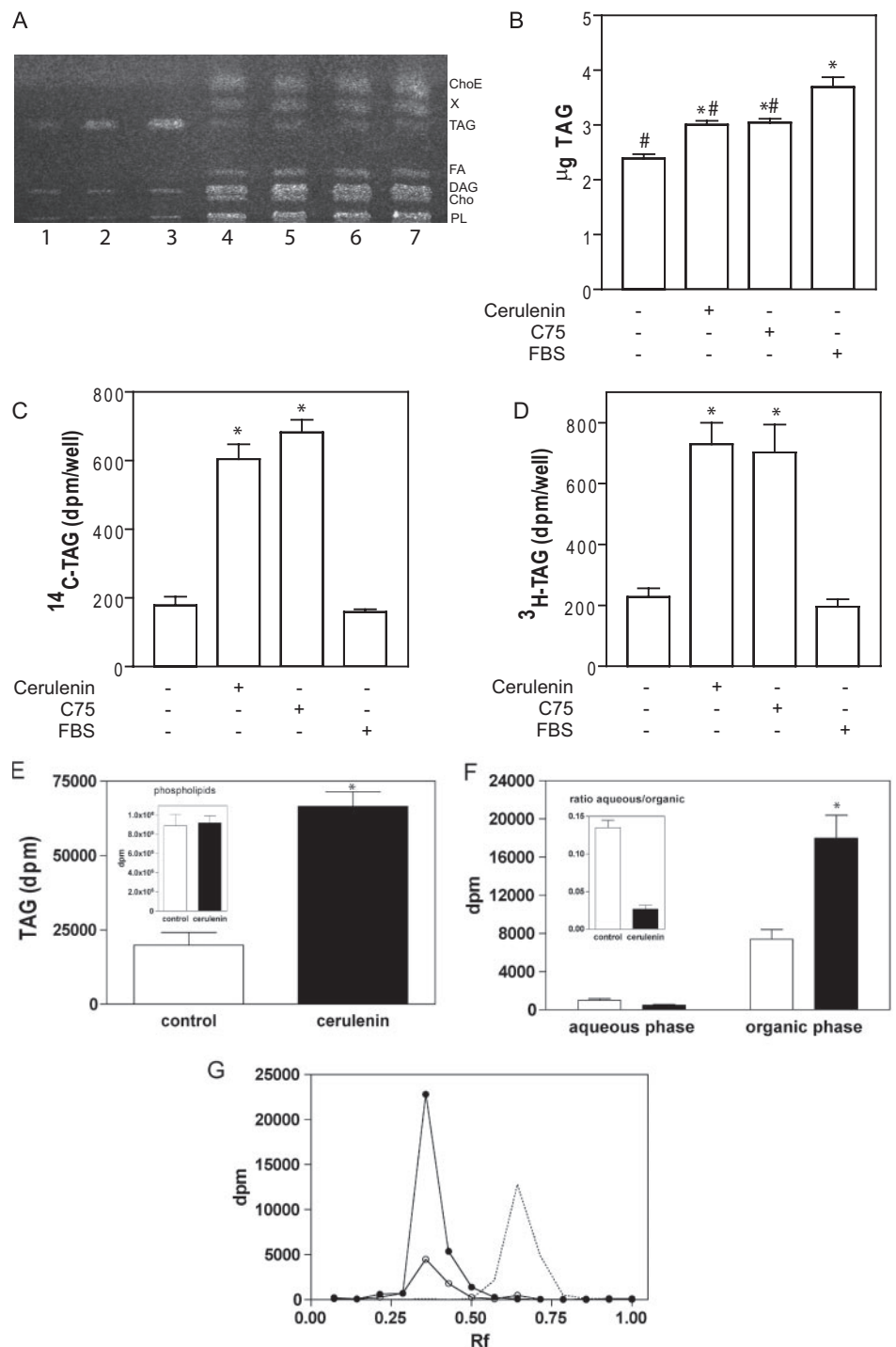


FIGURE 3. Cerulenin and C75 induce TAG synthesis from pre-existing fatty acids. *A*, cells were treated for 6 h with vehicle (*lane 4*), 30 μM cerulenin (*lane 5*), 20 μM C75 (*lane 6*), or 7.5% FBS (*lane 7*), and lipids were separated by TLC and stained with primuline. *Lanes 1–3* contain 1, 5, and 10 μg of tripalmitin standard. *PL*, phospholipids; *Cho*, cholesterol; *DAG*, 1,2-diacylglycerol. *B*, densitometric quantification of TAG (means \pm S.E. of four independent experiments each carried out in triplicate). *C* and *D*, cells were labeled for 24 h with 0.5 $\mu\text{Ci}/\text{ml}$ (8.6 μM) [¹⁴C]acetate (*C*) or 0.5 $\mu\text{Ci}/\text{ml}$ (10.2 nM) [³H]palmitic acid (*D*), washed, and treated with cerulenin, C75, or FBS for 6 h, then lipids were separated by TLC and radioactivity in TAG was counted. Results are means \pm S.E. of three independent experiments carried out in triplicate. *E* and *F*, cells were labeled for 24 h with 5 $\mu\text{Ci}/\text{ml}$ [³H]palmitic acid (1 μM), then treated with vehicle (*open bars*) or with cerulenin (*filled bars*) for 6 h. Radioactivity in TAG is shown in *panel E*, together with that in phospholipids (*inset*). After extraction of TAG from the silica, acyl-ester bonds were hydrolyzed by methanolic KOH treatment. *Panel F* shows radioactivity present in water-soluble and chloroform phases after alkaline deacylation of TAG. These chloroform phases were concentrated and developed in TLC as shown in *panel G*, which represents radioactivity from control (*open circles*) or cerulenin-treated samples (*filled circles*), together with that of a non-deacylated TAG sample (*dotted line*). Radioactivity at *R_f* between 0.30 and 0.40 co-migrated with palmitic acid standard. *, significantly different ($p < 0.01$) from control. #, significantly different ($p < 0.01$) from FBS.

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also counted for radioactivity to monitor [³H]AA incorporation into the cells.

Quantification of [³H]TAG—Serum-starved cells, seeded in 24-well plates, were labeled with [³H]palmitate (usually 0.5 μCi/ml unless indicated otherwise, equivalent to 10.2 nM) or with [¹⁴C]acetate (0.5 μCi/ml or 8.6 μM) for 24 h, washed twice with PBS and once with culture medium, and treated as described in each experiment. Cells were then harvested on ice and washed with 1 ml of PBS, and lipids were extracted (20). To separate the major lipid species, the chloroform phases were evaporated under vacuum, dissolved in 15 μl of chloroform/methanol (3:1, v/v), and spotted onto silica gel G TLC plates (Merck), which were developed in hexane/diethyl ether/acetic acid (70:30:1, v/v), and stained with primuline spray. Identification of the major species was made by co-migration with authentic standards. Quantification of radioactive TAG was done by scraping into vials the silica gel from regions corresponding to migration of the standards. Primuline-stained TAG was quantified by densitometric analysis after acquiring images under UV light.

Alkaline Hydrolysis of [³H]TAG—Serum-starved cells in 6-well plates were labeled with [³H]palmitate (5 μCi/ml or 0.1 μM) and treated with cerulenin. Pooled lipid extracts from 6 wells were separated by TLC. Silica gel containing [³H]TAG was scraped into glass tubes, and lipid was extracted with 1 ml of chloroform/methanol (1:1, v/v). 2 ml of chloroform and 0.5 ml of 1 M KOH in methanol/water (19:1, v/v) were added, and the tubes were left 1 h at room temperature. After neutralization with HCl, 2.5 ml of chloroform, 1.5 ml of methanol, and 1.5 ml of water were added to split organic and aqueous phases.

