Knockdown of Triglyceride Synthesis Does Not Enhance Palmitate Lipotoxicity or Prevent Oleate-Mediated Rescue in Rat Hepatocytes

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Abbreviations:

ATF3, activating transcription factor 3; CHOP, CCAAT/enhancer-binding protein homologous protein; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; FFA, free fatty acid; MUFA, monounsaturated fatty acid; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NTC, non-targeting siRNA control; OA, oleic acid; PA, palmitic acid; PC, phosphatidylcholine; PL, phospholipid; ROS, reactive oxygen species; SFA, saturated fatty acid; TG, triacylglycerol; UPR, unfolded protein response

Abstract

Experiments in a variety of cell types, including hepatocytes, consistently demonstrate the acutely lipotoxic effects of saturated fatty acids, such as palmitate (PA), but not unsaturated fatty acids, such as oleate (OA). PA+OA co-treatment fully prevents PA lipotoxicity through mechanisms that are not well defined but which have been previously attributed to more efficient esterification and sequestration of PA into triglycerides (TGs) when OA is abundant. However, this hypothesis has never been directly tested by experimentally modulating the relative partitioning of PA/OA between TGs and other lipid fates in hepatocytes. In this study, we found that addition of OA to PA-treated hepatocytes enhanced TG synthesis, reduced total PA uptake and PA lipid incorporation, decreased phospholipid saturation and rescued PA-induced ER stress and lipoapoptosis. Knockdown of diacylglycerol acyltransferase (DGAT), the rate-limiting step in TG synthesis, significantly reduced TG accumulation without impairing OA-mediated rescue of PA lipotoxicity. In both wild-type and DGAT-knockdown hepatocytes, OA co-treatment significantly reduced PA lipid incorporation and overall phospholipid saturation compared to PA-treated hepatocytes. These data indicate that OA's protective effects do not require increased conversion of PA into inert TGs, but instead may be due to OA's ability to compete against PA for cellular uptake and/or esterification and, thereby, normalize the composition of cellular lipids in the presence of a toxic PA load.

Key Words:

Saturated fatty acids; lipoapoptosis; endoplasmic reticulum stress; phospholipid metabolism; triglycerides; diacylglycerol acyltransferase

1. Introduction

Almost two decades ago, Unger *et al.* introduced the concept of lipoapoptosis [1-3], which involves cellular dysfunction and death due to the over-accumulation of lipids in non-adipose tissues [4]. Hepatocyte lipoapoptosis due to liver fat accumulation is one of the hallmark features of nonalcoholic steatohepatitis (NASH) in humans [5, 6]. The large body of literature published since the introduction of this concept indicates that lipid-induced hepatocyte toxicity is most acute when cells are exposed to elevated saturated fatty acids (SFAs) such as palmitate (PA) [7-10]. Clinical studies indicate that diets high in saturated fat may be linked to NASH progression while, conversely, unsaturated fat can impart a protective effect [4, 11]. *In vivo* studies using rodent diets high in saturated fat have shown increased hepatic inflammation, cell death, and markers of liver injury compared to normal high fat or chow diets [12-14]. Despite its potential disease relevance, however, the molecular mechanisms underlying the differential effects of saturated versus unsaturated fat on liver hepatocytes are not well understood and represent a critical area of investigation in order to develop more targeted and effective therapeutics for NASH and related disorders.

In vitro experiments in a wide variety of cell types including Chinese hamster ovary (CHO) cells [15-17], pancreatic beta cells [18-20], breast cancer cells [21], HeLa cells [22] and hepatic cells [7, 8, 10, 23-26] have consistently demonstrated the acute lipotoxic effects of saturated fatty acids but not unsaturated fatty acids. Palmitate-mediated lipotoxicity is characterized by endoplasmic reticulum (ER) stress, ER calcium release, reactive oxygen species (ROS) accumulation, caspase activation and cell death [9, 27, 28]. Cells treated with monounsaturated fatty acids (MUFAs), such as oleate (OA), exhibit none of these features but instead demonstrate significant increases in triglyceride (TG) synthesis and accumulation [7, 10, 15]. Adding OA to PA-treated cells can fully prevent PA lipotoxicity through mechanisms that are not well defined but which have been previously attributed to increased TG accumulation and sequestration of PA into neutral lipid stores [15, 18, 19]. However, this hypothesis has never been directly tested by experimentally modulating the relative partitioning of fatty acids between

TGs and other lipid fates in hepatocytes. Prior studies in *Dgat1*^{-/-} mouse embryonic fibroblasts (MEFs) [15], cardiomyocytes [29], and macrophages [30] do not fully address this question, since these cells are derived from non-lipogenic tissues that have lipid biosynthetic capacities and metabolic phenotypes that are quite different from hepatocytes.

The goal of this study was to better understand the possible mechanisms by which oleate rescues palmitate-induced lipotoxicity in hepatocytes and the role that triglyceride and phospholipid metabolism plays in this phenomenon. We show that addition of OA to PA-treated hepatocytes reduces PA uptake, PA lipid incorporation, and overall phospholipid saturation while rescuing PA-induced apoptotic cell death. As shown previously, this rescue effect is accompanied by enhanced TG synthesis and, specifically, increased relative partitioning of PA into TG stores. However, simultaneous knockdown of both liver isoforms of diacylglycerol acyltransferase (DGAT), the rate-limiting step in TG synthesis, significantly reduced TG accumulation but without altering the ability of OA to prevent PA lipotoxicity. In both wild-type and DGAT knockdown cells, co-treatment with OA significantly reduced PA lipid incorporation and overall phospholipid saturation in comparison to hepatocytes treated with PA alone. These data indicate that OA's protective effects do not require increased conversion of PA into inert TG stores, but instead may be attributable to OA's ability to compete against PA for cellular uptake and/or esterification and, thereby, normalize the composition of cellular lipids in the presence of a toxic PA load.

