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A Protective Role for Triacylglycerols during Apoptosis

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Abstract

Triacylglycerols (TAGs) are one of the major constituents of the glycerolipid family. Their main role in cells is to store excess fatty acids, and they are mostly found within lipid droplets. TAGs contain acyl chains that vary in length and degree of unsaturation, resulting in hundreds of chemically distinct species. We have previously reported that TAGs containing polyunsaturated fatty acyl chains (PUFA-TAGs) accumulate via activation of diacylglycerol acyltransferases during apoptosis. In this work, we show that accumulation of PUFA-TAGs is a general phenomenon during this process. We further show that the accumulated PUFA-TAGs are stored in lipid droplets. Because membrane-residing PUFA phospholipids can undergo oxidation and form reactive species under increased levels of oxidative stress, we hypothesized that incorporation of PUFAs into PUFA-TAGs and their localization within lipid droplets during apoptosis limit the toxicity during this process. Indeed, exogenous delivery of a polyunsaturated fatty acid resulted in a profound accumulation of PUFA phospholipids and rendered cells more sensitive to oxidative stress, causing reduced viability. Overall, our results support the concept that activation of TAG biosynthesis protects cells from lipid peroxide-induced membrane damage under increased levels of oxidative stress during apoptosis. As such, targeting triacylglycerol biosynthesis in cancer cells might represent a new approach to promoting cell death during apoptosis.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00975. Detailed experimental information and Figures S1–S3 (PDF)

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Author Contributions

N.L. and G.E.A.-G. designed the experiments. N.L. conducted the experiments. N.L. and G.E.A.-G. wrote the manuscript. Y.S. prepared the shFASN lentiviral particles. J.F. provided critical feedback during the studies and on the manuscript.

Notes

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Triacylglycerols (TAGs) are neutral glycerolipids that act as the primary storage molecules for fatty acids, especially when fatty acids are in excess.¹ TAG biosynthesis is a multistep process, the final and rate-determining step of which is catalyzed by diacylglycerol acyltransferases (DGATs).¹ TAG biosynthesis takes place at the endoplasmic reticulum (ER). After their synthesis, TAGs are mostly incorporated within discrete compartments that bud off from the ER membrane, known as lipid droplets.^{2,3}

There is growing appreciation of the structural diversity of lipids from a functional point of view.⁴ TAGs exhibit structural diversity depending on the length and degree of unsaturation of the fatty acyl chains that they contain, which include saturated, monounsaturated, and polyunsaturated (at least two double bonds) fatty acids. Along this line, a few recent studies have suggested that TAGs might exhibit structure-specific roles. For instance, a study has suggested an association between a TAG that contains a *cis*-7-hexadecenoic acyl chain (a positional isomer of palmitoleic acid) and the anti-inflammatory response in human monocytes.⁵ Another study demonstrated that perturbation of TAG biosynthesis causes cell division failure and that the level of an unusual short fatty acyl chain (C12:0 fatty acyl) containing TAG is elevated at the division site as cells divide, suggesting its localization at membrane structures.⁶

Accumulation of TAGs and lipid droplets has been associated with apoptosis.^{7–11} Although TAGs are typically assumed to reside within lipid droplets, that is not necessarily the case. The cellular localization of lipids is often crucial to their function, and the localization of TAGs that accumulate during apoptosis has yet to be studied. We recently showed polyunsaturated fatty acyl-containing TAGs (PUFA-TAGs) accumulate during 5-fluorouracil (5-FU)-induced apoptosis as a result of activation of DGATs.¹¹ We have envisioned that the accumulation of PUFA-TAGs and their storage within lipid droplets separate PUFAs from cellular membranes and might protect cells from lipid peroxide-induced membrane damage during apoptosis, which is accompanied by elevated levels of reactive oxygen species.¹²

In this work, we first demonstrate that PUFA-TAG accumulation occurs during apoptosis independent of the cytotoxic agents and cell lines used. We also provide evidence that DGATs, which are responsible for the accumulation of TAGs, could be downstream targets of p53 activation during apoptosis via mechanisms that are currently unknown. We then isolated lipid droplets from apoptotic and control cells and showed that PUFA-TAGs accumulated profoundly as compared to other TAG species in lipid droplets isolated from

apoptotic cells. These results suggest that PUFA-TAGs that accumulate during apoptosis are stored within lipid droplets. We further show that polyunsaturated fatty acid treatment results in the accumulation of polyunsaturated phospholipids and induces significant toxicity under oxidative stress. Overall, our results support the hypothesis that the conversion of polyunsaturated fatty acids to PUFA-TAGs and their storage in lipid droplets prevent their localization to cellular membranes, which likely is a measure to prevent lipid-mediated toxicity limiting cell death during apoptosis.

