Original Paper

Differential Lipotoxic Effects of Palmitate and Oleate in Activated Human Hepatic Stellate Cells and Epithelial Hepatoma Cells

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Key Words
Fatty acids • Lipotoxicity • Activated hepatic stellate cells • Hepatoma cells • NAFLD

Abstract
Background/Aims: Nonalcoholic fatty liver disease (NAFLD) progression to fibrosis, cirrhosis and hepatocellular carcinoma, alters the cellular composition of this organ. During late-stage NAFLD, fibrotic and possibly cancerous cells can proliferate and, like normal hepatocytes, are exposed to high concentrations of fatty acids from both surrounding tissue and circulating lipid sources. We hypothesized that primary human activated hepatic stellate cells and epithelial hepatoma (HepG2) cells respond differently to lipotoxic conditions, and investigated the mechanisms involved. Methods: Primary activated hepatic stellate cells and HepG2 cells were exposed to pathophysiological concentrations of fatty acids and comparative studies of lipid metabolic and stress response pathways were performed. Results: Both cell types remained proliferative during exposure to a combination of palmitate plus oleate reflective of the general saturated versus unsaturated fatty acid composition of western diets. However, exposure to either high palmitate or high oleate alone induced cytotoxicity in activated stellate cells, while only palmitate caused cytotoxicity in HepG2 cells. mRNA microarray and biochemical comparisons revealed that stellate cells stored markedly less fatty acids as neutral lipids, and had reduced capacity for beta-oxidation. Similar to previous observations in HepG2 cells, palmitate, but not oleate, induced ER stress and actin stress fiber formation in activated stellate cells. In contrast, oleate, but not palmitate, induced the inflammatory signal TXNIP, decreased cytoskeleton proteins, and decreased cell polarity preceding cell death in activated stellate cells. Conclusions: Palmitate-induced lipotoxicity was associated with ER stress pathways in both primary activated hepatic stellate cells and epithelial hepatoma cells, whereas high oleate caused lipotoxicity only in activated stellate cells, possibly through a distinct mechanism involving disruption of cytoskeleton components. This may have implications for optimal dietary fatty acid compositions during various stages of NAFLD.

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Introduction

Nonalcoholic fatty liver disease (NAFLD), the most common chronic liver disease worldwide, is coincident with visceral obesity, insulin resistance, and hypertriglyceridemia. It encompasses a range of disorders associated with lipid accumulation in hepatocytes, from simple steatosis to nonalcoholic steatohepatitis (NASH) with or without fibrosis, to cirrhosis [1, 2]. NASH occurs in as many as one third of NAFLD cases, and can advance further to cirrhosis and hepatocellular carcinoma in a smaller fraction of cases [2, 3]. All stages of NAFLD, including cirrhosis and hepatocellular carcinoma coincide with ongoing hepatocyte steatosis. NAFLD progression is understood to be partly a consequence of hepatocyte lipotoxicity, which occurs as fatty acid flux to the liver exceeds its ability to oxidize them, to export them into plasma, or to store them safely as triglycerides [4-7]. Hepatocyte injury ensues, promoting hepatic inflammation, hepatic stellate cell activation, and the onset of fibrosis [1, 4, 7, 8]. Thus with disease progression, the cellular composition of the liver shifts toward increased proportions of inflammatory and fibrotic cells, and possibly cancerous cells.

Studies of cultured hepatocytes and hepatocellular carcinoma lines have revealed that exposure to excess saturated fatty acids in particular triggers cellular stress responses that can lead to cell death [9-19]. For these experiments, cells are typically incubated with palmitate (0.2 to 1.0 mM) conjugated to bovine serum albumin (BSA). As the most abundant saturated fatty acid in our diet, these concentrations reflect serum levels observed in obese and metabolic syndrome individuals [20]. High palmitate induces oxidative stress [10-12, 18, 19] and ER stress [9, 15-17, 19, 21, 22] in cultured hepatocytes and hepatoma cells, leading to cell death. Corresponding observations of hepatocyte oxidative and ER stress, and hepatocyte death have been made in several rodent models of NAFLD [10-12, 23-27]. Studies of hepatic lipid composition during NAFLD suggest that, as in cultured hepatocytes, saturated fatty acids are primarily responsible for hepatic lipotoxicity in vivo [12, 24, 27].

In healthy liver, stellate cells comprise approximately 5-15% of total liver-resident cells, and are considered to have a quiescent (non-activated) phenotype [28-30]. Non-activated stellate cells proliferate slowly and synthesize a limited amount of extracellular matrix. Their predominant function is to regulate vitamin A homeostasis through its storage as retinyl palmitate in cytoplasmic lipid droplets, and its mobilization and transport into plasma [31, 32]. However, in response to liver injury, including exposure to excess lipids, lipid metabolites, and inflammatory cytokines released by hepatocytes and Kupffer cells in the setting of NAFLD, stellate cells become activated [33, 34]. They lose the ability to produce and store vitamin A [32, 35], acquire a proliferative myofibroblast-like phenotype, synthesize large amounts of extracellular matrix, are the principle cell type responsible for NASH-associated fibrosis [34, 36, 37], and may contribute to the subsequent development of hepatocellular carcinoma [30].