2. Materials and Methods

2.1. Materials and Reagents

Oleic and palmitic acids, bovine serum albumin (BSA), and Dulbecco's modified Eagle's medium (DMEM) were all purchased from Sigma-Aldrich (St. Louis, MO). CHOP primary (mouse) and goat anti-mouse secondary antibodies were purchased from Abcam (Cambridge, MA). β -Actin primary (goat) and donkey anti-goat secondary antibodies were procured from Santa Cruz Biotechnology. Cleaved PARP

primary (rabbit) and mouse anti-rabbit secondary antibodies were purchased from Cell Signaling (Danvers, MA). All other chemicals were purchased from standard commercial sources.

2.2. Preparation of Fatty Acid Solutions

Fatty acid treatment solutions were prepared by coupling free fatty acids to fatty acid-free BSA. Specifically, palmitate or oleate was fully dissolved in 200-proof ethanol for a concentration of 195 mM. This FFA stock solution was added to a pre-warmed BSA solution (10% w/w, 37°C) to a final FFA concentration of 3 mM. The solution was fully dissolved by warming at 37°C for an additional 10 minutes. The final ratio of FFA:BSA was 2:1. Vehicle control treatments were prepared using stocks of 10% w/w BSA with an equivalent volume of ethanol added to match that contained in the final FFA stock. The final concentration of ethanol was less than 1% in all experiments.

2.3. Cell Culture and siRNA Transfections

Rat hepatoma cells, H4IIEC3 (ATCC), were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/glutamine (2mM). Measurements were obtained at 70-80% confluency. Hepatocytes were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. The final concentration of siRNA was 25 nM. Multiple siRNA oligomers were initially screened to identify siDGAT1 and siDGAT2 sequences that consistently provided the greatest knockdown efficiencies. The two selected siRNA sequences were applied in all subsequent experiments, as it was not practical to include multiple sequences for each target gene in the final experimental design. Therefore, the siRNA transfections were only performed using a single siRNA sequence for each target gene.

2.4. Primary Hepatocyte Isolation, Culture and siRNA Transfections

Sprague Dawley rats were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in a temperature- and humidity-controlled environment with 12:12 h light-dark cycle and fed standard chow

diet ad libitum. Following a 1-week acclimation period, rats were used for primary hepatocyte isolation. Briefly, the hepatic cells of 5-6 week old rats were isolated using collagenase perfusion and first incubated in DMEM-based attachment media containing 20 mM glucose, 100 nM dexamethasone and 5 nM insulin on collagen I coated plates for 4 h (attachment period). The cells were washed once with PBS and the medium was changed to a DMEM-based growth medium containing 20 mM glucose, 100 nM dexamethasone and 1 nM insulin for 16h. Experimental treatments were performed after this period using the same growth medium. All experimental protocols were approved by the Animal Care and Use Committee at Vanderbilt University. siRNA experiments in primary cells were done similarly to those described in H4IIEC3 cells, namely using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. The final concentration of siRNA was 25 nM.

2.5. Cell Toxicity

Toxicity was assessed using propidium iodide as described previously [31]. Propidium iodide is an intercalating dye that can only permeate dead cells. It becomes highly fluorescent when embedded in the double-stranded DNA exposed after cell death. After culturing cells in 96-well plates with experimental treatments, the medium was removed and replaced with a solution of the dye and serum-free DMEM. Cells were incubated at 37°C for 1 h in the dark prior to fluorescence measurement at ex/em wavelengths of 530/645 nm.

2.6. Caspase Activation

The Apo-ONE Homogenous Caspase 3/7 Assay kit was used to measure apoptotic caspase activation. Cells were cultured in 96-well plates and incubated with desired treatments for 6 hours. The Apo-ONE kit uses a lysis buffer combined with a caspase 3/7 specific substrate (Z-DEVD-R110), which becomes fluorescent once these caspases remove its DEVD peptide. Fluorescence was measured at ex/em wavelengths of 485/535 nm.

2.7. Western Blotting

Cells were lysed with ice-cold RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) supplemented with Na-orthovanadate, protease inhibitor cocktail, and PMSF for 30 min on ice. Samples were centrifuged at 16,100 rcf and 4°C for 20 min, and the resulting supernatants constituted the total protein extracts. Protein concentrations were determined by BCA assay (Thermo Fisher Scientific, Rockford, IL). Samples were added in concentrations of 30 µg/lane for SDS-PAGE western blotting. Dilutions of the primary antibodies were Anti-CHOP (1:1000), anti-β-actin (1:1000) and anti-cleaved-PARP (1:1000), all dissolved in 5% BSA. Secondary antibodies (anti-mouse, anti-goat and anti-rabbit, respectively) were all dissolved in a 1:1 mix of TBST: 5% BSA at a dilution of 1:10,000.

2.8. Total RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol and then reverse transcribed using a cDNA reverse transcriptase kit (BioRad, Hercules, CA). Quantitative real-time PCR was done using iQ SYBR Green Supermix (BioRad, Hercules, CA) and BioRad CFX96 Cycler (BioRad, Hercules, CA). The sequences of the primers were as follows: C/EBP homologous protein (CHOP) forward (5'-CTCAGCTGCCATGACTGTA-3') and reverse (5'-CGTCGATCATACCATGTTGAAGA-3'), ATF3 forward (5'-CCATCCAGAACAAGCACCT-3') and reverse (5'-CGCCTCCTTTTTCTCTCATCT-3'), ribosomal 18s forward (5'-CGGACAGGATTGACAGAT-3') and reverse (5'-CGCTCCACCAACTAAGAA-3'), diacylglycerol acyltransferase 1 (DGAT1) forward (5'-TTACCGTGGTATCCTGAA-3') and reverse (5'-GCCATACTTGATAAGATTCTC-3'), diacylglycerol acyltransferase 2 (DGAT2) forward (5'-AGACACCATAGACTACTT-3') and reverse (5'-ATACCTCATTCTCTCCAA-3'). Target gene expression was normalized on the basis of 18s content.