METHODS

Lipid Droplet Isolation and Lipid Analysis

This procedure was modified on the basis of a previous protocol.¹³ Control and apoptotic HCT-116 cells were collected using a cell scraper and washed with phosphate-buffered saline (PBS). After PBS had been removed, cells were disrupted by homogenization on ice in a 2 mL Dounce homogenizer in hypotonic lysis medium (HLM) buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and protease inhibitor cocktail. The cell lysates were then centrifuged for 10 min at 1000g and 4 °C. The supernatant was collected and kept on ice. For the pellet, another cycle of homogenization and centrifugation was performed and the supernatants were collected and combined. Protein concentrations of the supernatants from control and apoptotic samples were then measured, and samples were normalized on the basis of protein concentration. The sucrose concentrations of samples were adjusted to 20% by adding HLM buffer containing 60% sucrose. Next, 2 mL of the sample was transferred to an ultracentrifuge tube, and 5 mL of HLM buffer containing 5% sucrose and 3 mL of HLM buffer containing 0% sucrose were then layered sequentially on top of the homogenate. Ultracentrifugation was performed using a Beckman Coulter Optima L-90K ultracentrifuge with a SW40 Ti swing rotor for 1 h at 100000g and 4 °C. After ultracentrifugation, each fraction (L1–L10, 1 mL each) was carefully collected from the top down using a 1 mL micropipette. The freshly collected fractions were then characterized by Nile red fluorescence and Western blotting (details can be found in the Supporting Information).

For lipid analysis of the lipid droplet-enriched layer, the top layer, L1, was collected. One milliliter of methanol and 2 mL of chloroform were added to L1, and lipids were extracted by vortexing. The mixture was then centrifuged at 4 °C and 500g for 15 min to separate the aqueous and organic layers. The organic layer was then carefully removed and dried using a rotatory evaporator. Dried lipids were then resuspended in chloroform containing 1 μ M TAG (13:0/13:0/13:0) as an internal standard and used for liquid chromatography–mass spectrometry (LC–MS) analysis.

C18:3 and C16:0 Fatty Acid Treatment under Oxidative Stress

The fatty acid–bovine serum albumin (FA–BSA) complex was initially prepared as described previously.^{14,15} A fatty acid (C18:3 or C16:0 FA) stock solution in ethanol was added to 10% BSA (w/v) prepared in Dulbecco's modified Eagle's medium (DMEM) to reach a final concentration of 4 mM. The mixture was sonicated at 55 °C for 30 min until it was optically clear to obtain the FA–BSA complex. Fatty acid synthase (FASN) knockdown

HCT-116 cells were plated in 96-well plates (40000 cells per well). Sixteen hours after the cells had been plated, the FA-BSA complex was diluted in regular culture medium and added to cells (final concentration of fatty acid of 10 μ M, final BSA concentration of 0.125%, 6 h treatment time). Control groups received regular culture medium with BSA only (final BSA concentration of 0.125%). After being subjected to fatty acid treatment for 6 h, cells were exposed to 200 μ M H₂O₂ for 1 h to induce oxidative stress. Cell viability was then measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (see the Supporting Information).

RESULTS AND DISCUSSION

PUFA-TAG Accumulation Is a General Phenomenon during Apoptosis

Functional roles of several membrane lipids, such as phosphatidylserines¹⁶ and ceramides,¹⁷ in apoptosis have been established. However, the functional involvement of TAGs, traditionally known as molecules for energy storage, in apoptosis remains to be elucidated. In a previous study, we showed that 5-FU treatment induced PUFA-TAG accumulation in a colon cancer cell line (HCT-116) during apoptosis.¹¹ On the basis of this observation, we hypothesized that PUFA-TAGs are functionally involved in apoptosis. If this hypothesis is correct, first, we expect that the accumulation of PUFA-TAGs would be observed regardless of the specific cytotoxic agent used to induce apoptosis. Second, we expect that PUFA-TAG accumulation would be observed in different cell lines undergoing apoptosis.

We first tested whether different cytotoxic agents indeed lead to PUFA-TAG accumulation during apoptosis. We induced apoptosis using various small molecules, including a kinase inhibitor (staurosporine) and chemotherapeutic DNA damage-inducing drugs (doxorubicin and etoposide),¹⁸ in HCT-116 cells. We evaluated apoptotic activity as demonstrated by poly(ADP-ribose) polymerase (PARP) cleavage¹⁹ in each of the cell lines that was treated with different cytotoxic agents (Figure 1A). We then collected apoptotic and control samples, extracted lipids, and performed LC-MS analysis to investigate the levels of different TAGs in apoptotic and control cells as described previously.¹¹ Specifically, we targeted TAGs with a total number of carbons on the acyl chains ranging from 44 to 62 and a total number of double bonds ranging from 0 to 8. The relative abundances (blue) of the species that were present in either control or apoptotic cells, along with the fold changes comparing the relative abundance of TAG species in apoptotic samples to control samples, are shown in Figure 1B.

Our results clearly show that PUFA-TAG accumulation during apoptosis is independent of the cytotoxic agent used. In all experiments, we observed significant accumulation of multiple PUFA-TAGs, which we defined as species that contain four or more double bonds. In staurosporine-treated samples, C48:4, C50:4, C52:6, C54:7, and C56:8 PUFA-TAGs exhibit the most profound accumulation among all the detected TAG species. Similarly, PUFA-TAGs including C52:6, C54:7, C56:8, C58:7, and C58:8 in doxorubicin-treated samples and PUFA-TAGs including C54:5, C56:5, C56:6, and C56:8 in etoposide-treated samples demonstrate significant accumulation. Overall, it is clear that PUFA-TAG accumulation is a central feature of apoptosis independent of the specific apoptotic stimuli.