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 20 μg of protein was separated by stand-

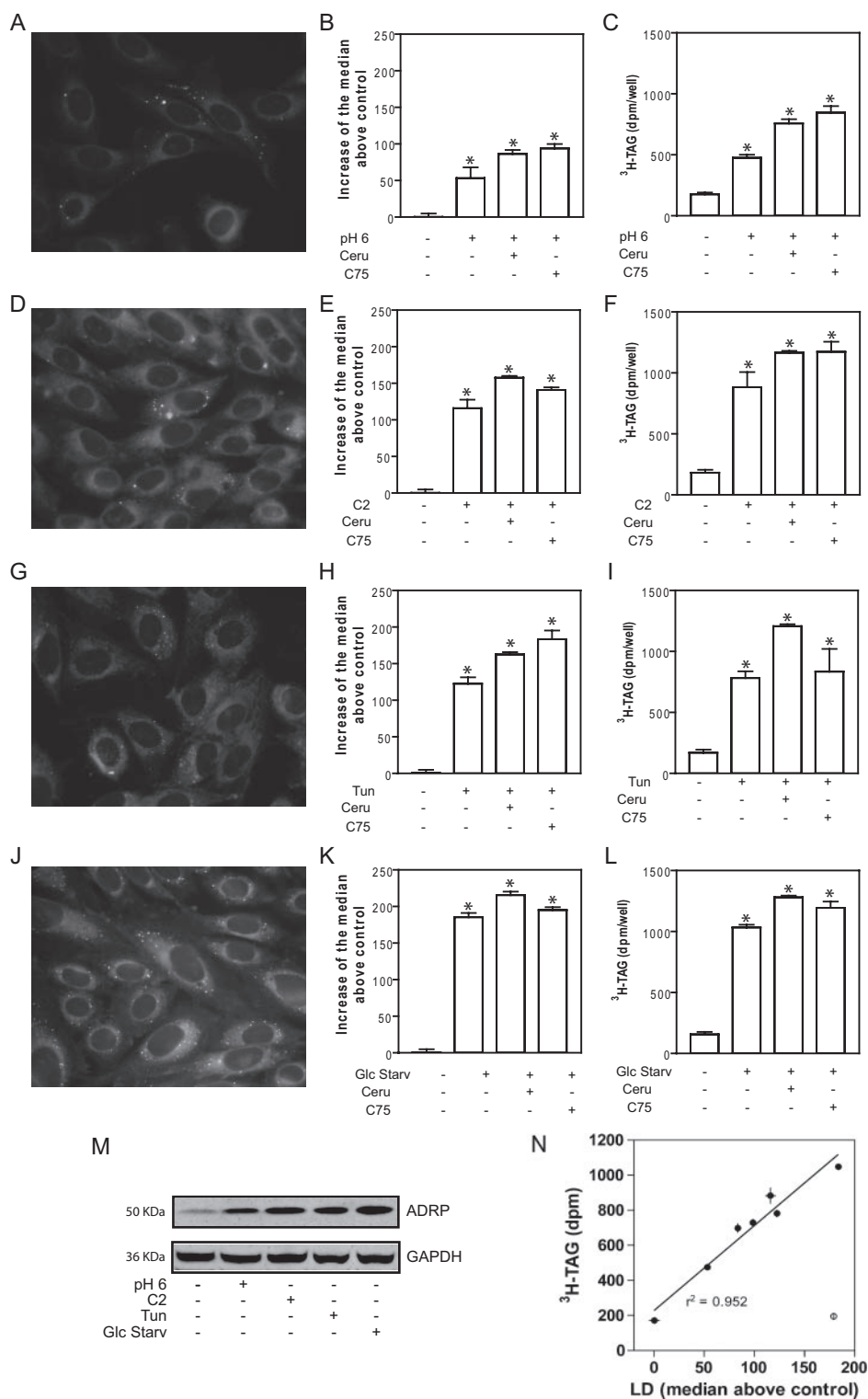


FIGURE 4. TAG synthesis and LD biogenesis are independent of fatty acid synthesis regardless of the kind of stress. Serum-starved cells (see Fig. 2A) were treated for 6 h with medium at pH 6 (A–C), with 20 μM C₂-ceramide (D–F), 5 μg/ml tunicamycin (G–I), or Krebs buffer without glucose (J–L), either in the absence or presence of cerulenin (30 μM) or C75 (20 μM). Cells were fixed, stained with Nile Red, and visualized in an epifluorescence microscope (A, D, G, and J) or processed for LD quantification by flow cytometry (B, E, H, and K). Photomicrographs and panel M show LD and ADRP occurrence, respectively, in the absence of fatty acid synthase inhibitors. Cells labeled for 24 h with 0.5 μCi/ml [³H]palmitic acid were treated as above, lipids were separated by TLC, and [³H]TAG were quantified (C, F, I, and L). Panel N shows the correlation between TAG synthesis and LD occurrence as induced by the six different treatments: cerulenin, C75, pH6, C₂-ceramide, tunicamycin, and glucose starvation, plus the control (closed symbols). LD induction with FBS did not stimulate the synthesis of TAG (open symbol). Results in panels G–J and K–N are means ± S.E. from three independent experiments. *, significantly different (*p* < 0.01) from controls.