2.9. Phospholipid Fatty Acid Profiles

Cells seeded in 10-cm Petri dishes at an initial density of 4×10^6 cells per plate were incubated in standard medium until reaching ~70-80% confluency, at which time experimental treatments were

administered. Cells were then trypsinized for 3 min and scraped using cold PBS. Cell suspensions were pelleted by centrifugation and resuspended in fresh PBS. Fatty acyl lipid analysis was performed by the Vanderbilt Hormone Assay and Analytical Services Core using thin-layer chromatography (TLC) and gas chromatography-flame ionization detection (GC-FID) techniques. Briefly, lipids were extracted from the aforementioned cell pellets using a modified Folch separation. An internal standard (1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine) for phospholipids was added to the lipid-containing chloroform phase. Total lipids were then extracted and separated by TLC using petroleum ether/ethyl ether/acetic acid (80/20/1, v/v/v) on silica plates. Spots corresponding to different lipid pools were visualized with rhodamine 6G in 95% ethanol and scraped individually into glass tubes for transmethylation. Transmethylation was performed using a boron trifluoride-methanol 10% (w/w) solution. Derivatized lipids were then analyzed using GC-FID, where standardized calibration curves were used to analyze fatty acid composition and quantify total content.

2.10. ³H-Palmitate Lipid Class Incorporation

Cells seeded in 6-well dishes at 1.5×10^5 cells per well were transfected with siRNA and incubated in standard medium until reaching ~70-80% confluency. Next, the cells were incubated for 12 h at 37°C in 400 μ M [9,10-³H] palmitic acid (1 μ Ci ³H/ μ mol PA), either in the presence or absence of 400 μ M oleic acid. Lipids were extracted using a modified Folch procedure; twice, 0.75 mL chilled methanol was added to each well and cells were scraped into 1:1 chloroform:water. Once vortexed and centrifuged, the lipid-containing chloroform phase was vacuum dried without heat. Lipid classes were separated by TLC, as described above. Each TLC spot was added to an individual vial and radioactivity was assessed by scintillation counting.

2.11. ³H-Palmitate Uptake, Oxidation, and Triglyceride Secretion

Cells seeded in 6-cm dishes at 0.5×10^6 cells per dish were transfected with siRNA and incubated in standard medium until reaching ~70-80% confluency. Next, the cells were incubated for 12 h at 37°C in

400 μM [9,10- ^3H] palmitic acid (1 μCi $^3\text{H}/\mu\text{mol}$ PA), either in the presence or absence of 400 μM oleic acid. After the incubation, the culture medium was collected and deproteinized by addition of perchloric acid followed by centrifugation. The precipitate contained albumin-bound ^3H -PA and the supernatant contained $^3\text{H}_2\text{O}$ dehydrated from ^3H -PA during beta-oxidation. To measure $^3\text{H}_2\text{O}$ production, the supernatants were neutralized with K_2CO_3 and applied to an individual AG1-X8 Resin column (BioRad, Hercules, CA) to remove any remaining palmitate from the sample. The column was allowed to empty under gravity flow and flushed twice with distilled water. The initial charge and all subsequent washes were collected in a scintillation vial, mixed with Ecolite scintillation cocktail, and read in a scintillation counter. The protein precipitates from the medium samples were washed using a 3% perchloric acid solution, and albumin-bound palmitate was extracted using a mixture of hexane and isopropanol (3:2). The solvent was transferred to a scintillation vial, dried by air flow, dissolved in 1 mL heptane, mixed with 1 mL water and 10 mL Ecolite scintillation cocktail, and read in a scintillation counter. To measure non-oxidized ^3H -PA incorporated into cellular lipids, cells were rinsed with 5 mL of D-PBS three times, harvested with 4 mL D-PBS, deproteinized by perchloric acid and analyzed for ^3H enrichment by the same procedure used to measure residual albumin-bound ^3H -PA precipitated from the culture medium. To measure incorporation of ^3H -PA into secreted triglycerides, culture medium was separated into lipid classes by TLC, as described in 2.9. *Phospholipid Fatty Acid Profiles*. The TG spot was added to an individual vial and radioactivity was assessed by scintillation counting. After the extraction of lipids, the cell pellets were dried and dissolved in 1 mL water for measurement of protein content.

2.12. LC-MS Analysis of Palmitate Uptake

Cells seeded in 6-well dishes at 1.5×10^5 cells per well were transfected with siRNA and incubated in standard medium until reaching ~70-80% confluency. Next, the cells were incubated at 37°C in 400 μM palmitic acid, either in the presence or absence of 400 μM oleic acid. After 12 h of incubation with FFA treatments, the spent medium was collected from each well and the viable cell count was obtained. A known concentration of uniformly ^{13}C -labeled palmitate was dissolved in methanol/acetonitrile and was

spiked into the medium samples to initiate extraction. Then, samples were vortexed and sonicated for 15 min prior to incubation at -20°C for 1 h. Insoluble material was removed by centrifugation. Samples were separated with a Luna Aminopropyl column (3 μ m, 150 mm $\times$ 1.0 mm I.D., Phenomenex) and analyzed by an Agilent Q-TOF 6530 mass spectrometer equipped with an AJS electrospray ionization source. The absolute concentrations of palmitate were determined by calculating the ratio between the fully unlabeled peak from medium samples and the fully labeled peak from the internal standard. Specific palmitate consumption rates were calculated by normalizing the measured palmitate uptake to viable cell numbers.

2.13. Statistical Analysis

All data are represented as mean $\pm$ standard error. Type I ANOVA (Student's t test) was used to assess statistical differences involving multiple (two) treatments. ANOVA was followed with Tukey-Kramer post-hoc testing to identify significant intergroup differences.

3. Results

3.1. DGAT knockdown effectively reduces triglyceride synthesis but does not sensitize hepatocytes to oleate lipotoxicity

DGAT plays an important role in determining the rate of cellular TG synthesis. Therefore, we sought to examine how inhibiting the expression of DGAT affects lipoapoptosis and cellular lipid composition. We chose to simultaneously inhibit both DGAT1 and DGAT2, since both isoforms have demonstrated activity in hepatocytes [32, 33]. Simultaneous transfection of siRNAs targeted against rat DGAT1 and DGAT2 (siDGAT1/2) efficiently reduced mRNA expression levels of both DGAT1 and DGAT2 by approximately 70% and 85%, respectively, in both H4IIEC3 cells and primary rat hepatocytes (Fig. 1A and B). Oleate is a potent activator of TG synthesis in hepatocytes through its binding and inactivation of the constitutive DGAT-inhibitory protein Ubxd8 [34]. Oleate administration was used to demonstrate the

functional inhibition of cellular TG synthesis in response to siDGAT1/2 transfection. siDGAT1/2 cells treated with 400 μ M OA showed significantly less TG accumulation than cells transfected with a non-targeted siRNA control (NTC) vector in both H4IIEC3 cells and primary rat hepatocytes (Fig. 1C and D). Despite these differences in TG synthesis, there was no difference in cell death between siDGAT1/2 and NTC cells after treatment with OA alone (Fig. 2A and B). These data indicate that reduced TG synthesis capacity does not sensitize hepatocytes to OA lipotoxicity.

