Cells exposed to stress of different origins synthesize triacylglycerols and generate lipid droplets (LD), but the physiological relevance of this response is uncertain. Using complete nutrient deprivation of cells in culture as a simple model of stress, we have addressed whether LD biogenesis has a protective role in cells committed to die. Complete nutrient deprivation induced the biogenesis of LD in human LN18 glioblastoma and HeLa cells and also in CHO and rat primary astrocytes. In all cell types, death was associated with LD depletion and was accelerated by blocking LD biogenesis after pharmacological inhibition of Group IVA phospholipase A2 (cPLA2α) or down-regulation of ceramide kinase. Nutrient deprivation also induced β-oxidation of fatty acids that was sensitive to cPLA2α inhibition, and cell survival in these conditions became strictly dependent on fatty acid catabolism. These results show that, during nutrient deprivation, cell viability is sustained by β-oxidation of fatty acids that requires biogenesis and mobilization of LD.

Background: Cellular stress leading to cell death induces the formation of lipid droplets.

Results: Nutrient deprivation induces LD biogenesis and mobilization, fueling fatty acid oxidation to sustain cell viability. 

Conclusion: β-Oxidation requires biogenesis and mobilization of LD.

Significance: The role of LD in cell survival and β-oxidation might provide new potential targets for antitumor therapy.
LD Fuel β-Oxidation during Nutrient Deprivation

Should this hypothesis hold, interfering with LD biogenesis might be a potential antitumor strategy.

Here we show that survival of different cell types (CHO, LN18 human glioblastoma, HeLa, or rat astrocytes) under complete nutrient deprivation depends on LD, which confer the capacity to degrade fatty acids through β-oxidation.

EXPERIMENTAL PROCEDURES

Materials—[9,10-3H]Palmitic acid (60 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. Lipofectamine™ RNAlMAX transfection reagent was from Invitrogen. cPLA2α inhibitor pyrrolidine-2 (py-2, catalogue number 525143) was from Calbiochem; carnitine palmitoyltransferase-1 (CPT1) inhibitor etomoxir (EX), autophagy inhibitor 3-methyladipine (3-MA), sodium oleate, primuline, Nile red, Oil Red O, and propidium iodide (PI) were from Sigma. Rabbit anti-cPLA2α and anti-phospho-Ser505 cPLA2α antibodies were from Cell Signaling; rabbit anti-perilipin 2, anti-perilipin 3, anti-CERK, anti-LC3B, and anti-CPT1 antibodies were from Abcam, and mouse anti-β-actin was from Sigma. 4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY® 493/503) and LysoTracker® Red DND-99 were from Molecular Probes.

Cells—Human glioblastoma LN18 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma), containing 10% fetal bovine serum (FBS; Sigma), 100 units/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). CHO-K1 cells were cultured in Ham’s F-12 medium (Sigma), containing 7.5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell passages were made once a week by trypsinization (Sigma). Primary astrocytes from rat cerebral cortex were cultured as described (26). For experiments, cells were seeded on 6- or 24-well plates at a density of 10^5 cells/ml and maintained for 24 h in culture medium in the absence of FBS in order to deplete cells of LD before the treatments, as described previously (22, 23). This plating density ensured that cells were far from confluence (60–70%) at the time of treatments, avoiding confluence-induced generation of LD (14, 15, 27). Stress was induced by complete nutrient deprivation, which was achieved washing twice with phosphate-buffered saline (PBS; Sigma) and substituting medium by Krebs-Henseleit (KH) buffer without glucose (116 mM NaCl, 4.7 mM KCl, 25 mM NaHCO3, 1.2 mM MgSO4, 1.2 mM KH2PO4, and 1.3 mM CaCl2, pH 7.4, equilibrated with O2/CO2 (95:5)).

Microscopy—For fluorescence microscopy, cells cultured on glass coverslips were washed with PBS, fixed with 3% paraformaldehyde (Sigma) for 10 min, and washed twice with PBS. The occurrence of LD was routinely checked by microscopic examination of cells stained with Nile red, BODIPY® 493/503, or Oil red-O. For Nile red staining, cells were overlaid with 0.5 ml of a working solution of PBS containing 2 μl/ml Nile red stock solution in acetonitrile (0.2 mg/ml). For BODIPY® 493/503 staining, cells were overlaid with 0.5 ml of a working solution of PBS containing 2.5 μl/ml of a BODIPY® 493/503 stock solution in 98% ethanol (1 mg/ml). Oil Red O staining of LD followed the recommendations of Koopman et al. (28). In most instances, nuclei were counterstained with DAPI. To label acidic compartments, unfixed cells were overlaid with warmed culture medium or buffer, containing 75 nm LysoTracker® Red DND-99. Cells were incubated for 30 min and then washed with PBS and fixed with 3% paraformaldehyde. Samples were kept in the dark until photographed in a Leica Qwin 500 microscope with a Leica DFC500 camera, using Leica DCviewer 3.2.0.0 software. Electron microscopy was carried out as described (23).