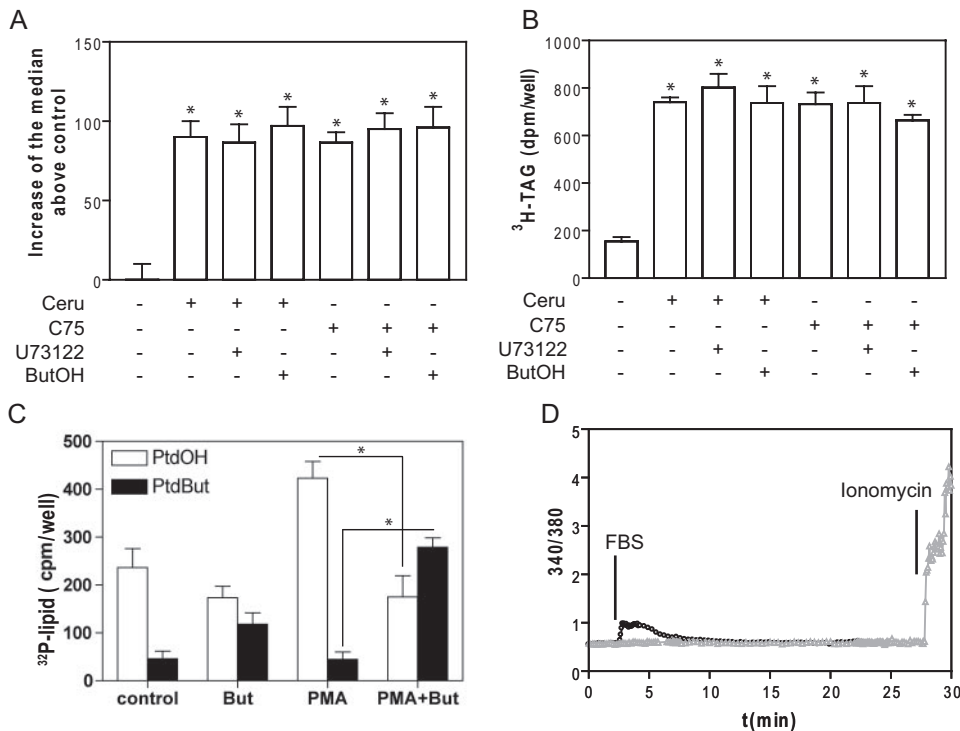


FIGURE 5. Phospholipases D and C are not involved in TAG synthesis and LD biogenesis induced by FAS inhibitors. *A*, cells were treated for 6 h with 30 μ M cerulenin or 20 μ M C75 in the presence or absence of 10 μ M U73122 or 25 mM 1-butanol (*ButOH*), fixed, stained with Nile Red, and analyzed by flow cytometry. *B*, cells were labeled for 24 h with 0.5 μ Ci/ml [³H]palmitic acid, and after the same treatments as in panel *A*, [³H]TAG was quantified after TLC separation of lipid extracts. *C*, to assess 1-butanol effects on phospholipase D-derived products, cells were labeled for 4 h with 2 μ Ci/ml [³²P]orthophosphate, then phospholipase D was stimulated with 100 nM phorbol 12-myristate 13-acetate in the presence or absence of 25 mM 1-butanol (*But*). Phosphatidic (*PtdOH*, open bars) acid and phosphatidylbutanol (*PtdBut*, filled bars) were quantified after TLC separation of lipid extracts. *D*, U73122 effect on PLC was tested in fura-2/AM-loaded cells treated sequentially with 7.5% FBS to stimulate the generation of an inositol 1,4,5-trisphosphate-mediated calcium signal, and with 5 μ M ionomycin, as indicated, in the absence (*black circles*) or presence (*gray triangles*) of 10 μ M U73122. Results are means \pm S.E. of six (*A*) or three (*B* and *C*) independent experiments carried out with triplicate determinations. Panel *D* shows an experiment representative of three with essentially the same outcome. *, significantly different ($p < 0.01$) from controls.

and 10% SDS-PAGE and transferred to nitrocellulose membranes. Primary (1:1000) and secondary antibodies (1:5000) were diluted in 25 mM Tris-HCl buffer, pH 7.4, containing 140 mM NaCl, 10% defatted dry milk, 0.1% bovine serum albumin, and 0.1% Tween 20. Membranes were developed using ECL detection reagents (Amersham Biosciences).

siRNA Transfection—We used three short interfering RNA (siRNA) duplexes (Gene Link) designed against human iPLA₂-VIA (GenBank™ accession number AF064594): PLA2G61-[693] (siRNA1), PLA2G62-[2295] (siRNA2), and PLA2G63-[2641] (siRNA3), with the following sequences: 5'-GAGAGACCGUCUCCAUAUATT-3' (sense) and 5'-TTCUCUCUGGCAGAAGGUAU-3' (antisense) for siRNA1; 5'-GUGACACAGUGCUGUCAATT-3' (sense) and 5'-TTCACUGUGACCGACCAGUU-3' (antisense) for siRNA2; and 5'-CCCUGACUGUCAAGGACAATT-3' (sense), and 5'-TTGGGACUGACAGUCCUGUU-3' (antisense) for siRNA3. Cells were transfected at 60% confluence by adding to each 35-mm well 1 ml of Opti-Mem (Amersham Biosciences) containing 1.5 μ l of the stock siRNA solution (20 μ M) and 5 μ l 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, then changed to serum-free medium for 24 h prior to

treatments. When appropriate, labeling with [³H]palmitate was done during these 24 h.

Calcium Imaging—Cells grown onto polylysine-coated coverslips were incubated with the calcium indicator Fura-2/AM at 4 μ M 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 HEPES, and 5 glucose, pH 7.4). After 1 h, cells were washed and coverslips were 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 4–10 s using a 12-bit charge-coupled device ERG ORCA Hamamatsu camera. A ratio image of cells was analyzed using the Metafluor software (Universal Imaging). 14–20 cells were analyzed in each experiment.

Phospholipase D—Cells were labeled for 4 h with 20 μ Ci/ml [³²P]orthophosphate (PerkinElmer Life Sciences), and phospholipase D was stimulated with 100 nM phorbol-12-myristate-13-acetate (Calbiochem) and with or without 25 mM 1-butanol. To quantify phosphatidic acid, lipid extracts were

separated by TLC using chloroform/methanol/acetone/acetic acid/water (50:10:20:10:5, v/v). Phosphatidylbutanol was separated using chloroform/methanol/acetic acid (65:15:2, v/v). [³²P]Phosphatidic acid and [³²P]phosphatidylbutanol were identified by co-migration with standards made after the incubation of phosphatidylcholine with cabbage phospholipase D (Sigma-Aldrich).

Confocal Microscopy—Serum-starved cells were labeled for 24 h with 1 μ M C₁-BODIPY 500/510-C₁₂. After washing with medium, cells were treated with drugs for 6 h, fixed, and photographed in a Leica TCS SP2 AOBs confocal microscope.

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 the Bonferroni multiple comparison test.

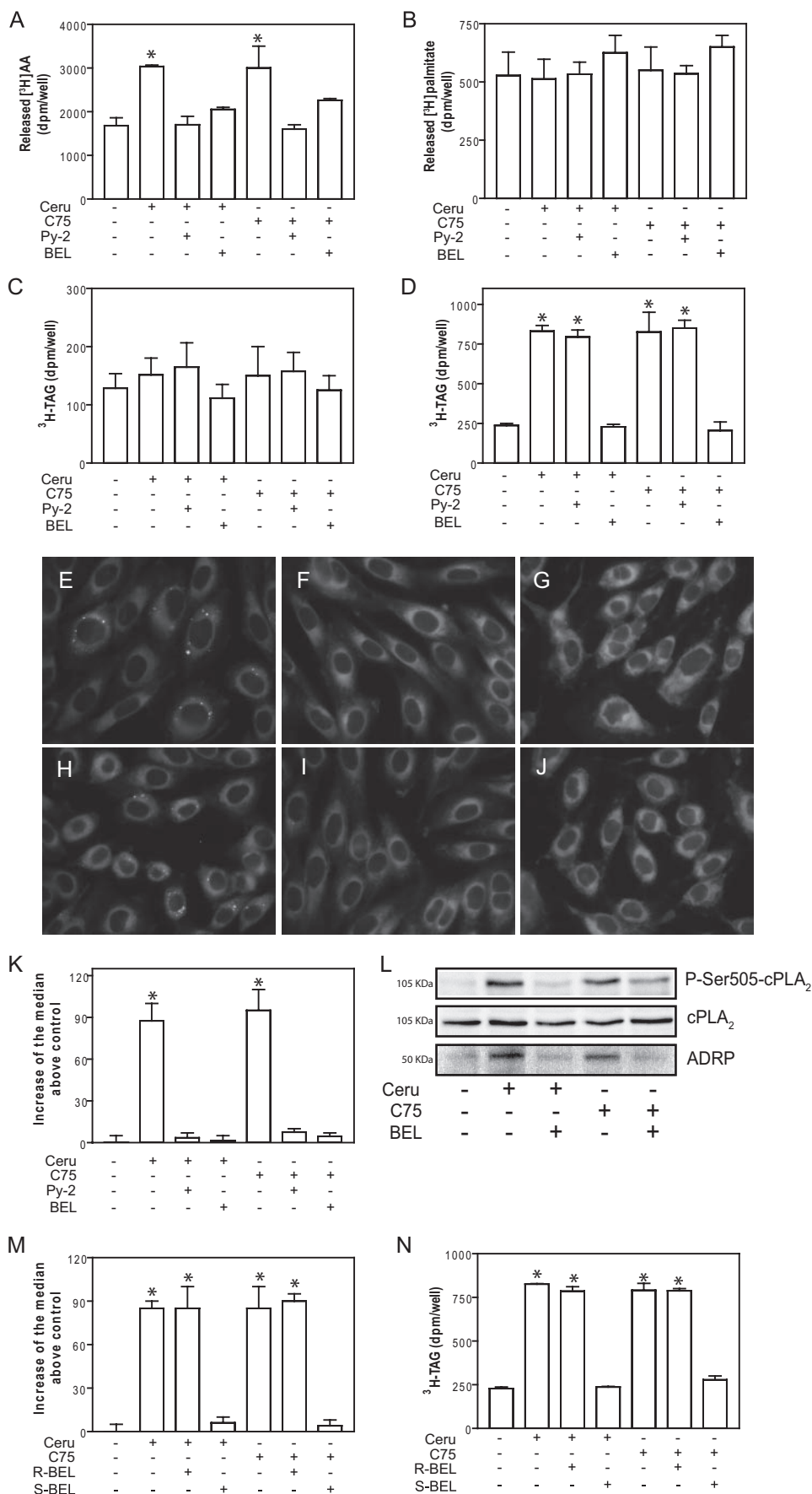
RESULTS

FAS Inhibitors Induce LD and TAG Synthesis—In preliminary experiments, we observed that the inhibitors of fatty acid synthase (FAS) cerulenin and C75 (21) induce LD biogenesis in CHO-K1 cells. To investigate the metabolic origin of TAG, we reasoned that stress induced by these drugs in cells kept in the absence of serum would provide a good working model: *de novo*