In these experiments, we found that TAG species with the same total carbon number but different unsaturation states accumulate at different levels during apoptosis. A distinct pattern can be observed in the fold changes of TAGs following staurosporine and doxorubicin treatment. For instance, in the case of TAGs C56:2–C56:8, accumulation is more profound with an increasing number of double bonds. On the other hand, the levels of phosphatidylcholines, the major component of cellular membranes,²⁰ did not show any particular trends depending on the unsaturation state of the acyl chains during apoptosis (Figure 2A and ref 11). We note that polyunsaturated fatty acyl-containing phosphatidic acids, phosphatidylethanolamines, phosphatidylinositols, and diacylglycerols did not show any accumulation during apoptosis (Figure S1, for staurosporine-induced apoptosis, and ref 11 for 5-FU-induced apoptosis), supporting the notion that among the detectable lipid species that we targeted, accumulation of PUFA-containing species is specific to TAGs during this process. Overall, we conclude that the accumulation of PUFA containing lipids is specific to TAGs and is not the outcome of a general, cellwide accumulation of unsaturated lipids.

After showing that the accumulation of PUFA-TAGs in HCT-116 cells is independent of the cytotoxic agents used, we tested whether the cell line affects PUFA-TAG accumulation. We investigated the changes in TAGs in a breast cancer cell line (MCF-7) during staurosporine-induced apoptosis (Figure 2B). Following the same experimental setup described above, we extracted lipids and analyzed TAGs in control and apoptotic MCF-7 cells. This analysis showed that PUFA-TAG species including C52:6, C54:7, C56:8, C58:7, and C58:8 accumulate to a significant extent in apoptotic MCF-7 cells (Figure 2B), similar to what we observed in HCT-116 cells (Figure 1B). Overall, our results suggest that PUFA-TAG accumulation is not specific to a particular apoptotic agent or cell line but rather can be generalized as a phenomenon associated with apoptosis. This association between apoptosis and PUFA-TAG accumulation further supported our hypothesis that these PUFA-TAGs could play a role in apoptosis.

PUFA-TAGs That Accumulate during Apoptosis Are Located in Lipid Droplets

The localization of biomolecules within cells informs their interaction with other cellular components and, thus, provides information about their function in cells. Traditionally known as molecules for energy storage, TAGs are mainly stored in lipid droplets.² However, other functions of TAGs are beginning to emerge. For example, a recent study has shown the localization of a TAG to the site of cell division.⁶ This suggests that TAGs can also be membrane-associated and that they can play roles other than energy storage in cellular processes. The targeted lipidomics approach we used clearly shows the accumulation of PUFA-TAGs during apoptosis. However, this approach is limited in providing information about the cellular localization of these species (i.e., their accumulation in specific membranes or organelles such as lipid droplets). As such, to investigate the spatial regulation of PUFA-TAGs during apoptosis, we used fractionation techniques to isolate lipid droplets and studied the changes in lipid composition in these structures during apoptosis.

Lipid droplets are less dense than other organelles because of their lipid core. As such, it is possible to obtain cellular fractions that are enriched with lipid droplets using fractionation-

based separations. Specifically, lipid droplets are enriched in the top fractions because of their buoyant density.¹³ We isolated lipid droplets from control and 5-FU-treated apoptotic cells that exhibited ~50% cell death and investigated their lipid composition. Briefly, we collected control and apoptotic cells and homogenized them in a hypotonic buffer using a Dounce homogenizer. After normalization based on overall protein content, we fractionated the lysates in a sucrose gradient using ultracentrifugation (Figure 3A; see Methods). After ultra-centrifugation, we collected 10 fractions (1 mL each), with L1 being the top fraction and L10 being the bottom fraction (Figure 3A). The bottom layer (L10) was a pellet and contained cellular membranes (i.e., the ER and Golgi apparatus) and other water-insoluble biomolecules.

We initially characterized different fractions by fluorescence spectroscopy using Nile red, an environmentally sensitive dye. The maximum emission of Nile red decreases as the polarity of the chemical environment decreases.²¹ Nile red shows a different emission maximum in the presence of cellular membrane lipids that are composed of more polar lipids (i.e., phospholipids) compared to that of lipid droplets that are enriched with more hydrophobic lipids (i.e., TAGs and cholesterol esters).^{21,22} We measured the emission spectra (500–700 nm) of the fractions obtained from ultracentrifugation from control and 5-FU-treated apoptotic samples after excitation at 480 nm (Figure 3B). In apoptotic samples, we found a decrease in the maximum emission wavelength of the top layer (L1) as compared to those of the bottom layers (shift from ~625 nm in bottom layers L9 and L10 to ~585 nm in top layer L1). This observation indicates that the top layer is more hydrophobic (i.e., enriched with lipid droplets) while the bottom layers contain phospholipid-rich membrane structures (Figure 3B).