Flow Cytometry—Indirect quantification of Nile red-stained LD by flow cytometry was performed as described (22, 23, 29) with the only difference that cells were not fixed. Briefly, harvested cells were transferred to tubes, together with their overlying medium or buffer, to prevent the loss of floating cells. After two washes with PBS, cells were resuspended in 0.5 ml of the Nile red working solution (0.4 μg/ml final concentration). Samples were kept 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, 10,000 events were acquired. For each assay, we obtained a side scatter versus forward scatter plot (SS/FS plot), a bidimensional representation of each event in terms of structural complexity (SS) and size (FS). Each sample appeared split into two populations differing in FS value. Staining with PI showed that the population with a lower FS value (smaller cells) consisted of dead cells. The shift of dead cells to lower FS values allowed us to quantify separately LD content in viable and dead cells in the same sample, although the Nile red emission spectrum does not allow co-staining with PI. Alternatively, cells were stained with PI and BODIPY® 493/503. To do this, cells were processed as described above and resuspended in 0.5 ml of the BODIPY® 493/503 working solution containing 2.5 μl of the PI stock solution (1 mg/ml in water). The working solution also contained 2.5 μl of RNase (Sigma) stock solution (1 mg/ml in water), to achieve specific DNA staining. Samples were kept in the dark at 4°C for 20 min before carrying out the analysis in the FL1 and FL3 channels.

Lactate Dehydrogenase (LDH) Assay—To confirm that PI staining monitored cell death accurately, we measured LDH activity released into the medium. Briefly, the medium was collected carefully, centrifuged for 10 min at 13,000 × g, and incubated in a reaction mix containing NADH, pyruvate, and sodium azide. NADH oxidation was followed after the decrease of absorbance at 340 nm, and its rate was referred to total LDH activity, which was estimated after hypotonic lysis of untreated cells.

β-Oxidation—Cells seeded in 24-well plates were maintained for 24 h in medium without FBS and then labeled overnight with about 1 μCi/ml [3H]palmitic acid (10–30 nM, final concentration) in culture medium containing 0.5% delipidated bovine serum albumin (BSA; Sigma). After labeling, cells were washed once with BSA-containing culture medium and twice with PBS and left for 30 min in culture medium. The β-oxidation assay was based on that described by Djouraudi et al. (30) and monitored the generation of [3H]water. Treatments were run with quadruplicate determinations and started by substitution of culture medium by 0.5 ml of fresh culture medium or KH buffer without glucose. After treatments, which proceeded for 1–8 h, 0.4 ml were transferred to a 5-ml polypropylene tube containing 1 ml of a 1:2 slurry of Dowex 1 × 2 (chloride form)
anionic exchange resin (Sigma) in water. Tubes were vortexed and centrifuged for 10 min at 1,000 rpm. The resin trapped radioactive metabolic intermediates other than $[^3]H$water, and 0.5 ml of the supernatants containing $[^3]H$water were counted for radioactivity. All experiments included a blank control, where $[^3]H$water was determined immediately after the addition of KH buffer without glucose.

Quantification of $[^3]H$-Labeled Lipids—Cells seeded in 24-well plates were labeled overnight with 1 μCi/ml $[^3]H$palmitic acid, washed, and treated as explained above. After treatment, lipids were extracted as described (31). 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 60 high performance thin layer chromatography glass plates with concentrating zone (Merck), which were developed in hexane/diethyl ether/acidic acid (70: 30:1, v/v/v) and stained with primuline spray (5 mg of primuline in 100 ml of acetone/water (80:20, v/v)). Identification of phospholipids, TAG, and cholesteryl esters was achieved by co-migration with 1-palmitoyl-2-oleoyl-phosphatidylcholine, tripalmitin, and cholesteryl palmitate (Sigma) standards, respectively. To quantify radioactive lipids, the silica gel from regions corresponding to migration of the standards was scraped into scintillation vials.