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synthesis of fatty acids is abolished by the treatments, and there is no external source of lipid, making it simpler to consider possible sources of LD content. In these conditions, 30 μ M cerulenin and 20 μ M C75 reduced cell viability 50% over 24 h, and inhibited acetate conversion to fatty acids 85–90% over a 6-h treatment (Fig. 1). The same 6-h treatments, over the duration of time at which cell viability was reduced 10% as assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay (not shown), induced the biogenesis of LD and increased levels of the LD-associated protein adipophilin (ADRP) (Fig. 2). Indirect quantification by flow cytometry showed that the occurrence of LD was about half that found after treatment with FBS (Fig. 2E). Unlike the induction of LD biogenesis with FBS, which depends on the uptake of serum lipoproteins (7), LD induction by FAS inhibitors in FBS-free medium necessarily has to take place from endogenous sources. To find out whether LD induction by FAS inhibitors takes place with synthesis of TAG, we measured total TAG content and synthesis. As shown in Fig. 3 (A and B), treatment with cerulenin or C75 induced little (~30%) but significant increases in TAG content. The same treatments in cells prelabeled for 24 h with [¹⁴C]acetate or with [³H]palmitic acid induced a 4-fold increase in [¹⁴C]- or [³H]TAG (Fig. 3, C and D), demonstrating new TAG synthesis from pre-existing fatty acids. In contrast, LD induction with serum did not involve synthesis of TAG from endogenous precursors. Alkaline deacylation of radioactive TAG confirmed its origin from pre-existing fatty acids and that the radioactive label was not incorporated into glycerol (Fig. 3, F and G).

Induction of LD Biogenesis and TAG Synthesis during Stress Does Not Require Fatty Acid Synthesis—The observation that LD biogenesis and TAG synthesis occur in the absence of fatty acid synthesis made us consider whether this would hold



in other models of cellular stress that do not involve the inhibition of FAS. To test this, we exposed the cells to various kinds of stress: pH 6, which has been shown to induce LD (22, 23), C₂-ceramide, which induces cell death after the early inhibition of phosphatidylcholine synthesis (9, 10), tunicamycin, which inhibits *N*-glycosylation and induces unfolded protein response (24, 25) and autophagy (26), and deprivation of glucose, a condition that induces apoptosis and autophagy (27–29). As shown in Fig. 4, all the treatments induced LD and stimulated the synthesis of TAG, and there was a high correlation ($r^2 = 0.952$) between TAG synthesis and LD promoted by all the stress inducers (Fig. 4N). Treatment with stress inducers in the presence of FAS inhibitors did not decrease LD formation or TAG synthesis but actually increased both effects. These results confirm that LD biogenesis and TAG synthesis during cellular stress do not require fatty acid synthesis.

Phospholipases D and C Are Not Involved in TAG Synthesis and LD Biogenesis during Stress—To address the origin of TAG we considered the role of phospholipases D and C, which generate phosphatidic acid and 1,2-diacylglycerol (DAG), respectively. Formation of these products could account for the incorporation of phospholipid-linked fatty acids into TAG, either directly or after the action of phosphatidate phosphohydrolase (PAP). To test this, we treated serum-starved cells with cerulein or C75 and in the presence of the phospholipase C inhibitor U73122 (30) or 1-butanol (31), which competes with water in a phospholipase D-catalyzed transphosphatidyl transfer reaction, generating phosphatidylbutanol instead of phosphatidic acid. These compounds did not affect LD biogenesis or TAG synthesis (Fig. 5, A and B), yet butanol inhibited phospholipase D-generated phosphatidic acid while increasing phosphatidylbutanol, and U73122 blocked inositol 1,4,5-trisphosphate-mediated calcium responses (Fig. 5, C and D).

Groups VIA and IVA Phospholipase A₂ (iPLA₂-VIA and cPLA₂α) Are Involved in TAG Synthesis and LD Biogenesis, Respectively—The release of fatty acids by a PLA₂-mediated process might also account for TAG synthesis from pre-formed fatty acids. Fig. 6A shows that treatment with cerulein or C75 stimulated the release of AA, in a manner that was sensitive to the cPLA₂α-selective inhibitor py-2 at 1 μM and to the iPLA₂ inhibitor BEL at 10 μM, suggesting at first glance the activation of cPLA₂α and iPLA₂. AA, however, was not incorporated into TAG (Fig. 6C). In contrast, palmitic acid was not released to the medium (Fig. 6B) but instead was used for TAG synthesis in a manner that was inhibited by BEL but not by py-2. This pharmacological evidence suggests the role of iPLA₂ but not cPLA₂α in providing fatty acids for TAG synthesis. On the other hand, py-2 inhibited LD biogenesis induced by cerulein or C75 (Fig. 6K). This agrees with our previous finding that LD biogenesis from exogenous lipid requires cPLA₂α (7) and further validates it when LDs are induced by cellular stress. Interestingly, also

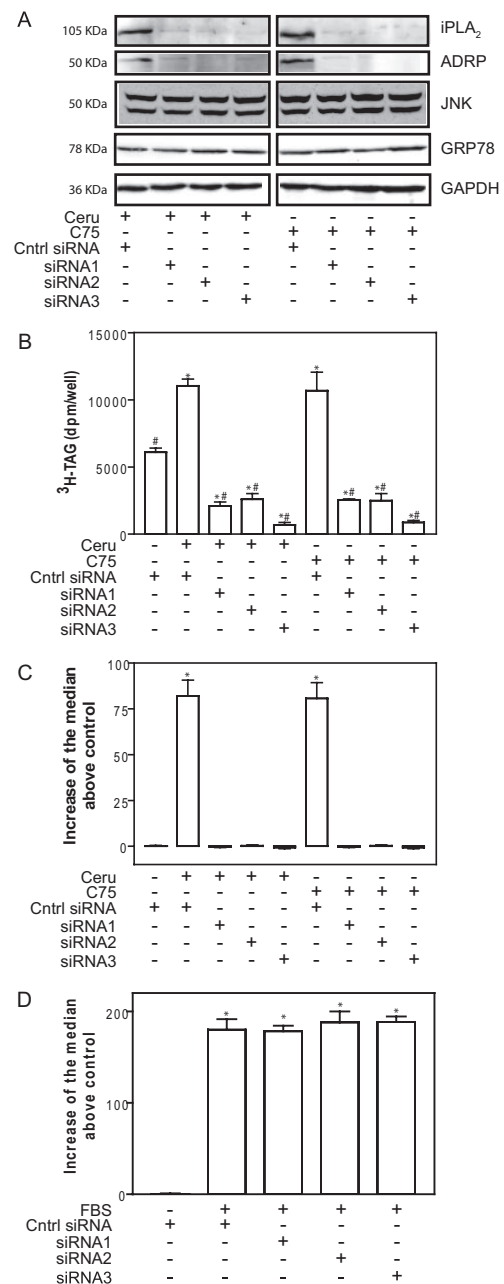


FIGURE 7. Silenced expression of iPLA₂-VIA inhibits TAG synthesis and LD biogenesis during stress, but it does not affect LD induction by FBS. Cells were transfected with siRNA as described under “Experimental Procedures,” and maintained in 7.5% FBS-containing medium during 48 h, then in medium without FBS for 24 h, either in the presence (B) or absence (A, C, and D) of 1 μCi/ml [³H]palmitate, prior to the 6-h treatments with FAS inhibitors (A–C) or 7.5% FBS (D). At the end of the 6-h treatments, radioactivity in TAG (B) and the occurrence of LD (C and D) were quantified. *Panel A* shows that down-regulation of iPLA₂-VIA with all three siRNA is mirrored by the inhibition of LD biogenesis, as indicated by reduced ADRP levels. *, significantly different ($p < 0.01$) from non-treated cells transfected with control siRNA. #, significantly different ($p < 0.01$) from cerulein- or C75-treated cells transfected with control siRNA.