3.2. Inhibition of TG synthesis does not enhance palmitate-induced apoptosis or prevent oleate-mediated rescue

Similar to prior studies with untransfected cells [7, 8, 10], PA treatment of hepatocytes transfected with NTC siRNA resulted in significant increases in cell death (Fig. 2A and B). PA toxicity was also associated with increased activity of caspases 3 and 7 (Fig. 2C) and accumulation of cleaved PARP (Fig. 2D) in H4IIEC3 cells, indicative of apoptosis-mediated cell death. Co-supplementation of OA to PA-treated NTC cells reduced toxicity markers to levels that were not significantly different from BSA-treated control cells. Interestingly, knockdown of DGAT1/2 in both H4IIEC3 cells and primary rat hepatocytes did not alter the lipotoxic response to PA and/or OA treatments. Palmitate was toxic to siDGAT1/2 cells while OA was not, and most surprisingly, cells supplemented with PA were still rescued by the addition of OA even when TG synthesis was substantially inhibited (Figure 2A and B). Furthermore, OA co-supplementation was still able to protect against caspase 3/7 activation and accumulation of cleaved PARP in siDGAT1/2 H4IIEC3 cells, indicating a rescue from PA-mediated apoptosis.

3.3. DGAT1/2 knockdown does not affect palmitate- and oleate-induced markers of ER stress

Next, we sought to examine whether DGAT1/2 knockdown affected other aspects of PA-induced lipotoxicity aside from cell death and apoptosis. Previous studies have shown that PA exposure induces acute ER stress pathways, also known as the unfolded protein response (UPR), as part of the lipotoxicity

cascade [7, 35]. The purpose of UPR is to respond to deleterious changes in ER function, which can result from accumulation of misfolded proteins [36] or changes in ER membrane composition [22, 23, 37]. We measured expression of CCAAT/enhancer-binding protein homologous protein (CHOP) and activating transcription factor 3 (ATF3), two genes associated with downstream UPR signaling, as surrogate markers of ER stress during lipotoxic treatments. Quantitative real-time PCR analysis showed a marked increase in CHOP and ATF3 mRNA expression in response to PA but not OA treatment in both NTC vector and siDGAT1/2 transfected H4IIEC3 cells (Fig. 3A and B). Co-treatment with OA in both control and knockdown cells restored these markers of ER stress to basal levels. The same trend was observed in measurements of CHOP protein expression (Fig. 3C). These results indicate that elevated TG synthesis is not required for OA to inhibit PA-induced ER stress in hepatocytes.

3.4. DGAT1/2 knockdown alters partitioning of exogenous palmitate amongst intracellular lipid pools

We next examined the effects of DGAT1/2 knockdown on PA incorporation into the main intracellular lipid pools. As expected, DGAT knockdown in H4IIEC3 cells significantly reduced the relative partitioning of radiolabeled ^3H -PA into the TG pool, both alone and in the presence of OA (Fig. 4). There was a small, but significant, increase in accumulation of ^3H -PA into the diacylglycerol pool in the siDGAT1/2 cells. However, the majority of the radiolabeled PA was alternatively channeled into the phospholipid pool in these knockdown cells. It is important to note that the reduction in relative ^3H -PA incorporation into TG parallels the reduction in total TG accumulation exhibited by siDGAT1/2 cells (Fig. 1C). Taken together, these results indicate that DGAT knockdown leads to re-routing of palmitate away from TG and into alternative lipid end-products. Interestingly, there were no significant differences in the relative intracellular ^3H -PA partitioning or total TG accumulation of siDGAT1/2 cells co-treated with PA+400 μM OA versus NTC cells treated with PA alone, and yet the former cells were protected from lipotoxicity while the latter cells were not. This finding is inconsistent with the hypothesis that OA's ability to prevent PA lipotoxicity is simply a consequence of its ability to divert PA into neutral TG stores.

3.5. DGAT1/2 knockdown does not alter oleate-mediated changes in phospholipid composition

To determine whether changes in PA partitioning were also associated with changes in phospholipid composition, we measured the relative abundance of various fatty acyl chains in the total cellular phospholipids of H4IIEC3 cells (Table 1). Administration of PA for 12 h resulted in significant increases in the abundance of palmitic acid (16:0) compared to BSA treatment in both NTC cells ($48.73 \pm 0.62\%$ versus $12.87 \pm 0.14\%$) and DGAT1/2 knockdown cells ($53.06 \pm 2.66\%$ versus $13.58 \pm 0.18\%$). This correlated with a significant increase in total phospholipid saturation in PA-treated cells versus BSA control cells. Similar to previous reports in untransfected cells [7], PA/OA co-treatment of NTC cells significantly reduced both the abundance of PA in phospholipids ($30.05 \pm 0.24\%$ versus $48.73 \pm 0.62\%$) and overall phospholipid saturation ($40.80 \pm 0.27\%$ versus $61.55 \pm 0.85\%$) compared to cells treated with PA alone. Most interestingly, siDGAT1/2 cells exhibited similar shifts in PA abundance ($30.77 \pm 0.22\%$ versus $53.06 \pm 2.66\%$) as well as total saturation level of phospholipids ($40.62 \pm 0.24\%$ versus $64.94 \pm 2.77\%$) when PA/OA co-treated cells were compared to cells treated with PA alone. In fact, there were no statistical differences in 16:0 abundance or phospholipid saturation level between the NTC and siDGAT1/2 cells when co-treated with PA/OA. Similar trends were observed in phospholipid composition of siDGAT1/2 and NTC primary rat hepatocytes in response to these same fatty acid treatments, although the changes were less pronounced after 12 h (Supplemental Table A). This is consistent with our prior observation that lipoapoptosis and associated changes in phospholipid composition progress more gradually in primary rat hepatocytes than in H4IIEC3 cells [7]. In summary, these data suggest that inhibition of TG synthesis does not alter the ability of oleate to normalize the composition of cellular phospholipids in PA-treated hepatocytes.