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 SDS-PAGE and transferred to nitrocellulose membranes. Primary (1:1,000) and secondary (1:5,000) antibodies were diluted in 25 mm Tris-HCl buffer, pH 7.4, containing 140 mm NaCl, 0.5% defatted dry milk, bovine serum albumin according to the manufacturer’s advice, and 0.1% Tween 20. Membranes were developed using ECL detection reagents from Amersham Biosciences.

siRNA Transfection—Expression of cPLA$_2$α was silenced with a predesigned siRNA (Gene Link) directed against human cPLA$_2$α. CHO 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™ RNAiMAX transfection reagent (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 for 24 h prior to treatment. Expression of CERK and perilipins 2 and 3 was silenced in human glioblastoma LN18 cells, using predesigned siRNAs from Gene Link (CERK) and Invitrogen (perilipins 2 and 3). Transfection was performed using siPORT NeoFX reagent (Invitrogen).

Statistical Analysis—Data analysis was carried out with Prism software (GraphPad). Values are expressed as mean ± S.E. Differences between mean values were compared using the unpaired Student’s one-tailed $t$ test and were considered significant if $p$ was <0.05.

RESULTS

LD Sustain Cell Viability—To test the hypothesis that LD generated in stress have a prosurvival role, we used complete nutrient deprivation as a stressor stimulus, because among the different types of stress we previously analyzed, this was the strongest in terms of TAG synthesis and LD occurrence (22). Fig. 1 shows that LN18 (A and B), CHO (C and D), rat astrocytes (E and F), and HeLa cells (G and H) in KH buffer without glucose generated LD. In all cell types, LD occurrence was sensitive to inhibition of cPLA$_2$α with the specific inhibitor (32) py-2. CHO cells under complete nutrient deprivation generated LD that reached a maximum at 8–12 h of treatment and decreased thereafter (Fig. 1J). Nutrient-deprived LN18 and HeLa cells and rat astrocytes also generated LD with a bell-shaped time course (not shown). In agreement with previous results from our laboratory (22, 23, 29) and others (24, 33, 34), LD biogenesis took place after activation of cPLA$_2$α by phosphorylation (Fig. 1K) and was abrogated by py-2 (Fig. 1J) or by down-regulation of the enzyme (Fig. 1, I and L). Similar to cPLA$_2$α, down-regulation of CERK, which participates in cPLA$_2$α activation during the biogenesis of LD (29), also decreased LD occurrence in LN18 cells under nutrient deprivation (Fig. 1J). We also tried to reduce LD levels by down-regulating the LD-associated proteins perilipins 2 and 3. In contrast to CERK or cPLA2α, combined knockdown of perilipins 2 and 3 (Fig. 1N) did not affect the overall LD occurrence induced by nutrient deprivation, as monitored by flow cytometry (Fig. 1M), but LD were clearly larger (Fig. 1O), reflecting a fusion process (35).

To assess cell death during nutrient deprivation, we used flow cytometry to quantify cell shrinkage, as evidenced by the left shift in SS/FS plots shown in Fig. 2 (A–C). Smaller cells in the low FS values were permeable to PI, as shown by the higher FL3 fluorescence in the low FS population (Fig. 2, D–F) and therefore considered dead, whereas those in the high FS values had a lower FL3 fluorescence and were scored viable. Using this criterion, LN18 cells maintained viability during 24 h of complete nutrient deprivation, as shown in G. However, the same treatment in the presence of 1 μM py-2 induced cell death after a lag of 8 h, leaving only about 10% viable cells after 24 h. This contrasts with the complete lack of toxicity of the cPLA2α inhibitor in cells kept in culture medium. Cell death as monitored by flow cytometry agreed closely with LDH release to the medium, shown in Fig. 2H. Death of starved CHO and HeLa cells proceeded at a faster pace, whereas astrocytes maintained viability longer than LN18 cells. Again, py-2 consistently accelerated death in all cell types under starvation, but it did not affect viability in culture medium (not shown). Similar to pharmacological inhibition of cPLA2α, reduced expression of CERK accelerated death of nutrient-deprived LN18 cells but did not affect the viability of cells in culture medium (Fig. 2I). In contrast, simultaneous knockdown of perilipins 2 and 3 did not alter the death rate of nutrient-deprived cells (not shown).