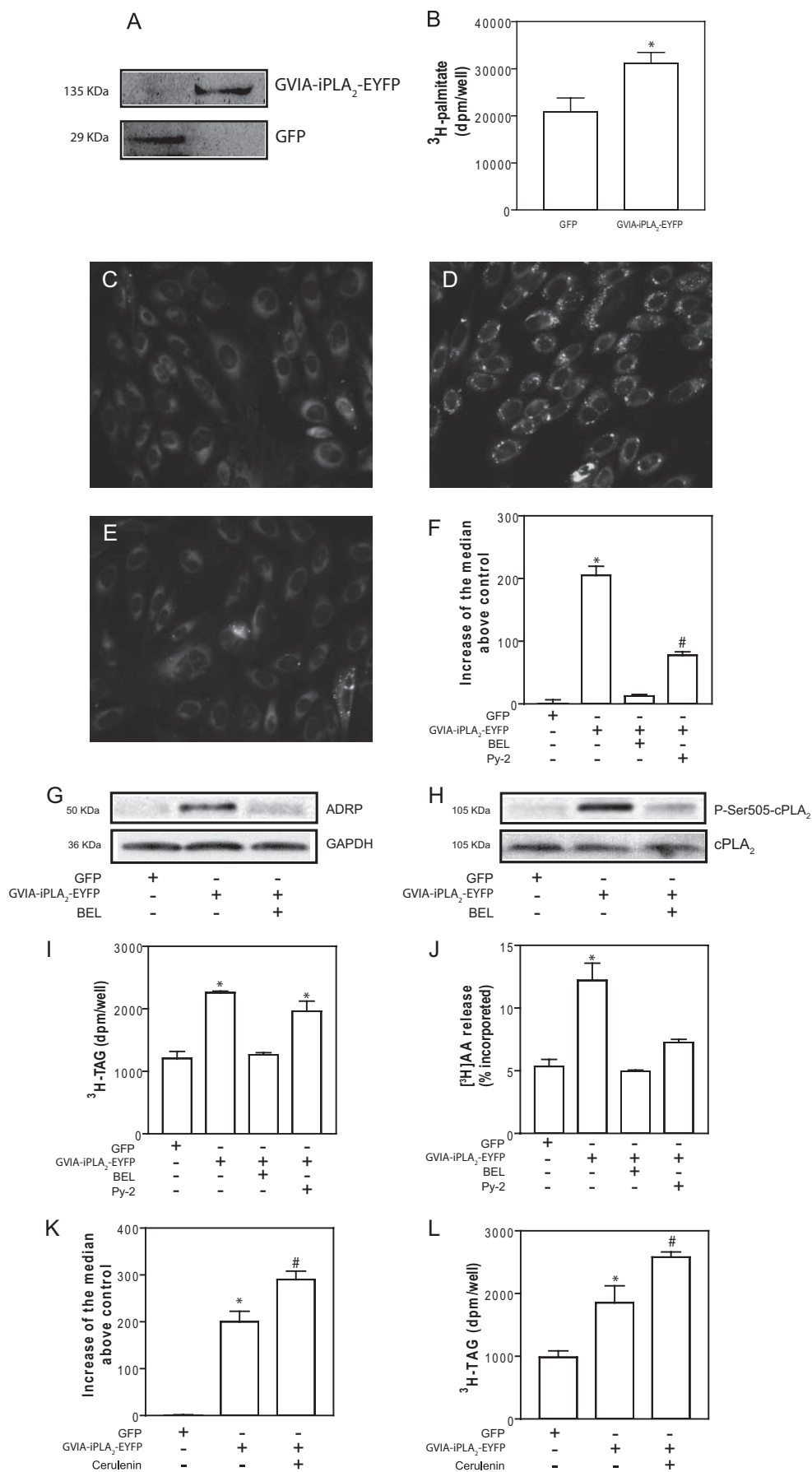
FIGURE 6. TAG synthesis and LD biogenesis during stress require the activities of iPLA₂-VIA and PLA₂ IVA. A–D, cells were labeled for 24 h with 0.5 μCi/ml [³H]arachidonic acid (A and C) or [³H]palmitic acid (B and D), washed, and treated for 6 h with cerulein or C75 in the absence or presence of 1 μM py-2 or 10 μM BEL. A and B show radioactivity released to the medium; C and D, radioactivity in TAG. E–K, cells were treated 6 h with cerulein (E–G) or C75 (H–J) alone (E and H) or with 1 μM py-2 (F and I) or 10 μM BEL (G and J), fixed, stained with Nile Red, and photographed (E–J). Alternatively, LDs were indirectly quantified with flow cytometry (K). BEL inhibition of LD biogenesis as induced by cerulein or C75 was mirrored by the expression of ADRP and phosphorylation of cPLA₂α at Ser⁵⁰⁵ (L). Unlike R-BEL, which was without effect, 10 μM S-BEL inhibited LD biogenesis (M) and TAG synthesis (N) as stimulated by cerulein or C75. Results in A–D, K, M, and N are means ± S.E. of three independent experiments each carried out in triplicate. *, significantly different ($p < 0.01$) from controls.

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BEL inhibited LD formation induced by cerulenin or C75, monitored by microscopic examination (Fig. 6, E–J), flow cytometry (Fig. 6K), or by the expression of ADRP (Fig. 6L). Inhibition of iPLA₂ also precluded TAG synthesis and LD biogenesis induced by acidic pH, C₂-ceramide, tunicamycin, or deprivation of glucose (not shown). The iPLA₂ family consists of two members in mammals, namely Group VIA PLA₂ (iPLA₂-VIA) and Group VIB PLA₂ (iPLA₂-VIB) (14). The *S*-enantiomer of BEL has been shown to be more potent on iPLA₂-VIA than on iPLA₂-VIB, whereas the *R*-isomer was more potent on iPLA₂-VIB (32). In our system, 10 μM *S*-BEL blocked incorporation of palmitate into TAG, and the generation of LD as stimulated by cerulenin or C75, but the same concentration of *R*-BEL was without effect (Fig. 6, M and N). Taken together, these data suggest the implication of iPLA₂-VIA in the release of fatty acids required for TAG synthesis in our model.

Cerulenin and C75 promoted the phosphorylation of cPLA₂α at Ser⁵⁰⁵, which is necessary for enzyme activation during LD biogenesis (7), and it was inhibited to some degree by BEL (Fig. 6L), suggesting that iPLA₂-VIA acts upstream of cPLA₂α. With this in mind, and taking into account that cPLA₂α is specific for AA whereas iPLA₂ shows no substrate specificity for the fatty acid at the *sn*-2 position (14), the inhibition of AA release by BEL (Fig. 6A) agrees with iPLA₂-VIA playing a cPLA₂α-activating role.