3.6. Oleate co-treatment reduces total PA uptake and PA lipid incorporation independently of DGAT1/2 expression level

To examine the effect of OA co-treatment on PA uptake and disposal, we incubated H4IIEC3 cells with ^3H -PA and examined the total rate of ^3H -PA depletion from the medium and the rate of ^3H -PA accumulation in cellular lipids and secreted TGs. We found that oleate co-treatment significantly reduced total PA uptake in both NTC and siDGAT1/2 cells (Fig. 5A). This trend was confirmed using an LC-MS assay of palmitate depletion, although the effect of oleate addition was not significant in siDGAT1/2 cells (Supplemental Fig. S1). The reduction in PA uptake was associated with a roughly proportional decrease in PA incorporation into cellular lipids (Fig. 5B) and into secreted TGs (Supplemental Fig. S2), indicating that oleate competes with palmitate for cellular uptake and esterification into complex lipids. More than half of the ^3H -PA consumed was incorporated into cellular lipids, which represents the major fate of exogenous palmitate under all conditions studied. In contrast, the relative ^3H -enrichment of secreted TGs was very low under all conditions (<1% of total ^3H -PA uptake), indicating that TG secretion did not contribute significantly to overall PA disposal.

3.7 DGAT1/2 knockdown enhances PA oxidation while inhibiting PA lipid incorporation

In addition to measuring ^3H -PA uptake and lipid incorporation, we also assessed the rate of $^3\text{H}_2\text{O}$ enrichment as an indicator of palmitate oxidation. DGAT1/2 knockdown significantly increased PA oxidation in H4IIEC3 cells treated with palmitate alone (Fig. 5C), despite decreases in total PA uptake (Fig. 5A and Supplemental Fig. S1) and cellular lipid incorporation (Fig. 5B). The same trends were observed in cells co-treated with oleate. DGAT1/2 knockdown also reduced the incorporation of ^3H -PA into secreted TGs, although the effect was not significant. As a result, the fraction of PA oxidized (relative to total uptake) approximately doubled in both PA-treated and PA/OA-treated cells in response to DGAT1/2 knockdown (Fig. 5D). Oleate co-treatment also significantly increased the fraction of PA oxidized, although this was primarily due to its effect to reduce total PA uptake rate while leaving PA oxidation unchanged (NTC cells) or slightly lower (siDGAT1/2 cells) in comparison to cells treated with palmitate alone.

4. Discussion

Currently, it is a widely held hypothesis that increases in TG accumulation are responsible for the ability of OA and other unsaturated fatty acids to rescue cells from PA-induced lipotoxicity [15, 34, 35, 38, 39]. This hypothesis is supported by the finding that *Dgat1*^{-/-} primary mouse MEFs showed significantly increased sensitivity to OA-induced lipotoxicity in comparison to *Dgat*^{+/+} MEFs [15]. We therefore sought to examine the effects of OA supplementation on PA-treated hepatocytes to assess whether DGAT knockdown would similarly sensitize these cells to FFA-induced lipotoxicity. Surprisingly, while DGAT knockdown effectively reduced intracellular TG accumulation (Fig. 1) and decreased incorporation of exogenous PA into intracellular lipids (Fig. 5B) and secreted TGs (Supplemental Fig. S2), it did not modify the differential effects of fatty acid treatments on cell viability or apoptosis in either H4IIEC3 rat hepatoma cells or primary rat hepatocytes (Fig. 2). In contrast to prior reports in other cell types, these data suggest that increased TG synthesis is not necessary for OA-mediated rescue of PA lipotoxicity in liver hepatocytes. Furthermore, DGAT knockdown alone was insufficient to prevent PA lipotoxicity, suggesting that reduced PA uptake was not solely responsible for the rescue effects of oleate co-treatment.

We have shown previously [7] that addition of PA to hepatocytes rapidly induces ER stress, which is correlated with increases in ROS, apoptosis, and cell death. In that prior study, addition of OA to PA-treated cells prevented ER stress and correlated with a change in PA distribution within cellular lipid pools: specifically, PA incorporation into the phospholipid fraction was reduced and was redirected into the TG fraction. In the current study, we therefore examined the impact that inhibition of TG synthesis would have on PA partitioning and overall phospholipid composition in hepatocytes. DGAT knockdown cells incorporated a higher percentage of exogenous PA into the phospholipid fraction and less into TGs than vector control cells, in both the presence and absence of OA (Fig. 4). However, this change in relative PA partitioning had no impact on ER stress markers (Fig. 3). This is possibly explained by the

lack of any meaningful differences in phospholipid saturation between DGAT knockdown and vector control cells co-treated with equimolar concentrations of PA and OA (Table 1). The ability of oleate to compete with palmitate for cellular uptake and esterification into complex lipids was independent of DGAT expression level (Fig. 5A and B), which explains why phospholipid composition was largely unaffected by DGAT knockdown despite substantial changes in palmitate partitioning between intracellular lipid fates.