Cell Death Is Associated with LD Depletion—The bell-shaped profile of LD occurrence over time suggests an ongoing process of biogenesis and mobilization that seems relevant for cell survival, given the toxic effects of py-2 and CERK knockdown, and is in keeping with a possible catabolic use of LD generated in response to starvation. As a first approach to test this idea, we loaded LN18 cells with LD using a 16-h treatment with exogenous lipid in DMEM (10% FBS and 100 μM sodium olate). As expected, LD-preloaded cells survived nutrient deprivation longer than cells that were depleted of LD at the beginning of the treatment, which died rapidly between 24 and 32 h of star-
These results agree with those of Katchadourian and Maysinger (36), who showed that oleic acid preconditioning increases survival of PC12 cells to starvation. If cell viability during starvation is sustained by the catabolic use of LD, conceivably cell death should take place after LD depletion, and this is what we found when analyzing LD content in viable and dead cells. Viable and dead cells can be separated after SS/FS flow cytometry plots, as shown in Fig. 2, and therefore...
Nile red-stained cells can be gated in two different populations from the same sample to quantify LD occurrence. Fig. 3, B–E, represents the quantification of LD in viable and dead LN18 (B), CHO (C), HeLa (D), and astrocyte (E) cells over time, showing that, regardless of cell type or the time of treatment, dead cells were consistently depleted of LD as compared with viable cells.

Nutrient Deprivation Induces β-Oxidation of Fatty Acids That Requires LD—To address whether LD generated in complete nutrient deprivation provide catabolic substrates, we labeled endogenous lipids by incubating LN18 cells overnight in culture medium in the absence of serum and with [3H]palmitic acid at concentrations (10–30 nM) low enough to avoid LD biogenesis (22, 23). After washing out the tracer, we switched the cells to nutrient-deprived conditions and monitored the generation of tritiated water as an index of fatty acid oxidation (30). As shown in Fig. 4A, [3H]palmitate-prelabeled LN18 cells generated [3H]water in a manner that was sensitive to a 30 μM concentration of the CPT1 inhibitor EX, indicating that it was an index of mitochondrial β-oxidation of fatty acids. [3H]Water generation by cells maintained in culture medium was similar to that in cells where β-oxidation was inhibited with EX, showing that nutrient deprivation did induce the oxidation of fatty acids. In agreement with this, fatty acid oxidation was associated with increased expression of CPT1A (Fig. 4A, inset). As with LN18 cells, nutrient deprivation also stimulated fatty acid oxidation in CHO and HeLa cells (Fig. 4, B and C). More importantly, inhibition of LD biogenesis with py-2 in LN18, CHO, and HeLa cells decreased β-oxidation down to a level close to that defined by EX (Fig. 4, A–C) and within a time frame that excluded the possibility that the effect was secondary to cell death. Down-regulation of perilipins 2 and 3, however, did not affect significantly β-oxidation or TAG synthesis (not shown). Analysis of the major lipid species revealed that [3H]palmitate labeling went mainly to phospholipids (Fig. 4D), and nutrient deprivation induced a small but non-significant decrease. Nutrient deprivation induced TAG accumulation that was potentiated after inhibition of β-oxidation with EX (Fig. 4E). Accumulation of cholesteryl esters, the other main lipid contained in LD, was 10-fold lower than that of TAG, and nutrient deprivation induced a 2-fold increase (not shown), probably reflecting increased availability of [3H]palmitate, and it was not affected by EX. Fig. 4G shows the aggregated values of [3H]TAG + [3H]water, the latter shown in Fig. 4F, and illustrates that nutrient deprivation stimulated synthesis of TAG that led to β-oxidation.