To further characterize the isolated structures, we collected proteins from different fractions and examined the presence of protein markers for lipid droplets and ER using Western blotting. This is informative because it allows confirmation of lipid droplet enrichment in different layers. Because of the low protein content in certain layers, we performed an acetone precipitation method²³ to concentrate proteins. We first investigated the presence of adipose differentiation-related protein (ADRP/ADFP), a protein that localizes to lipid droplets²⁴ and has been widely used as a marker for these structures. A representative Western blot is shown in Figure 3C. ADRP/ADFP was present in L1, L9, and L10, but not in the middle fractions. We attribute the strong ADRP/ADFP signal in L1 to the enrichment of lipid droplets in this layer. The presence of this protein in L9 and L10 can probably be due to its association with other membrane structures such as the ER from which lipid droplets originate. In parallel, we used calnexin as an ER marker.²⁵ Calnexin was detected in the bottom pellet portion, L10, containing the insoluble membrane structures, but not in L1. This indicates that L1 is enriched with lipid droplets, whereas ER membranes are present in L10. Altogether, the enhanced hydrophobicity of the L1 layer as compared to those of other fractions and the presence of ADRP/ADFP strongly support the idea that L1 is enriched with lipid droplets. Isolation of this layer allowed us to investigate the accumulation of PUFA-TAGs in lipid droplets during apoptosis.

We then conducted lipid analysis of the top layer, L1, which is enriched with lipid droplets, from control and apoptotic samples. Specifically, we obtained L1 from control and 5-FU-

treated apoptotic cells, extracted lipids, and analyzed changes in the levels of different TAG species using LC–MS. We targeted TAGs with a total number of acyl carbons ranging from 44 to 62 and a total number of double bonds ranging from 0 to 8. Figure 4 shows the relative abundances (blue) and fold changes (red) of the TAG species that we detected in control and apoptotic samples. TAG species, in general, showed overall accumulations in apoptotic samples because of the increased level of lipid droplets induced by apoptosis.^{8,11} Specifically, PUFA-TAGs, which we distinguished as containing 4 or more double bonds, showed higher levels of accumulation compared to those of other TAG species (Figure 4). Intriguingly, C46:4, C48:4, C52:5, C52:6, C54:6, and C54:7 PUFA-TAGs, which we previously identified as the major lipids that accumulate during 5-FU-induced apoptosis via a global lipidomics analysis,¹¹ were among the species that exhibited the largest fold changes. We note that we did not detect any PUFA-TAGs in the membrane fraction (L10) from control and apoptotic cells, supporting their storage in lipid droplets. Overall, these results strongly support the possibility that the PUFA-TAGs that accumulate during apoptosis are stored in lipid droplets, suggesting their function can be linked to their localization in lipid droplets.

DGAT Activation Correlates with p53 Activation

We have previously shown that the accumulation of PUFA-TAGs caused by elevated TAG biosynthesis during apoptosis occurs downstream of p53 activation.¹¹ p53 is a key tumor suppressor that controls oncogenic activity.²⁶ In addition to this gatekeeper role during oncogenic transformations, p53 also governs the expression of a wide range of downstream proteins and plays a role in several cellular metabolic processes such as glycolysis, lipid biosynthesis, and metabolism.^{27,28} Previous studies have suggested that p53 suppresses lipid biosynthetic pathways.^{28,29} For instance, p53 downregulates sterol regulatory element-binding protein-1 (SREBP-1), a family of transcription factors that control the expression of genes involved in lipogenesis.²⁹ Given the downregulatory effect of p53 on lipid biosynthesis, the link between p53 activation and TAG accumulation during apoptosis is surprising.

Our previous studies suggest that DGATs are involved in the accumulation of PUFA-TAGs during apoptosis.¹¹ Given the p53-dependent accumulation of PUFA-TAGs,¹¹ it is plausible that p53 might be involved in the activation of DGATs. To test this, we investigated the change in gene expression of *DGATs* (*DGAT-1* and *DGAT-2*) caused by 5-FU treatment in HCT-116 cells that lacked p53 activity [p53^{-/-} (see the Supporting Information for details)]. We observed that the levels of expression of *DGAT-1* and *DGAT-2* were increased by 4- and 2-fold, respectively, in wild type HCT-116 (p53^{+/+}) cells during apoptosis (Figure 5A and ref 11). In contrast, *DGAT-1* showed a significantly lower level of accumulation (~2-fold), and that of *DGAT-2* remained the same in p53^{-/-} HCT-116 cells following 5-FU treatment. We then assessed the level of DGAT-1 at the protein level using Western blotting (Figure 5B). Consistent with the gene expression results, 5-FU treatment resulted in a 2-fold increase in the level of DGAT-1 at the protein level in wild type HCT-116 (p53^{+/+}) cells, while no change was observed in p53^{-/-} HCT-116 cells. These results support the potential involvement of p53 in the activation of DGATs; however, the mechanism by which p53 regulates DGAT activation remains to be elucidated.

Polyunsaturated Fatty Acids Sensitize Cells to Oxidative Stress-Induced Cell Death

Polyunsaturated fatty acids and other PUFA-containing lipids are more amenable to oxidation and formation of unstable lipid peroxides, which are toxic to cells.^{30,31} They can react with other biomolecules, influence lipid packing, and cause distortions in cellular membranes.^{32–34} The formation of these lipid peroxides could be accelerated under increased levels of oxidative stress. Previous studies have suggested that polyunsaturated fatty acids are diverted to TAG biosynthesis in multiple processes involving elevated levels of oxidative stress, including *Drosophila* development,³⁰ apoptosis,^{10,11} and replicative senescence.³⁵ In this work, we show that PUFA-TAGs are specifically stored in lipid droplets during apoptosis. On the basis of these observations, we constructed a model in which an increased level of oxidative stress during apoptosis leads cells to utilize TAG biosynthesis as a mechanism to sequester polyunsaturated fatty acids as PUFA-TAGs and store them in lipid droplets. This compositional and spatial regulation of PUFA-containing structures physically separates them from cellular membranes. As a result, the formation of lipid peroxides in cellular membranes is prevented, limiting lipid peroxide-induced toxicity during apoptosis.