The fatty acyl chain composition and degree of unsaturation in phospholipids determines the biophysical characteristics of cellular membranes, including fluidity and assembly, which in turn influence their function [40]. The ER membrane typically contains unsaturated phosphatidylcholine (PC) as its major phospholipid component [41], and its composition is tightly regulated: even small changes in fatty acyl composition may induce ER stress. Exogenous saturated fatty acids have been shown to rapidly incorporate into phospholipids of the ER membrane and alter its structure through increased order and reduced fluidity [7, 35, 37, 42], which may explain why markers of ER stress appear rapidly after treatment with PA but not OA [7, 35]. The precise nature of the link between reduced membrane fluidity and ER stress has not been fully elucidated, but recent literature suggests that changes in lipid composition substantially affect ER membrane proteins [43]. Volmer *et al.* have recently demonstrated that increased lipid acyl-chain saturation of the ER membrane can directly activate ER transmembrane proteins IRE α and PERK, whose stimulation initiates two of the three main arms of the UPR [42]. This is consistent with our results in PA-treated hepatocytes showing upregulation of ER stress markers CHOP and ATF3, both of which are activated through the PERK pathway in the UPR. While activation of UPR signaling proteins provides a sensitive indicator of ER stress, these mechanisms do not fully explain the association between PA-induced ER stress and cell death [44]. Increased phospholipid saturation may also impair the function of sarco-endoplasmic reticulum calcium ATPase (SERCA), which is critical for maintaining calcium homeostasis within the ER lumen [43, 45]. Interestingly, we have recently shown

that increased Ca^{2+} efflux from the ER can promote overactivation of mitochondrial metabolism leading to ROS accumulation and apoptosis in response to PA treatments [26].

Our results confirm that hepatocytes readily incorporate exogenous PA into phospholipids leading to increased membrane saturation (Table 1), which is associated with initiation of ER stress (Fig. 3). When OA was supplemented to PA-treated cells, oleate efficiently competed with palmitate for cellular uptake and esterification (Fig. 5 and Supplemental Fig. S1–S2). In addition, PA/OA co-treatment partially restored the composition of cellular phospholipids to that of BSA-treated cells (Table 1). This ability of OA to counteract PA's effects on phospholipid saturation correlated with a reduction in markers of ER stress and lipoapoptosis, irrespective of DGAT expression levels and TG biosynthetic capacity. Another possible explanation for OA's ability to rescue PA lipotoxicity could be attributed to the increase in fractional PA oxidation we observed in PA/OA co-treated cells (Fig. 5D). There has been much prior debate about the role of fatty acid oxidation in modulating lipotoxicity. One view holds that impaired or incomplete beta-oxidation leads to accumulation of toxic lipid intermediates that activate apoptosis [46]. The other view holds that fatty acid overload results in excessive beta-oxidation that fuels ROS accumulation and cellular dysfunction [47]. However, we showed in a prior study that blocking beta-oxidation with etomoxir did not affect ROS accumulation or the appearance of apoptotic markers in H4IIEC3 cells treated with PA [8]. Furthermore, the current study showed that DGAT knockdown increased fractional PA oxidation to a similar extent as PA/OA co-treatment but without rescuing palmitate lipotoxicity. These results imply that the enhancement in fractional PA oxidation induced by PA/OA co-treatment is probably not the primary mode by which oleate acts to rescue palmitate lipotoxicity.

Our study demonstrates that any increased capacity for PA disposal via esterification into TGs is not necessary for OA to exert its protective effects to inhibit PA lipotoxicity in rat hepatocytes. However, the results of this study appear to contradict previous results in other cell types, such as the study by Listenberger et al. that demonstrated increased cellular sensitivity to oleate in response to DGAT

knockout in MEFs [15]. Additional studies involving yeast screens for fatty acid sensitive strains support those results [48, 49]. There are several possible explanations for these seemingly contradictory findings. All of the prior studies were done in non-hepatic cells and thus may involve quite different metabolic phenotypes. Additionally, these studies utilized complete knockouts of lipogenic enzymes, which may lead to non-physiological accumulation of lipid intermediates or death by a mechanism that is completely different from the mechanism of palmitate lipotoxicity in normal hepatocytes. Another complication is that some of the yeast knockouts, such as the ARV1 mutant [49], exhibited high levels of basal ER stress in the absence of fatty acid treatments [50]. Further studies are needed to identify the important differences between these lipotoxicity models and our own, in order to fully elucidate the mechanism by which OA can prevent PA-induced lipotoxicity in hepatocytes.

Studying the role of DGAT in hepatocytes has been complicated by the fact that both DGAT1 and DGAT2 are highly expressed in liver [51] and because *Dgat2*^{-/-} mice die soon after birth [52]. On the other hand, *Dgat1*^{-/-} mice are viable and have enabled studies of complete DGAT knockout in cell types that do not express DGAT2. Unlike hepatocytes, however, these other cell types do not normally synthesize and secrete large amounts of lipids and therefore exhibit different metabolic responses to an exogenous FFA load. For example, accumulation of ceramides has been linked to palmitate lipotoxicity in skeletal muscle [53] and cardiomyocytes [54], but recent studies have shown that palmitate induces apoptosis independently of ceramide synthesis in hepatocytes [10, 25]. Furthermore, cardiomyocytes from *Dgat1*^{-/-} mice exhibited decreased rates of PA oxidation due to downregulation of *Ppar* target genes [29], which was opposite to the effect we observed when knocking down DGAT in hepatocytes. Finally, DGAT1 overexpression protected macrophages against M1 inflammatory activation in response to palmitate treatment, but the mechanism likely involved signaling through toll-like receptor pathways that are important in immune cells but not in hepatocytes [30]. Therefore, it is important to avoid generalization of lipotoxicity mechanisms discovered in other cell types to hepatocytes, and vice versa.

One limitation of our current study is that it did not attempt to identify individual phospholipid species associated with PA-induced lipotoxicity, since all polar lipids eluted together and were not resolved by the TLC method we used for lipid class separation. We were also unable to detect significant changes in the total abundance of phospholipid or diacylglycerol (DAG) species in response to FFA treatments or DGAT knockdown, although the total phospholipid abundance trended downward in siDGAT1/2 cells under all conditions (Supplemental Fig. S3). By applying a more sensitive LC-MS/MS lipid profiling approach, we previously found that DAG and phospholipid species were significantly elevated in PA-treated H4IIEC3 cells [7]. This prior study found that the abundance of saturated phosphatidylcholine and phosphatidic acid species were most significantly altered by PA treatment [7]. Alterations in other phospholipid species have also been proposed as contributing factors for PA-induced lipotoxicity. For example, aberrant formation of phosphatidylglycerol and its decreased conversion into cardiolipin has been proposed to initiate apoptosis in cardiomyocytes [55]. Cardiolipin interacts with cytochrome c within the mitochondrial inner membrane, and inhibition of cardiolipin synthesis may trigger apoptosis as a result of cytochrome c release and mitochondrial dysfunction. However, our previous work demonstrated that markers of ER stress were apparent in PA-treated hepatocytes prior to any indicators of mitochondrial dysfunction [7]. Furthermore, our prior lipid profiling results failed to reveal any significant changes in synthesis or accumulation of phosphatidylglycerol species under these conditions [7]. Further work is necessary to identify individual phospholipid species that could possibly mediate lipotoxic responses to palmitate overload in hepatocytes.