β-Oxidation of Fatty Acids Sustains Cell Viability—The preceding results are consistent with a necessary role of TAG synthesis and LD biogenesis in fatty acid oxidation during complete nutrient deprivation. Alternatively, because starvation leads ultimately to cell death, LD might accumulate as a consequence of increased fatty acid availability secondary to impaired mitochondrial function, as has been shown in cells undergoing etoposide-induced apoptosis (12). Although this possibility seems unlikely in our case, we analyzed the effect of inhibition of β-oxidation with EX on cell viability and LD content. Fig. 5 (A and B) shows FL3/FS flow cytometry plots of PI and BODIPY 493/503-stained HeLa cells that had been maintained for 10 h under nutrient-deprived conditions in the absence (A) or presence of 30 μM EX (B). Increased FL3 fluorescence reflects cell permeability to PI and shows that 30 μM EX potentiated starvation-induced cell death. LD content in dead cells was clearly higher in EX-treated samples, as evidenced by the stronger BODIPY FL1 fluorescence, shown as a right shift of FL3/FL1 plots in the panel insets. EX was not toxic to LN18 (Fig. 5C) or CHO cells (not shown) maintained in culture medium (Fig. 5C), which agrees with our data showing no induction of β-oxidation in these conditions (Fig. 4) and is also consistent with the lack of toxicity of py-2 in culture medium (Fig. 2). In contrast, inhibition of β-oxidation accelerated death of nutrient-deprived LN18 cells with a time course similar to that induced by py-2, and the same was true for CHO and HeLa cells (not shown). Moreover, EX increased the overall LD occurrence in LN18 cells induced by nutrient deprivation (Fig. 5, D and E), and this was apparent both in viable and death LN18 cells (Fig. 5, F and G) and also in viable and death CHO cells (not shown). Taken together, the results presented so far show that cell viability during nutrient deprivation is sustained by β-oxidation of fatty acids that requires biogenesis and mobilization of LD.

Autophagy Does Not Seem to Be Involved in LD Mobilization for β-Oxidation—Mobilization of LD by autophagy to fuel fatty acid oxidation has been shown to take place in hepatocytes and liver (37) and also in hypothalamic neurons (38). Autophagy is also involved in the mobilization of LD-stored cholesteryl esters in macrophages for efflux of cholesterol (39), and we addressed whether this mechanism was involved in LD mobilization in our model. As shown in Fig. 6, nutrient deprivation of LN18 cells induced the appearance of autophagosomes (Fig. 6A), microtubule-associated protein light chain 3 (LC3) puncta (Fig. 6B), and the conversion of LC3-I to LC3-II (Fig. 6C), indic-
The autophagy inhibitor 3-MA blocked the appearance of LC3 puncta and also conversion of LC3-I to LC3-II (Fig. 6, B and C). In contrast, inhibition of LD biogenesis with py-2 did not affect LC3 conversion (Fig. 6C). Regarding β-oxidation of endogenous fatty acids, 3-MA did not affect the EX-sensitive generation of [3H]water induced by nutrient deprivation (Fig. 6D). Unlike the deleterious effects of pharmacological inhibition of LD biogenesis or β-oxidation, 3-MA did not accelerate LN18 cell death; rather, it increased viability at long treatment times (Fig. 6E), and similar results were found.
with CHO cells (not shown). These results suggest that the autophagic machinery is involved in cell death, but it does not participate in the mobilization of LD for $\beta$-oxidation that is required to sustain cell viability in starvation. We did not find association between LD and acidic compartments (Fig. 6G), as one would expect for an autophagic processing of LD. However, cytometry analysis of Nile red-stained cells revealed that treatment with 3-MA increased LD content in LN18 (Fig. 6F) and CHO cells (not shown), suggesting that, although not involved in LD mobilization for $\beta$-oxidation and cell survival, autophagy may account for part of the LD processing activity.

**DISCUSSION**

This work shows that cells under complete nutrient deprivation synthesize TAG and pack them in LD, which are mobilized to fuel $\beta$-oxidation of fatty acids. Biogenesis of LD has been shown in many experimental models of cellular stress and also appears associated with diverse pathological conditions. In order to develop new antitumor or cell-protecting therapeutic strategies, it is of interest to assess their relevance for cell survival. LD have been shown to accumulate in cells undergoing etoposide-induced apoptosis, as a consequence of decreased fatty acid oxidation secondary to impaired mitochondrial function (13). LD formation in tumors, which has long been known to take place in the anoxic core (40–42), might also be secondary to impaired $\beta$-oxidation. In these cases, synthesis of TAG and LD biogenesis could buffer excess fatty acids and prevent their toxicity. In this regard, LD formed in response to extracellular fatty acids and ER-linked TAG synthesis appear to relax ER stress (12) and protect cells from lipotoxicity. The potential use of the fatty acid catabolic pathway as a source of new targets for cancer therapy has been highlighted recently (43). Regardless of whether LD in perinecrotic tumor areas develop secondary to mitochondrial dysfunction or, as in our experimental model, they provide catabolic substrates to overcome nutrient starvation, it might be worth pursuing further studies to test whether pharmacological inhibition of cPLA$_2$ with py-2 could prove useful as an adjuvant antitumor therapy to target fatty acid oxidation. Also, LD formed in ischemic heart and neurons appear to have a cell-protective role (24, 25), as lipid-buffering structures and/or as providers of metabolic fuel, and interventions aimed at increasing LD biogenesis could contribute to decrease cell damage.