On the basis of our model, we hypothesized that diversion of polyunsaturated fatty acids to phospholipid biosynthesis will reduce cell viability because of the increased level of lipid peroxidation under oxidative stress. To test this hypothesis, we investigated the effect of exogenous addition of fatty acids on cell viability under oxidative stress. To promote lipid uptake and incorporation of these fatty acids into downstream lipids, we deactivated lipid biosynthesis in HCT-116 by knocking down fatty acid synthase (FASN) (Figure 5C) and performed lipid add-back experiments in this cell line, shFASN HCT-116. Accumulation of lipid droplets occurs when high concentrations of fatty acids (e.g., 200 μM) are available for extended periods of time.^{36,37} We exogenously delivered 10 and 200 μM C18:3 fatty acid and investigated the accumulation of lipid droplets using Nile red staining to predict the extent to which C18:3 fatty acid will be incorporated into lipid droplets. Representative images of Nile red staining of control and C18:3 fatty acid-treated cells are shown in Figure S2. We quantified the accumulation of lipid droplets by counting the number punctae that formed after Nile red staining (Figure S2). We did not observe an increase in the number of lipid droplets with 10 μM treatment ($p = 0.4457$, Student's t test), whereas 200 μM treatment caused a significant ($p < 0.001$, Student's t test) and profound increase of lipid droplets (Figure S2). To recapitulate conditions whereby C18:3 fatty acid will mostly be incorporated into phospholipids, we treated shFASN HCT-116 cells with 10 μM C18:3 fatty acid and performed LC–MS-based lipid analysis of control and fatty acid-treated shFASN HCT-116 cells, specifically targeting PCs, the major membrane phospholipids. We found a profound accumulation of PUFA PCs (containing at least three double bonds, highlighted in gray in Figure 5D), supporting our hypothesis that C18:3 fatty acids are mostly incorporated into phospholipid pools in cells.

We then investigated cell viability in this system under oxidative stress. We treated cells with C18:3 fatty acid as we described above and exposed them to H_2O_2 for 1 h to induce oxidative stress. Cell viabilities are shown in Figure 5E. The treatment with only fatty acid slightly decreased cell viability. However, in the presence of H_2O_2 , addition of C18:3 fatty

acid caused a significant decrease in cell viability [H_2O_2 alone, 93% cell viability; C18:3 with H_2O_2 , 66% cell viability ($p < 0.001$, Student's t test)]. A similar effect could also be observed when C20:4 fatty acid was used [$p < 0.01$, Student's t test (Figure S3)]. On the other hand, addition of C16:0 and C18:1 fatty acid in the presence of H_2O_2 did not induce a significant change in cell viability [$p > 0.05$, Student's t test (Figure 5E and Figure S3)]. Our results suggest that polyunsaturated fatty acids (i.e., C18:3 and C20:4 fatty acid) sensitize cells to oxidative stress and promote cell death. We propose that the synergistic effect between C18:3 or C20:4 fatty acid and H_2O_2 treatment is due to the increased levels of PUFA phospholipids (Figure 5C), which can be oxidized to reactive lipid peroxides under oxidative stress, compromising membrane integrity and inducing additional toxicity. These results strongly support our hypothesis that incorporation of PUFAs into non-membrane-associated PUFA-TAGs prevents cells from lipid-induced membrane damage and limits cell death during apoptosis.

CONCLUSIONS

Previous studies have associated the accumulation of TAGs and lipid droplets under different cellular stress conditions, including apoptosis. We have previously shown profound accumulation of PUFA-TAGs during 5-FU-induced apoptosis via the activation of diacylglycerol acyltransferases. In this work, we investigated the spatial regulation and functional involvement of these species during apoptosis. We first show that the accumulation of PUFA-TAGs could be generalized regardless of the cell line or the cytotoxic agent used to induce apoptosis. We provide evidence that diacylglycerol acyltransferases might be activated by p53 during apoptosis, resulting in the accumulation of PUFA-TAGs during this process. However, it is important to note the possible involvement of other enzymes, including regulators or downstream targets of p53, in the regulation of glycerolipid biosynthesis during apoptosis. For instance, a recent study²⁷ has highlighted a relationship between p53 and diacylglycerol kinases (DGKs), which catalyze the formation of phosphatidic acid, regulating the pool of phospholipids and glycerolipids in cells,³⁸ by showing that p53 is inactivated by the ϵ form of DGK (DGK ϵ).²⁷ Given this link between p53, DGK ϵ , and the activation of TAG biosynthesis during apoptosis, it is possible that DGK ϵ is involved in the accumulation of PUFA-TAGs during apoptosis. However, our lipidomics data suggest otherwise. If TAG accumulation were due to the decreased activity of DGK ϵ and the corresponding increase in cellular DAG pools, we would expect to observe decreased levels of phospholipids during apoptosis. This was not the case for 5-FU-induced¹¹ or staurosporine-induced (Figure S1) apoptosis. However, the role of DGK ϵ in regulating lipid pools during apoptosis is an exciting research direction that should be further explored.