Down-regulation of the Expression of iPLA₂-VIA Abrogates TAG Synthesis and LD Biogenesis—A major precursor of DAG for TAG synthesis is phosphatidic acid, which is dephosphorylated by PAP. Although BEL is very specific for the Group VI enzymes of the PLA₂ family, the drug also inhibits PAP (18, 33, 34). A more specific approach to inhibit iPLA₂-VIA came from the use of three siRNA



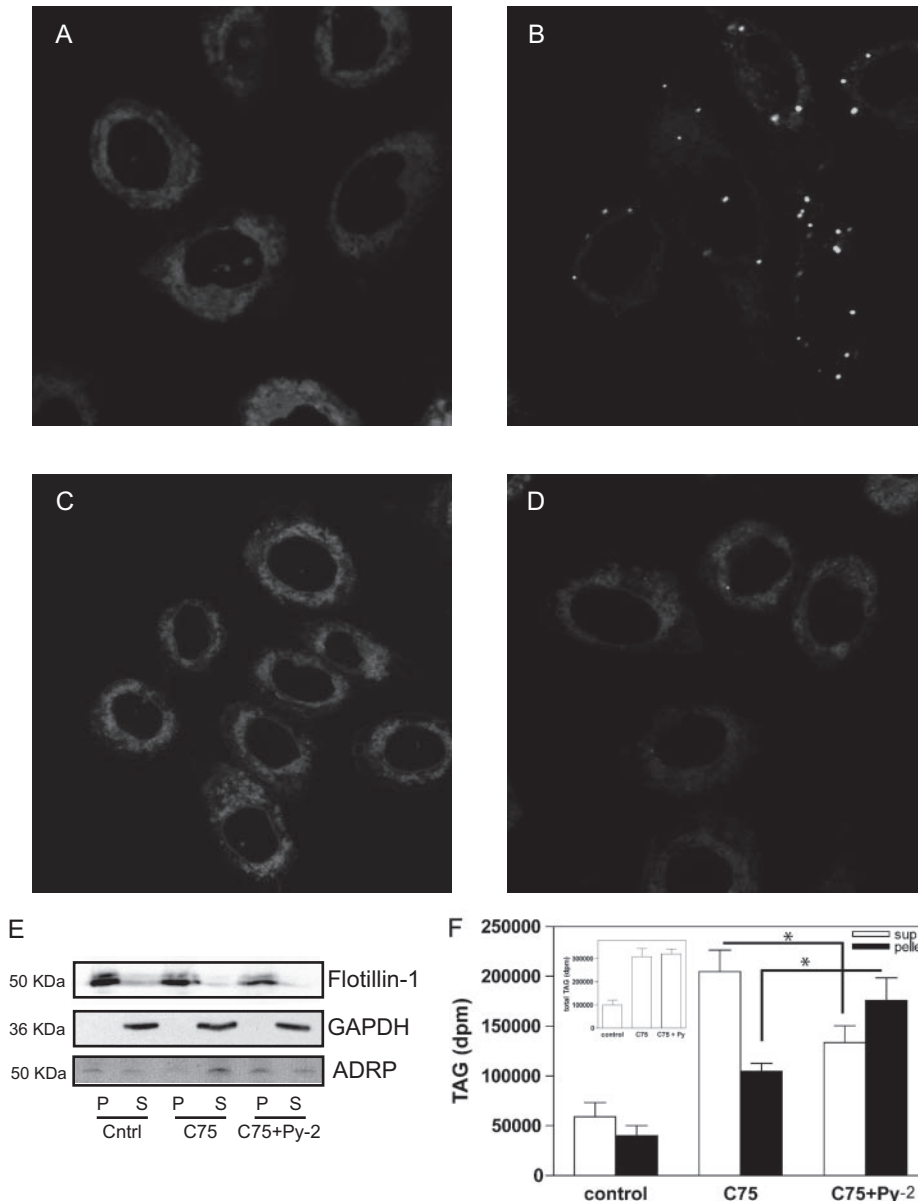


FIGURE 9. LD content during stress is formed after iPLA₂-VIA. A–D, cells were incubated during 24 h with 1 μ M C₁-BODIPY[®] 500/510-C₁₂. After washing, cells were left untreated (A) or treated for 6 h with 20 μ M C75 (B), C75 together with 10 μ M BEL (C), or with 7.5% FBS (D), and visualized in the confocal microscope. E and F, after labeling cells during 24 h with 50 μ Ci/ml [³H]palmitate, they were washed with medium and left untreated (control) or treated for 6 h with 20 μ M C75 alone or in combination with 1 μ M py-2. Cells were then disrupted by a 10-s sonication and centrifuged 60 min at 20,000 \times g to obtain pellet and supernatant fractions that were enriched in flotillin-1 and GAPDH, respectively (E). Radioactive TAG in the supernatant increased 4-fold due to C75 treatment, but this was reduced by half after the inhibition of LD biogenesis with py-2 (F). Inset in panel F shows that py-2 treatment did not affect total [³H]TAG. *, significantly different ($p < 0.05$).

designed against the human sequence. iPLA₂-VIA occurs in human tissues in at least five different splicing variants but only two have enzymatic activity, termed VIA-1 and VIA-2 (14). Although iPLA₂-VIA-1 appears to be the only variant expressed

A-EYFP-transfected cells also mirrored the expression levels of ADRP (Fig. 8G) and TAG synthesis (Fig. 8I). Again, increased TAG synthesis was blocked by BEL but not by py-2 (Fig. 8I),

in CHO cells (35), the siRNA sequences were designed against the common exons to ensure down-regulation of both variants.

As shown in Fig. 7A, all three siRNA reduced iPLA₂-VIA expression. Unlike cells transfected with control siRNA, which when challenged with cerulenin or C75 increased expression of ADRP (Fig. 7A), synthesis of TAG (Fig. 7B), and LD (Fig. 7C), knockdown of iPLA₂-VIA rendered the cells unresponsive to cerulenin or C75 in terms of ADRP expression (Fig. 7A), TAG synthesis (Fig. 7B), and LD biogenesis (Fig. 7C), but it did not affect LD biogenesis induced by FBS (Fig. 7D) or increased expression of ADRP (not shown). Again, the results show that, unlike the paradigm of LD formation from exogenous lipid, iPLA₂-VIA is required for TAG synthesis taking place during LD biogenesis under stress.

Overexpression of iPLA₂-VIA Increases TAG Synthesis and LD Occurrence—Transient transfection of a construct encoding a fusion protein consisting of iPLA₂-VIA2 followed by EYFP (iPLA₂-VIA2-EYFP) (Fig. 8A) increased free fatty acid availability, as evidenced in the presence of the acyl-CoA synthetase inhibitor triacsin C (Fig. 8B). In contrast to serum-deprived GFP-transfected cells, which had little LD content (Fig. 8, C and F), overexpression of iPLA₂-VIA-EYFP induced LD in serum-deprived conditions (Fig. 8, D and F) to levels higher than those attained in non-transfected cells under stress (compare with Fig. 4). As expected, this high LD content was abolished by 10 μ M BEL (Fig. 8, E and F). Increased LD occurrence under basal conditions in iPLA₂-VI-

FIGURE 8. Overexpression of iPLA₂-VIA increases TAG synthesis and LD biogenesis. Cells were transfected with GFP or with a fluorescent fusion protein containing group VIA2 human iPLA₂ followed by EYFP (GVIA2-iPLA₂-EYFP), as shown in panel A. GVIA2-iPLA₂-EYFP-transfected cells that were labeled 24 h with [³H]palmitate had increased free fatty acid content after a 6-h treatment with triacsin C (B). Unlike cells transfected with GFP, which had little LD content in the absence of stress inducers (C), LD occurrence in GVIA2-iPLA₂-EYFP-transfected cells was well apparent (D) and sensitive to BEL inhibition (E). GVIA2-iPLA₂-EYFP-transfected cells also had increased ADRP expression that was BEL-sensitive (G). LD indirect quantification by flow cytometry (F) shows that increased LD occurrence in GVIA2-iPLA₂-EYFP-transfected cells was inhibited also by the cPLA₂ inhibitor py-2. Radioactive TAG also increased in [³H]palmitate-labeled cells (I), but unlike LD occurrence, this effect was not inhibited by py-2. Increased LD in GVIA2-iPLA₂-EYFP-transfected cells was mirrored by increased cPLA₂ activity (J) and phosphorylation at Ser⁵⁰⁵ (H) that were inhibited by BEL. Overexpression of iPLA₂-VIA also enhanced LD biogenesis (K) and TAG synthesis (L) during a 6-h treatment with cerulenin.