Although based primarily on the use of py-2 to target LD biogenesis, a relevant finding of this work is that, under complete nutrient deprivation, the main source of fatty acids for $\beta$-oxidation is LD. Leaving aside sterol metabolism, the meta-
FIGURE 4. Nutrient deprivation induces β-oxidation that requires LD biogenesis. A, LN18 cells were prelabeled overnight with [3H]palmitate prior to their treatment with DMEM (gray bar) or KH without glucose in the absence (solid symbols) or presence of 1 μM py-2 (open symbols) or 30 μM EX (open bar). β-Oxidation was monitored after the generation of [3H]water. The inset shows increased expression of CPT1 after 16 h of starvation. B and C, [3H]palmitate-prelabeled CHO (B) or HeLa (C) cells were treated for 4 h with culture medium (gray bar) or KH without glucose in the absence (solid bar) or presence of 1 μM py-2 (open bar). β-Oxidation was defined as EX-sensitive production of [3H]water. Results are means ± S.E. (error bars) of an experiment carried out with determinations in quadruplicate and are representative of four (A) or three (B and C) independent experiments with similar outcome. *, significantly different from KH without glucose. D–G, [3H]palmitate-prelabeled LN18 cells were maintained in culture medium (gray bars) or KH without glucose in the absence (solid bars) or presence of 30 μM EX (open bars). After 8 h, lipids were extracted and separated by high performance thin layer chromatography, and radioactivity in phospholipids (PL) and TAG was quantified. D, nutrient deprivation did not induce a significant change in phospholipid content. E, nutrient deprivation induced TAG accumulation that was potentiated after inhibition of β-oxidation with EX. F, nutrient deprivation stimulated the generation of [3H]water as an index of β-oxidation, which was inhibited by EX. G, aggregated values of [3H]TAG + [3H]water, illustrating that nutrient deprivation induced a process of lipogenesis that led to the oxidation of fatty acids. Results are means ± S.E. of one experiment with quadruplicate determinations that was repeated once with a similar outcome. *, significantly different from KH without glucose; **, significantly different from DMEM.