Saturated fatty acids induce lipotoxicity in a variety of cell types and are postulated to be a contributing factor to pathological disorders including metabolic syndrome, insulin resistance and type 2 diabetes [56, 57]. Increasing evidence points toward ER stress as an integral player in both acute lipotoxicity and chronic metabolic disease. Recent literature suggests that not only do obese individuals have chronic underlying levels of ER stress [45, 58-60], but that ER stress pathways are activated in fatty liver and may play an important role in the development and progression of NASH [61, 62]. Aberrant

phospholipid metabolism and composition has been previously postulated as the link between ER stress and lipotoxicity [7, 22, 23, 45]. It is possible that SFAs initiate lipotoxicity through direct perturbations to the ER membrane. Specifically, exogenous PA rapidly incorporates into ER membrane phospholipids, increasing membrane saturation and impairing ER integrity [7, 35]. Interventions intended to prevent palmitate-mediated saturation, such as overexpressing the lysophosphatidylcholine acyltransferase 3 (LPCAT3) enzyme to increase unsaturated fatty acid incorporation in phospholipids [23], have shown beneficial effects toward reducing ER stress. Our study suggests that co-supplementing OA to PA-treated hepatocytes may act in a similar manner, by reversing oversaturation and preventing PA-mediated ER stress and lipotoxicity. Although this hypothesis was not directly tested, it is consistent with our observations. In contrast, our data clearly demonstrate that the effect of OA to increase PA partitioning into neutral TGs is not required to attenuate lipotoxicity in hepatocytes. Overall, these findings suggest that modulating alternative pathways of lipid metabolism, other than TG synthesis, may represent a promising direction for developing novel therapeutic strategies against nonalcoholic fatty liver disease (NAFLD). This is particularly relevant as there are currently no FDA-approved pharmacotherapies for NAFLD or NASH [17].

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6. Disclosures

None relevant to this study.

7. References

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Figure Legends

Figure 1. siRNA mediated knockdown of DGAT1/2 significantly reduced TG accumulation in H4IIEC3 cells and primary rat hepatocytes. Simultaneous siRNA mediated knockdown of DGAT1 and DGAT2 expression (siDGAT1/2) reduced mRNA transcript levels relative to non-targeted control (NTC) siRNA in both H4IIEC3 cells (A) and primary rat hepatocytes (B). siDGAT1/2 cells demonstrated significantly reduced TG accumulation in comparison to NTC cells when supplemented with identical fatty acid loads for 12 h in H4IIEC3 cells (C) and for 24 h in primary rat hepatocytes (D). TGs were separated by thin layer chromatography and analyzed by gas chromatography-flame ionization detection (GC-FID). Amounts were normalized to mg protein for absolute quantification. Data represent mean $\pm$ SE of n=4 replicate qPCR runs on RNA isolated from a single experiment. The results are representative of data obtained from multiple independent experiments. Data for each transcript are normalized to the NTC-BSA condition. *, $p<0.05$, **, $p<0.01$, vs. cells supplemented with the same treatment; ^a, $p<0.05$, vs BSA-control cells with same genetic manipulation (NTC or siDGAT1/2); ^b, $p<0.05$, vs cells with same treatment.

Figure 2. OA-mediated rescue of PA-induced lipoapoptosis was not inhibited by DGAT1/2 knockdown. siDGAT1/2 cells demonstrated statistically identical responses to treatment with 400 μ M PA and/or OA in comparison to BSA-treated controls. Cell death was assessed in H4IIEC3 cells (A) and primary rat hepatocytes (B) by propidium iodide (PI) staining at 12 and 24 h, respectively. Consistent trends were observed in cell apoptosis assessed by caspase 3/7 activity (C) and expression level of cleaved PARP (D, representative blot) in H4IIEC3 cells with 400 μ M of either PA and/or OA at 12 h. “si” refers to siDGAT1/2 knockdown cells with specified treatment. Data represent mean $\pm$ SE of n=4. **, $p<0.01$, vs BSA-control cells with same genetic manipulation (NTC or siDGAT1/2).

Figure 3. PA-induced increases in markers of ER stress were completely prevented by co-treatment with OA regardless of DGAT knockdown. Both NTC and siDGAT1/2 (“si”) in H4IIEC3 cells were treated with 400 μ M PA with or without 400 μ M OA supplementation to determine markers of ER stress. Relative mRNA transcript levels of CHOP (A) and ATF3 (B) were assessed at 6h. Levels of CHOP protein expression were assessed using Western blotting techniques at 8h, a representative blot of which is shown in (C). Thapsigargin (Thaps) was used as a positive control. Data represent mean $\pm$ SE of n=3. **, $p<0.01$, vs BSA-control cells.

Figure 4. Knockdown of DGAT1/2 increases the incorporation of exogenous PA into the phospholipid pool while reducing the amount incorporated into triglycerides. H4IIEC3 cells were treated with 400 μ M 3 H-PA with or without 400 μ M OA supplementation for 12 h in both NTC-vehicle control and siDGAT1/2 cells. Lipid class incorporation was determined using TLC separation and scintillation counting of each lipid spot. PL, phospholipid; DG; diacylglyceride; FFA, free fatty acid; TG, triglyceride; CE, cholesterol ester. Data represent mean $\pm$ SE of n=3. **, $p<0.01$ NTC vs siDGAT1/2 cells with same treatment.