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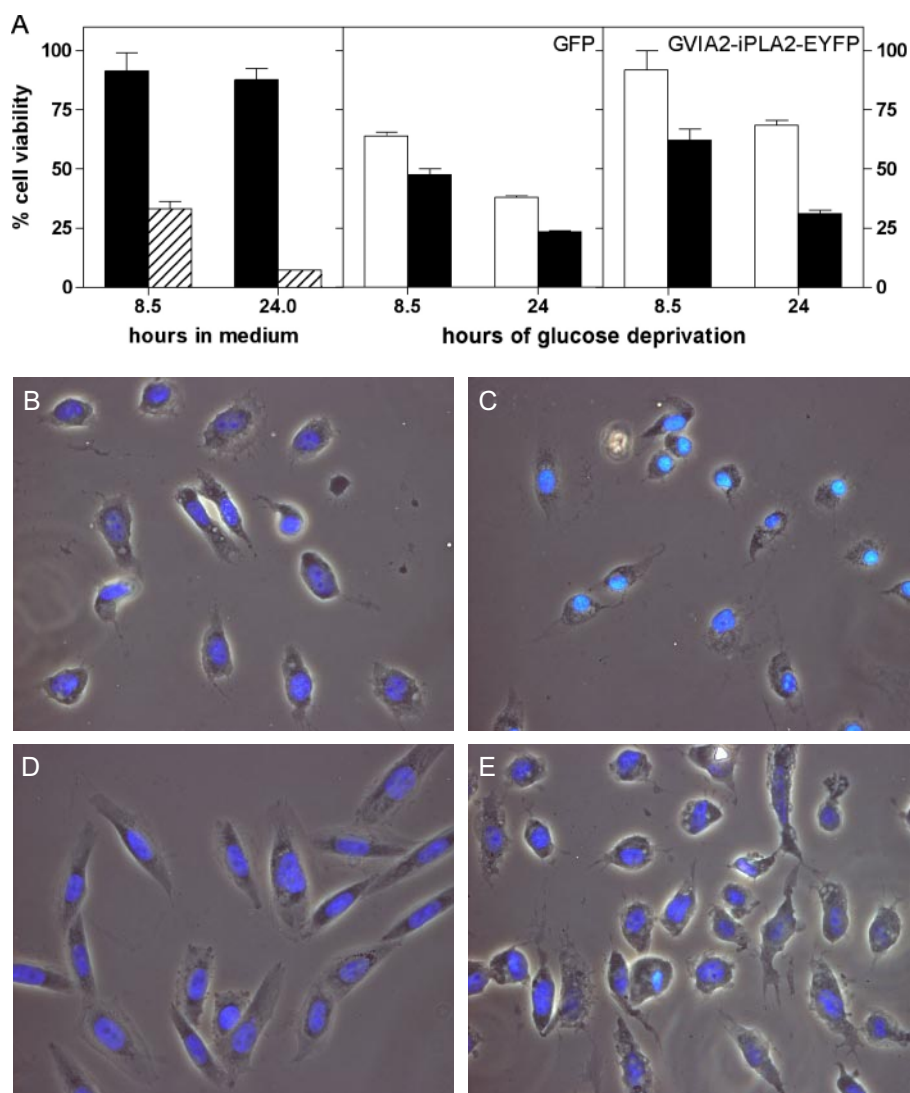


FIGURE 10. Cell viability during glucose deprivation is compromised after the inhibition of LD biogenesis. In *A*: *Left panel*, cells were treated for 8.5 or 24 h in FBS-free culture medium with 1 μM py-2 (filled bars) or with 10 μM BEL (striped bars). *Middle and right panels*: cells transfected with GFP or with GVIA2-iPLA₂-EYFP, as indicated, were maintained in glucose-free Krebs buffer for 8.5 or 24 h, in the absence (open bars) or presence (filled bars) of 1 μM py-2. In all cases, viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction. *B–E*, cells transfected with GFP (*B* and *C*) or with GVIA2-iPLA₂-EYFP (*D* and *E*) were maintained for 20 h in glucose-free Krebs buffer, and with (*C* and *E*) or without (*B* and *D*) 1 μM py-2. The images are merged 4',6-diamidino-2-phenylindole-fluorescence and phase-contrast photomicrographs.

whereas increased LD occurrence was inhibited by BEL and py-2 (Fig. 8F). Importantly, overexpression of iPLA₂-VIA increased the phosphorylation of cPLA₂α at Ser⁵⁰⁵ (Fig. 8H) and the release of AA (Fig. 8J), in a BEL-sensitive fashion, the latter effect being also sensitive to py-2. Treatment of iPLA₂-VIA-EYFP-transfected cells with cerulenin further increased LD occurrence and TAG synthesis (Fig. 8, K and L). Taken together, these results confirm the implication of iPLA₂-VIA in TAG synthesis and LD biogenesis, and suggest again a role for the enzyme in the events leading to phosphorylation and activation of cPLA₂α.

TAGs in LD Arise from iPLA₂-VIA—The previous results demonstrate that LD biogenesis during stress takes place with TAG synthesis after fatty acids released by iPLA₂-VIA and strongly suggest that the newly formed TAGs are present in LD. To further assess this, we labeled cells for 24 h with the fluores-

cent fatty acid C₁-BODIPY® 500/510-C₁₂ and treated them with C75. As shown in Fig. 9 (A–D), C75 induced the formation of fluorescent LD, whereas iPLA₂-VIA inhibition retained the fluorescent label in perinuclear membranes. Similarly, when LD biogenesis was induced by FBS, which is an iPLA₂-VIA-independent process, fluorescence remained associated to membranes. A more direct evidence was obtained from a fractionation experiment in [³H]palmitate-labeled cells: after a 24-h labeling period, LD were induced with C75, and cells were disrupted and centrifuged to obtain crude supernatant and membrane fractions. ADRP was induced by C75 treatment, and it was found mainly in the supernatant (Fig. 9E), indicating the enrichment of this fraction in LD. We have reported that inhibition of cPLA₂α during exogenous lipid loading results in the retention of TAG in membrane fractions (7). In agreement with this, the increase in [³H]TAG in the LD-enriched supernatant induced by C75 was partially inhibited by py-2, whereas the converse was true regarding the membrane fraction (Fig. 9F). Taken together, the results show that TAG in LD formed during cellular stress arise, at least in part, from phospholipid hydrolysis by iPLA₂-VIA.

LD Biogenesis during Glucose Deprivation May Be a Survival Strategy—To address a physiologi-

cal significance of LD biogenesis during stress, we monitored cell viability after deprivation of glucose under conditions that prevent LD formation. As shown in the preceding experiments, this can be done either by inhibiting iPLA₂-VIA, which prevents TAG synthesis and cPLA₂α activation, or by inhibiting cPLA₂α, which does not affect TAG synthesis but blocks LD biogenesis. Other than iPLA₂, BEL also inhibits PAP, which that can account for the toxicity of this compound (12, 13). In fact, in CHO-K1 cells maintained in serum-free medium, 10 μM BEL decreased cell viability some 70% in 8.5 h (Fig. 10A). We therefore chose cPLA₂α inhibition with 1 μM py-2, which is not toxic over a 24-h period (Fig. 10A, left panel). When GFP-transfected cells were switched to glucose-free Krebs buffer, viability decreased 30% at 8.5 h and the presence of py-2 decreased it further 20% (Fig. 10A, middle panel). Glucose deprivation during 8.5 h did not affect viability of cells transfected with iPLA₂-

VIA-EYFP (Fig. 10A, right panel), but py-2 still was toxic (Fig. 10A, right panel). Also, glucose deprivation over 20 h induced cellular shrinkage in GFP-transfected cells (Fig. 10B), which underwent further shrinkage and nuclear condensation in the presence of the cPLA₂α inhibitor (Fig. 10C). During glucose starvation, py-2 also induced cellular shrinkage in iPLA₂-VIA-EYFP-transfected cells (Fig. 10E), which were morphologically unaltered in the absence of the inhibitor (Fig. 10D). These results suggest that LD biogenesis during glucose deprivation has a survival effect.