bolic functions of LD as revealed or confirmed by proteomic studies can be grouped into fatty acid synthesis and activation, TAG biosynthesis, and fatty acid mobilization (44). Fatty acid synthesis does not take place in our glucose-devoid experimental setting, as evidenced by the ability of the fatty acid synthase inhibitors cerulenin and C75 to promote synthesis of TAG and LD biogenesis (22). Because LD are formed in the absence of exogenous lipoproteins or fatty acids, synthesis of TAG required for LD formation is at the expense of preexisting, phospholipid-linked fatty acids by a iPLA2-mediated process (22). This leaves fatty acid activation and TAG synthesis and mobilization as the relevant events involved in cell survival to complete nutrient deprivation. Accumulation of TAG within the ER membranes seems to be associated with LD formation (12, 44). Inhibition of cPLA2α, however, allows the dissociation of the two events, because it precludes LD biogenesis but does not affect synthesis of TAG (22, 23). Activation of cPLA2α for LD biogenesis requires phosphorylation by JNK (29, 33), and this may well account for the leaner phenotype of cPLA2α-defective (47, 48) or JNK1-defective mice (49). Pharmacological inhibition of cPLA2α with py-2 is a useful tool, therefore, to tackle the function of LD as providers of catabolic fuel. Our results show that cell survival to complete nutrient deprivation becomes strictly dependent on the catabolism of fatty acids. In this regard, similar to the inhibition of β-oxidation with EX, precluding the formation of LD with py-2 accelerates the death of starved cells, but it does not affect the viability of cells that do not depend on fatty acid catabolism. The observation that β-oxidation of fatty acids needs the formation of LD implies a dedicated metabolic channeling lipid droplet-mitochondrion, ensuring that fatty acyl-CoA derived from LD mobilization does not mix with other acyl-CoA pools within the cytosol, adding to the complexity of acyl-CoA synthetases in directing fatty acids into different metabolic pathways (50). Otherwise, neither synthesis of TAG after fatty acyl-CoA nor LD biogenesis after TAG would be needed for β-oxidation, and the release of fatty acids from phospholipids by iPLA2 and their activation by acyl-CoA synthetases would meet the requirement to fuel mitochondrial catabolism. Our results, therefore, underline the relevance of LD for fatty acid oxidation, in keeping with the idea...
FIGURE 5. Under nutrient deprivation, inhibition of β-oxidation accelerates cell death and leads to the accumulation of LD. A and B, HeLa cells were kept for 10 h in KH without glucose and in the absence (A) or presence of 30 μM EX (B). After treatment, they were harvested and stained with PI and BODIPY 493/503 to measure viability and LD content. A and B show the event distribution for each treatment in terms of PI permeability (FL3) and size (FS). Inhibition of β-oxidation (B) induced death of nutrient-deprived cells. Insets show the event distribution of the dead cell population for each treatment in terms of FL3 and LD occurrence (FL1). The increased FL1 signal induced by EX is apparent. C, inhibition of β-oxidation with 30 μM EX accelerated the death of LN18 cells maintained in KH without glucose but had no effect on cells maintained in culture medium (DMEM), as denoted by a gray bar. Treatment with EX led to an overall accumulation of LD (D) that was very apparent in Nile red-stained cells after a 16-h treatment (E). Analysis of viable (F) and dead cells (G) shows increased LD occurrence in EX-treated samples. Results are means ± S.E. (error bars) of six independent experiments carried out with duplicate determinations. *, significantly different from KH without glucose.
Inhibition of autophagy does not accelerate death of nutrient-deprived cells. Treatment with KH without glucose induced autophagy in LN18 cells. A, electronic microscope image of a late autophagosome after 16 h in KH without glucose. B and C, LN18 cells were treated for 6 h with DMEM or KH without glucose (w/o gl) and with or without 1 μM py-2 or the autophagy inhibitor 3-MA (10 mM). Nutrient deprivation induced autophagy, as monitored by the appearance of LC3 puncta (B, left) or by conversion of LC3-I to LC3-II (C). This process was blocked by 3-MA (B, right; C, top) but not by inhibition of LD biogenesis with py-2 (C, bottom). D, LN18 cells were prelabeled overnight with [3H]palmitate prior to their treatment for 5 h with DMEM or KH without glucose and with or without 3-MA or EX. Inhibition of autophagy with 3-MA had no effect on β-oxidation of fatty acids, which was defined as EX-sensitive production of [3H]water. E, at long treatment times, blocking autophagy had a significant cytoprotective effect. F, flow cytometry analysis of Nile red-stained cells showed an overall accumulation of LD in cells treated with 3-MA. G, LN18 cells were kept 16 h in DMEM (left) or KH without glucose (right); acidic compartments were labeled with LysoTracker® Red DND-99, and LD were stained with BODIPY 493/503. Results in D are means ± S.E. (error bars) of one experiment in quadruplicate that is representative of three experiments with similar results. Results in E and F are means ± S.E. of 4–6 independent experiments carried out with duplicate determinations, and asterisks in these panels denote significance as compared with KH without glucose.
that fatty acids liberated from LD may be used more efficiently for some metabolic pathways (45). In this regard, the possibility of direct contacts between LD and mitochondria has been considered (51). Among the LD-associated perilipin proteins, our data confirm that perilipins 2 and 3 shield LD from fusion with each other but do not affect the overall amount of TAG and, importantly, do not affect the rate of fatty acid catabolism, in agreement with the results of Bell et al. (35). LD- and mitochondria-associated perilipin 5 is expressed in highly oxidative tissues (52) and regulates TAG mobilization and flux of fatty acids to the mitochondria by a mechanism involving direct physical contact between the two organelles (53–55). Although we have been unable to detect perilipin 5 in our system, probably due to the unavailability of good antibodies, it is tempting to speculate that the metabolic adaptation to fatty acid oxidation that follows full nutrient deprivation, as described here, could be accompanied by expression of perilipin 5. A similar induction of specific LD-associated proteins may account in part for the high LD content together with a high oxidative capacity found in trained muscle, the so-called athlete’s paradox (33, 56).

In any case, our model system of total nutrient deprivation, in the absence of extracellular lipoproteins and free fatty acids and where cell viability is tightly dependent on LD mobilization, constitutes a simple setting to dissect the functional coupling between LD and mitochondria.

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