Figure 5. DGAT1/2 knockdown and PA/OA co-treatment independently decrease total PA uptake and PA lipid incorporation and increase the fraction of PA oxidized. H4IIEC3 cells were treated with 400 μ M 3 H-PA with or without 400 μ M OA supplementation for 12 h in both NTC-vehicle control and siDGAT1/2 cells. Total PA uptake (A) was assessed by depletion of protein-bound 3 H-PA from the cell culture medium. PA incorporation into cellular lipids (B) was determined by measuring 3 H enrichment in the lipid fraction of cell extracts. PA oxidation was measured by accumulation of 3 H₂O in the cell culture medium and expressed as an absolute rate (C) or as a fraction of total PA uptake (D). Rates were normalized to mg protein for absolute quantification. Data represent mean $\pm$ SE of n=3. ^a, $p<0.05$, vs

BSA-control cells with same genetic manipulation (NTC or siDGAT1/2); ^b, $p < 0.05$, vs cells with same treatment.

Fatty Acid	NTC PA	NTC PA+OA	NTC BSA	NTC OA	siDGAT1/2 PA	siDGAT1/2 PA+OA	siDGAT1/2 BSA	siDGAT1/2 OA
14:0	0.00 ± 0.00	0.00 ± 0.00	0.89 ± 0.02	0.48 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.69 ± 0.35	0.00 ± 0.00
16:0	48.73 ± 0.62	30.05 ± 0.23 ^a	12.87 ± 0.14 ^a	8.29 ± 0.10 ^a	53.06 ± 2.66 ^b	30.77 ± 0.22 ^a	13.58 ± 0.18 ^a	7.18 ± 0.11 ^a
16:1	8.82 ± 0.24	6.50 ± 0.06 ^a	6.71 ± 0.10 ^a	2.40 ± 0.08 ^a	9.70 ± 0.72	5.37 ± 0.09 ^a	5.88 ± 0.05 ^a	1.47 ± 0.02 ^a
18:0	12.82 ± 0.58	10.75 ± 0.12 ^a	18.75 ± 0.55 ^a	12.64 ± 0.42	11.88 ± 0.77	9.84 ± 0.10 ^a	18.92 ± 0.31 ^a	10.77 ± 0.20
18:1w9	11.06 ± 0.07	34.34 ± 0.40 ^a	28.19 ± 0.74 ^a	58.03 ± 0.05 ^a	12.28 ± 0.30	37.68 ± 0.14 ^{a,b}	30.22 ± 0.66 ^{a,b}	66.40 ± 0.22 ^{a,b}
18:1w7	3.22 ± 0.02	2.83 ± 0.02	8.01 ± 0.42 ^a	4.17 ± 0.08	2.92 ± 0.09	2.97 ± 0.02	7.36 ± 0.20 ^a	4.00 ± 0.05
18:2	1.80 ± 0.08	2.08 ± 0.01	4.08 ± 0.14 ^a	1.75 ± 0.05	1.10 ± 0.55	1.76 ± 0.04 ^a	3.82 ± 0.14 ^a	1.42 ± 0.01 ^a
20:3w6	0.66 ± 0.33	0.96 ± 0.01	1.51 ± 0.03	0.60 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.85 ± 0.42	0.00 ± 0.00
20:4	8.52 ± 0.07	7.73 ± 0.14	10.56 ± 0.03 ^a	6.64 ± 0.07	6.72 ± 0.29	7.30 ± 0.02	10.51 ± 0.15 ^a	5.77 ± 0.11
20:5	0.00 ± 0.00	0.12 ± 0.12	0.34 ± 0.17	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:4w6	0.00 ± 0.00	0.87 ± 0.05	1.38 ± 0.01	0.59 ± 0.01	0.00 ± 0.00	1.03 ± 0.09	1.84 ± 0.05	0.00 ± 0.00
22:5w6	0.00 ± 0.00	0.00 ± 0.00	0.65 ± 0.42	1.05 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.81 ± 0.40	0.51 ± 0.26
22:5w3	2.34 ± 0.09	1.75 ± 0.05	2.83 ± 0.03	1.43 ± 0.01	1.17 ± 0.59	1.73 ± 0.01	2.76 ± 0.04	1.22 ± 0.03
22:6	2.02 ± 0.01	2.01 ± 0.04	3.23 ± 0.01	1.91 ± 0.01	1.74 ± 0.06	1.56 ± 0.03	2.78 ± 0.02	1.25 ± 0.01
% SFA	61.55 ± 0.85	40.80 ± 0.26 ^a	32.52 ± 0.57 ^a	21.42 ± 0.44 ^a	64.94 ± 2.77 ^b	40.62 ± 0.24 ^a	33.18 ± 0.50 ^a	17.96 ± 0.22 ^{a,b}
% UFA	38.45 ± 0.44	59.20 ± 0.45 ^a	67.48 ± 0.98 ^a	78.58 ± 0.15 ^a	35.06 ± 1.16 ^b	59.38 ± 0.20 ^a	66.82 ± 0.93 ^a	82.04 ± 0.36 ^{a,b}

Table 1. Phospholipid fatty acid composition of DGAT1/2 knockdown H4IIEC3 cells treated with PA and/or OA for 12 h (% of total composition). H4IIEC3 cells were incubated with 400 µM PA and/or 400µM OA for 12 h. Phospholipids were separated by TLC and analyzed by GC-FID. Data represent mean fatty acid % ± SE of n=4. ^a, p<0.05 vs cells treated with PA alone and same genetic manipulation (NTC-vehicle or siDGAT1/2); ^b, p<0.05 NTC-vehicle vs siDGAT1/2 cells with same treatment. SFA, saturated fatty acids; UFA, unsaturated fatty acids.

Figure 1. siRNA mediated knockdown of DGAT1/2 significantly reduced TG accumulation in H4IIEC3 cells and primary rat hepatocytes.

A.

B.

C.

D.

Figure 2. OA-mediated rescue of PA-induced lipopapoptosis was not inhibited by DGAT1/2 knockdown.

Figure 3. PA-induced increases in markers of ER stress were completely prevented by co-treatment with OA regardless of DGAT knockdown

Figure 4. Knockdown of DGAT1/2 increases the incorporation of exogenous PA into the phospholipid pool while reducing the amount incorporated into triglycerides.

Figure 5. DGAT1/2 knockdown and PA/OA co-treatment independently decrease total PA uptake and PA lipid incorporation and increase the fraction of PA oxidized.