DISCUSSION

Our results show that in the absence of *de novo* synthesis and with no external source of fatty acids, induction of cellular stress leads to the synthesis of TAG from pre-existing fatty acids in a process requiring iPLA₂-VIA, and to LD biogenesis, which requires cPLA₂α. In contrast, induction of LD by FBS is independent of iPLA₂-VIA. Further, phosphorylation and activity of cPLA₂α decreases after iPLA₂-VIA inhibition and increases after iPLA₂-VIA overexpression, suggesting that enzyme control mechanisms for TAG synthesis and LD generation operate in a concerted manner.

LD biogenesis and synthesis of TAG is a hallmark of cellular stress, and it was suggested that alterations in phosphatidylcholine metabolism could be the source of TAG (8, 36). TAG synthesis takes place from DAG and a CoA-activated fatty acid, by the action of DAG acyltransferases (37). In proliferating cells, acetyl-CoA carboxylase and fatty acid synthase control the supply of *de novo* DAG, which can be converted either to TAG or phospholipids. In fact, when phosphatidylcholine biosynthesis decreases, TAG accumulates (38). Iorio and colleagues (8) proposed that the mechanism for TAG synthesis during programmed cell death could involve a reduction in phospholipid biosynthetic pathways and/or the activation of specific phospholipases. Both mechanisms could increase DAG that would account for LD biogenesis at the expense of phospholipid pools. As for the second mechanism, we show that phospholipases C and D are not implicated. Regarding the first mechanism, a very early inhibition of the CDP-choline pathway for phosphatidylcholine synthesis has been shown to occur in some models of toxicity with the amphiphilic drugs ET-18-OCH₃ (39), hexadecylphosphocholine (40), or C₂-ceramide (9, 10), or with cerulenin (38), and in fact we show that the two latter drugs induce TAG synthesis and LD formation. Further, CHO-MT58 cells, which express a thermosensitive CTP:phosphocholine cytidyltransferase, divert newly synthesized DAG to the TAG pool when cultured at the restrictive temperature (38), and we have observed that under these conditions they develop LD.⁶ Nevertheless, it is unlikely that all kinds of cellular stress inhibit phospholipid synthesis. Also, it is difficult to envisage how cerulenin or C75-mediated inhibition of fatty acid synthase could increase *de novo* formation of DAG unless fatty acids come from alternative sources. In this regard, our results show that (a) fatty acid availability increases after transfection of iPLA₂-VIA, and (b) TAG synthesis takes place from pre-existing fatty acids and is dependent on iPLA₂-VIA. We have been unable,

however, to detect decreases in total phospholipids mirroring the increase in TAG synthesis (Fig. 3E), even with an additional 24-h incubation of the cells in fresh medium after labeling with [³H]palmitate, to allow full incorporation of the tracer (not shown). This suggests that iPLA₂-VIA provides fatty acids from a minor phospholipid pool.

Little is known about the control iPLA₂-VIA (14). Various splice variants coexist in cells, and two of them (VIA-1 and VIA-2) have enzymatic activity. The enzyme contains eight (VIA-1) or seven (VIA-2) ankyrin repeats, which may allow oligomerization and full enzymatic activity. These two variants work similarly in our hands, because iPLA₂-VIA-1 is the main form expressed in CHO cells, and transfection of iPLA₂-VIA-2 has the same effect regarding TAG synthesis for the biogenesis of LD. Another two variants lack enzymatic activity but may act as dominant-negative inhibitors by precluding association of the active variants (41). It would be interesting to determine whether differential expression of active and inactive variants may account for LD generation during stress. In U937 leukemia cells undergoing apoptosis, iPLA₂-VIA is cleaved by caspase-3, generating a fragment responsible for increased activity (42). This is not the mechanism taking place in our model, because we have observed that (a) treatment of CHO-K1 cells with 2 μM actinomycin D, which induces strong caspase-3 proteolytic processing and activity, does not induce LD formation, and (b) LD induction by cerulenin is not inhibited by co-treatment with the pan-caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone at 100 μM (not shown).

Among the different groups of PLA₂ enzymes, iPLA₂-VIA plays a significant role in the Lands cycle of basal phospholipid remodeling through deacylation/reacylation reactions (18, 43). An account of the role of iPLA₂-VIA in phospholipid homeostasis is the observation that overexpressing CTP:phosphocholine cytidyltransferase increases phosphatidylcholine content to a much lesser extent than expected, due to accelerated degradation by iPLA₂-VIA, which appears to respond to excess phospholipid synthesis (44, 45). This housekeeping role of iPLA₂-VIA, which opposes that of CTP:phosphocholine cytidyltransferase in membrane phospholipid turnover, is important for cell cycle progression (46). Also, iPLA₂-VIA-mediated phospholipid remodeling allows mitochondrial membrane repair during oxidative damage (47). Release of fatty acids in U937 cells undergoing apoptosis is mediated by iPLA₂-VIA (42, 48), and in this case the enzyme works promoting cell death. Also, overexpression of iPLA₂-VIA in the same cells accelerates apoptosis induced by oxidative stress (49). In both cases, however, pharmacological inhibition or down-regulated expression did not rescue at longer times, showing that the enzyme is not determinant for apoptosis. Rather, it is responsible for the generation of lysophosphatidylcholine, which is recognized by macrophages for clearance of dying cells (13–17). Transgenic mice expressing human iPLA₂γ (iPLA₂-VIB) in a cardiac myocyte-selective manner accumulate great amounts of TAG during a brief caloric restriction, however this enzyme contributes to mitochondrial dysfunction (50). Our data show that overexpression of iPLA₂-VIA delays cell death under glucose deprivation; further, preventing LD biogenesis after the inhibition of cPLA₂α opposes the protection elicited by iPLA₂-VIA. Taken

⁶ A. Riihimäki, M. Barceló-Torns, A. Gubern, and E. Claro, unpublished results.

together, the results suggest a surviving role of LD biogenesis during glucose deprivation.

LD biogenesis has a protecting role against lipotoxicity derived from fatty acid accumulation (51, 52). This condition requires the overload of high concentrations of exogenous fatty acids and does not apply to our experimental paradigm. Mammals survive starvation by activating proteolysis and lipolysis. Other than muscle, where protein is degraded mainly by the proteasome system and is the major source of precursors for hepatic gluconeogenesis, fasting activates autophagy in most tissues (53). Lipolysis, on the other hand, takes place primarily in adipocytes, which are the major source of circulating free fatty acids. This mechanism has received most attention over the past years (1), but all tissues and cells types must be able to release fatty acids from TAG packed in LD (54), conceivably to supply local energetic needs through β -oxidation. In this regard, a recent report (55) shows that neurons from female rats form LD and survive nutrient deprivation better than neurons from male animals, which undergo autophagic death. Our results agree essentially with this report, suggesting that LD biogenesis in cells under stress may represent a lipid counterpart of autophagy: fatty acids used for structural functions (membrane phospholipids) could be reused for TAG synthesis in a process mediated by iPLA₂-VIA and packed in LD for energetic functions in a process requiring cPLA₂ α .

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