We isolated lipid droplets from control and apoptotic cancer cells and found that PUFA-TAGs that accumulate during apoptosis are stored in lipid droplets. This finding supports our hypothesis that spatial regulation of PUFA-TAGs is likely a means for the cell to physically sequester PUFAs from other membrane structures to avoid toxicity. More specifically, the storage of polyunsaturated fatty acids as PUFA-TAGs within lipid droplets might be a mechanism for protecting cells from lipid peroxide-induced membrane damage under increased levels of oxidative stress. Indeed, exogenous delivery of polyunsaturated fatty

acids under conditions where they were mostly incorporated into phospholipids rendered cells more susceptible to oxidative stress-induced cell death. Altogether, these results strongly support a protective role for PUFA-TAGs against lipid-mediated toxicity, limiting cell death during apoptosis. As such, future studies elucidating the biochemical machinery that is responsible for these accumulations would present new therapeutic opportunities for modulating cell death by targeting TAG biosynthesis in the presence of apoptotic stimuli.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Yen CL, Stone SJ, Koliwad S, Harris C, Farese RV Jr. Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. *J Lipid Res*. 2008; 49:2283–2301. [PubMed: 18757836]
2. Thiam AR, Beller M. The why, when and how of lipid droplet diversity. *J Cell Sci*. 2017; 130:315–324. [PubMed: 28049719]
3. Welte MA. Expanding roles for lipid droplets. *Curr Biol*. 2015; 25:R470–481. [PubMed: 26035793]
4. Kimura T, Jennings W, Epand RM. Roles of specific lipid species in the cell and their molecular mechanism. *Prog Lipid Res*. 2016; 62:75–92. [PubMed: 26875545]
5. Guijas C, Meana C, Astudillo AM, Balboa MA, Balsinde J. Foamy monocytes are enriched in cis-7-hexadecenoic fatty acid (16:1n-9), a possible biomarker for early detection of cardiovascular disease. *Cell Chem Biol*. 2016; 23:689–699. [PubMed: 27265749]
6. Atilla-Gokcumen GE, Muro E, Relat-Goberna J, Sasse S, Bedigian A, Coughlin ML, Garcia-Manyes S, Eggert US. Dividing cells regulate their lipid composition and localization. *Cell*. 2014; 156:428–439. [PubMed: 24462247]
7. Al-Saffar NM, Titley JC, Robertson D, Clarke PA, Jackson LE, Leach MO, Ronen SM. Apoptosis is associated with triacylglycerol accumulation in Jurkat T-cells. *Br J Cancer*. 2002; 86:963–970. [PubMed: 11953830]
8. Boren J, Brindle KM. Apoptosis-induced mitochondrial dysfunction causes cytoplasmic lipid droplet formation. *Cell Death Differ*. 2012; 19:1561–1570. [PubMed: 22460322]
9. Di Vito M, Lenti L, Knijn A, Iorio E, D'Agostino F, Molinari A, Calcabrini A, Stringaro A, Meschini S, Arancia G, Bozzi A, Strom R, Podo F. 1H NMR-visible mobile lipid domains correlate with cytoplasmic lipid bodies in apoptotic T-lymphoblastoid cells. *Biochim Biophys Acta, Mol Cell Biol Lipids*. 2001; 1530:47–66.
10. Pan X, Wilson M, McConville C, Arvanitis TN, Griffin JL, Kauppinen RA, Peet AC. Increased unsaturation of lipids in cytoplasmic lipid droplets in DAOY cancer cells in response to cisplatin treatment. *Metabolomics*. 2013; 9:722–729. [PubMed: 23678346]
11. Li N, Lizardo DY, Atilla-Gokcumen GE. Specific triacylglycerols accumulate via increased lipogenesis during 5-FU-induced apoptosis. *ACS Chem Biol*. 2016; 11:2583–2587. [PubMed: 27428610]

12. Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radical Biol Med*. 2010; 48:749–762. [PubMed: 20045723]
13. Brasaemle DL, Wolins NE. Isolation of lipid droplets from cells by density gradient centrifugation. *Current Protocols in Cell Biology*. 2006; 3 Chapter 3.15.
14. Kim JY, Lee HJ, Lee SJ, Jung YH, Yoo DY, Hwang IK, Seong JK, Ryu JM, Han HJ. Palmitic Acid-BSA enhances Amyloid-beta production through GPR40-mediated dual pathways in neuronal cells: Involvement of the Akt/mTOR/HIF-1alpha and Akt/NF-kappaB pathways. *Sci Rep*. 2017; 7:4335. [PubMed: 28659580]
15. Lin Y, Schuurbiens E, Van der Veen S, De Deckere EA. Conjugated linoleic acid isomers have differential effects on triglyceride secretion in Hep G2 cells. *Biochim Biophys Acta, Mol Cell Biol Lipids*. 2001; 1533:38–46.
16. Segawa K, Nagata S. An apoptotic 'eat me' signal: phosphatidylserine exposure. *Trends Cell Biol*. 2015; 25:639–650. [PubMed: 26437594]
17. Hannun YA, Obeid LM. Many ceramides. *J Biol Chem*. 2011; 286:27855–27862. [PubMed: 21693702]
18. Yadav N, Kumar S, Marlowe T, Chaudhary AK, Kumar R, Wang J, O'Malley J, Boland PM, Jayanthi S, Kumar TK, Yadava N, Chandra D. Oxidative phosphorylation-dependent regulation of cancer cell apoptosis in response to anticancer agents. *Cell Death Dis*. 2015; 6:e1969. [PubMed: 26539916]
19. Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res*. 1993; 53:3976–3985. [PubMed: 8358726]
20. van Meer G, Voelker DR, Feigenson GW. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol*. 2008; 9:112–124. [PubMed: 18216768]
21. Greenspan P, Fowler SD. Spectrofluorometric studies of the lipid probe, Nile red. *J Lipid Res*. 1985; 26:781–789. [PubMed: 4031658]
22. Epand RM. Introduction to membrane lipids. *Methods Mol Biol*. 2015; 1232:1–6. [PubMed: 25331123]
23. Brasaemle DL, Dolios G, Shapiro L, Wang R. Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *J Biol Chem*. 2004; 279:46835–46842. [PubMed: 15337753]
24. Akil A, Peng J, Omrane M, Gondeau C, Desterke C, Marin M, Tronchere H, Taveneau C, Sar S, Briolotti P, Benjelloun S, Benjouad A, Maurel P, Thiers V, Bressanelli S, Samuel D, Brechot C, Gassama-Diagne A. Septin 9 induces lipid droplets growth by a phosphatidylinositol-5-phosphate and microtubule-dependent mechanism hijacked by HCV. *Nat Commun*. 2016; 7:12203. [PubMed: 27417143]
25. Fujimoto Y, Itabe H, Sakai J, Makita M, Noda J, Mori M, Higashi Y, Kojima S, Takano T. Identification of major proteins in the lipid droplet-enriched fraction isolated from the human hepatocyte cell line HuH7. *Biochim Biophys Acta, Mol Cell Res*. 2004; 1644:47–59.
26. Kruiswijk F, Labuschagne CF, Voudsen KH. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. *Nat Rev Mol Cell Biol*. 2015; 16:393–405. [PubMed: 26122615]
27. So V, Jalan D, Lemaire M, Topham MK, Hatch GM, Epand RM. Diacylglycerol kinase epsilon suppresses expression of p53 and glycerol kinase in mouse embryo fibroblasts. *Biochim Biophys Acta, Mol Cell Biol Lipids*. 2016; 1861:1993–1999.
28. Parrales A, Iwakuma T. p53 as a regulator of lipid metabolism in cancer. *Int J Mol Sci*. 2016; 17:2074.
29. Yahagi N, Shimano H, Matsuzaka T, Najima Y, Sekiya M, Nakagawa Y, Ide T, Tomita S, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, Gotoda T, Nagai R, Kimura S, Ishibashi S, Osuga J, Yamada N. p53 Activation in adipocytes of obese mice. *J Biol Chem*. 2003; 278:25395–25400. [PubMed: 12734185]
30. Bailey AP, Koster G, Guillermier C, Hirst EM, MacRae JI, Lechene CP, Postle AD, Gould AP. Antioxidant role for lipid droplets in a stem cell niche of *Drosophila*. *Cell*. 2015; 163:340–353. [PubMed: 26451484]

31. Rysman E, Brusselmans K, Scheys K, Timmermans L, Derua R, Munck S, Van Veldhoven PP, Waltregny D, Daniels VW, Machiels J, Vanderhoydonc F, Smans K, Waelkens E, Verhoeven G, Swinnen JV. De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation. *Cancer Res.* 2010; 70:8117–8126. [PubMed: 20876798]
32. Gaschler MM, Stockwell BR. Lipid peroxidation in cell death. *Biochem Biophys Res Commun.* 2017; 482:419–425. [PubMed: 28212725]
33. Dixon SJ, Stockwell BR. The role of iron and reactive oxygen species in cell death. *Nat Chem Biol.* 2014; 10:9–17. [PubMed: 24346035]
34. Ademowo OS, Dias HKI, Burton DGA, Griffiths HR. Lipid (per) oxidation in mitochondria: an emerging target in the ageing process? *Biogerontology.* 2017; 18:859–879. [PubMed: 28540446]
35. Lizardo DY, Lin YL, Gokcumen O, Atilla-Gokcumen GE. Regulation of lipids is central to replicative senescence. *Mol BioSyst.* 2017; 13:498–509. [PubMed: 28128379]
36. Lecchi C, Invernizzi G, Agazzi A, Modina S, Sartorelli P, Savoini G, Cecilian F. Effects of EPA and DHA on lipid droplet accumulation and mRNA abundance of PAT proteins in caprine monocytes. *Res Vet Sci.* 2013; 94:246–251. [PubMed: 23102647]
37. Plotz T, Hartmann M, Lenzen S, Elsner M. The role of lipid droplet formation in the protection of unsaturated fatty acids against palmitic acid induced lipotoxicity to rat insulin-producing cells. *Nutr Metab.* 2016; 13:16.
38. Shulga YV, Topham MK, Epan RM. Regulation and functions of diacylglycerol kinases. *Chem Rev.* 2011; 111:6186–6208. [PubMed: 21800853]

Figure 1.

(A) Western blots of control (C) and apoptotic samples. Apoptosis was induced by various apoptotic agents, including staurosporine (STS), doxorubicin (Dox), and etoposide (Etop), in HCT-116 cells and MCF-7 cells. PARP cleavage was used as an apoptosis marker; α -tubulin was used as a loading control. (B) Targeted analysis of triacylglycerols (TAGs) in control and apoptotic HCT-116 cells. Fold change (FC) was determined as (average relative abundance in apoptotic samples)/(average relative abundance in control samples) (see the Supporting Information for details). An asterisk indicates that the lipid was not detected in control samples; thus, a value for fold change could not be calculated. Instead, a large value was assigned to fold change to represent the accumulation of that lipid in apoptotic samples.

Figure 2.

(A) Targeted analysis of phosphatidylcholines (PC) in control (C) and staurosporine-treated (STS) and doxorubicin-treated (Dox) apoptotic HCT-116 cells. (B) Targeted analysis of triacylglycerols (TAGs) in control and staurosporine-treated (STS) apoptotic MCF-7 cells. The fold change (FC) was determined as (average relative abundance in apoptotic samples)/(average relative abundance in control samples) (see the Supporting Information for details). An asterisk indicates that the lipid was not detected in control samples; thus, a numeric value for fold change cannot be calculated. Instead, a large value was assigned to the fold change to represent the accumulation of that lipid in apoptotic samples.

Figure 3.

(A) Workflow of the sucrose gradient ultracentrifugation method for isolating lipid droplet-enriched layer (L1) from control and apoptotic HCT-116 cells. (B) Nile red emission spectra in the range of 500–700 nm (480 nm excitation) in different fractions (L1–L3, L6, L9, and L10) after ultracentrifugation in control and 5-FU-treated apoptotic HCT-116 cells. RFU, relative fluorescence units. A decrease in the emission maximum in the top layer (L1, ~585 nm) compared to that in the bottom layers (L9 and L10, ~625 nm) was found in apoptotic samples, suggesting the presence of more hydrophobic structures, i.e., lipid droplets, in the top layer of apoptotic cell lysates. (C) Western blots of ADRP/ADFP and calnexin for different fractions (L1–L3, L6, L9, and L10) after ultracentrifugation in 5-FU-treated apoptotic HCT-116 cells. ADRP/ADFP is a lipid droplet marker, while calnexin is an ER marker. The presence of ADRP/ADFP and the absence of calnexin in top layer L1 support the idea that the top layer contains lipid droplets.

Figure 4.

Targeted analysis of triacylglycerols (TAGs) in lipid droplet-enriched fractions (L1) from control (C) and 5-FU-treated apoptotic HCT-116 cells. The fold change (FC) was determined as (average relative abundance in apoptotic samples)/(average relative abundance in control samples) (see the Supporting Information for details). An asterisk indicates that the lipid was not detected in control samples because of the low level of lipid droplets; thus, a numeric value for the fold change could not be calculated. Instead, a large value was assigned to the fold change to represent the accumulation of that lipid in apoptotic samples.

Figure 5.

(A) Change in the expression of *diacylglycerol acyltransferase-1* and *-2* (*DGAT-1* and *-2*, respectively) in control vs 5-FU-treated p53^{+/+} (wild type) and p53^{-/-} HCT-116 cells. mRNA levels of genes of interest were measured and normalized on the basis of the expression level of the housekeeping gene (*hypoxanthine phosphoribosyltransferase-1*) *HPRT-1*. The fold change was calculated by dividing the normalized levels of gene expression of *DGATs* under 5-FU-treated conditions by the average normalized levels of gene expression of *DGATs* under control conditions. The fold change is shown as the mean $\pm$ the standard deviation ($n = 3$). (B) Western blot of DGAT-1 in control vs 5-FU-treated p53^{+/+} (wild type) and p53^{-/-} HCT-116 cells. A 2-fold increase in the level of DGAT-1 at the protein level was observed for 5-FU-treated p53^{+/+} cells compared to that of control p53^{+/+} cells. α -Tubulin was used as a loading control. (C) Western blot of fatty acid synthase (FASN) in wild type (control), red fluorescent protein knockdown (shRFP), and FASN knockdown (shFASN) HCT-116 cells. shRFP was used as a negative control. (D) Targeted analysis of phosphatidylcholines (PCs) in control and 10 μ M C18:3 fatty acid-treated shFASN HCT-116 cells. The fold change was calculated by dividing the abundance of an individual PC under the fatty acid-treated condition by the average abundance of that species under control conditions. The fold change is represented as the mean $\pm$ the standard deviation ($n = 3$). We note that C34:6 PC was not detected in control samples and a value for the fold change can not be calculated, thus its fold change is shown as high. C18:3 treatment caused a profound accumulation of polyunsaturated fatty acyl-containing PCs (PUFA-PCs, with at least three total double bonds), which are highlighted in gray. (E) Cell viability of control, 10 μ M C18:3 FA-treated, and 10 μ M C16:0 FA-treated shFASN HCT-116 cells in the absence or presence of H₂O₂. C18:3 fatty acid treatment induced more cell death under oxidative stress than treatment with only H₂O₂ did, while C16:0 FA did not have such an effect ($n = 5$). The dashed line indicates the average cell viability after treatment with only H₂O₂.