Based on extensive studies of hepatoma cell cultures, fatty acid conditions that promote lipotoxicity in these cells, and the process itself, are relatively well understood and have been the subject of recent reviews [38, 39]. However, our knowledge of the responses of activated hepatic stellate cells to lipotoxic conditions is limited, despite the abundance of these cells in advanced NAFLD liver, their known contribution to disease progression, and their ongoing exposure to a high lipid environment during all stages of NAFLD. Here, we determined the sensitivity of primary human activated hepatic stellate cells (HSteC) to fatty acid-induced toxicity relative to a commonly used human epithelial hepatoma cell line (HepG2) and performed comparative studies of lipid metabolic and stress response pathways in these cell types. We found that, in contrast to HepG2 cells, HSteC underwent lipotoxicity upon exposure to high concentrations of either palmitate or oleate. Moreover, the predominant cell stress mechanisms leading to cell death appeared to differ between these fatty acids in HSteC.
Materials and Methods

Cell culture and fatty acid treatments

Cryopreserved primary human activated hepatic stellate cells (HSteC) were obtained from ScienCell (Carlsbad, CA) and grown and sub-cultured according to the manufacturer’s recommendations using their proprietary reagents, on poly-L-lysine coated culture dishes. Cells were maintained in SteCM medium containing 5.5 mM glucose, 2% fetal bovine serum (FBS), cell growth supplement (2 ng/ml each of EGF, IGF, and FGF), and penicillin/streptomycin solution (Sciencell). HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in Eagles minimum essential medium (EMEM) (Lonza Biowhittaker) containing 5.5 mM glucose, 10% FBS, 2 mM L-glutamine, and penicillin/streptomycin solution. All cultures were incubated at 37°C and 5% CO₂. For fatty acid treatments, SteCM and EMEM complete growth media were supplemented with 1.0 mM palmitate, or 1.0 mM oleate, or a combination of palmitate and oleate (2:3 ratio, to achieve a total concentration of 1.0 mM fatty acids). All fatty acids were complexed to bovine serum albumin (BSA) at a molar ratio of 2:1 prior to addition to culture medium, as previously described [40]. BSA supplemented medium was used for all control conditions. The concentration of fatty acids used reflects pathological circulating fatty acid levels (from both free and triglyceride-rich lipoprotein derived sources) observed in obesity and metabolic syndrome [20] and NAFLD [41].

Cell proliferation and cell viability

To determine population growth rates, cells were seeded at known densities and incubated under control or high fatty acid conditions for up to 48 h, as indicated. Cells were harvested at the indicated time points and counted with a hemocytometer. Population doublings were calculated using the following equation: log₂(number of cells harvested) – log₂(number of cells seeded) × (log₂2)⁻¹. To determine cell viability, cells were incubated for 48 h under control or high fatty acid conditions followed by staining with annexin V (AnnV) and propidium iodide (PI) (Molecular Probes). Stained cells were analyzed by flow cytometry (FACS Calibur). For subsequent quadrant analyses, apoptotic cells were defined as AnnV positive and PI negative, indicating intact plasma membranes. Dead cells were defined as AnnV positive and PI positive plus PI positive alone, indicating compromised plasma membrane integrity. Live cells were defined as negative for both AnnV and PI.

Fatty acid uptake

Initial rates of palmitate and oleate uptake were determined by incubating near confluent HSteC for 1 min with growth media containing either 1.0 mM palmitate with [9,10-³H(N)]palmitic acid (Perkin Elmer) or 1.0 mM oleate with [1-¹¹C]oleate (Perkin Elmer) at equivalent specific activities (determined for each lot of radioisotope). Reactions were stopped by incubation on ice with PBS containing phloretin plus BSA, followed by three washes with PBS containing phloretin alone. Cell monolayers were solubilized using 0.1 N sodium hydroxide, and radioactivity was quantified by liquid scintillation counting.

mRNA microarray

For mRNA microarray analyses, RNA was prepared as previously described [42]. All sample labeling and GeneChip (Human Gene 2.0 ST arrays) processing was performed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca), as previously described [42]. Microarray file data was imported into Partek Genomics Suite. Statistically significant differences between HSteC and HepG2 samples were determined by ANOVA (p < 0.01), and data were filtered for changes in expression exceeding two-fold. Gene ontology (GO) category over-representation analyses and KEGG pathway analyses were performed on lists of down- and up-regulated transcripts using Expression Analysis Systematic Explorer (EASE). All primers, reagents, and protocols were from Applied Biosystems.

qRT-PCR

Selected changes in gene expression determined by mRNA microarray analyses were independently confirmed by qRT-PCR using TaqMan assays for CPT1A (Hs00912671_m1), CPT1C (Hs00380581_m1), ACOX1 (Hs01074241_m1), LPIN1 (Hs00299515_m1), DGAT1 (Hs01017541_m1), and DGAT2 (Hs01045913_m1). Following 6 h and 24 h incubations with fatty acid supplemented media, HSteC gene expression was determined by qRT-PCR using TaqMan assays for CHOP (Hs00358796_g1), and TXNIP (Hs01006900_g1). For all reactions, GAPDH was used as an endogenous control. All assays and protocols were from Applied Biosystems.
Fatty acid oxidation

Palmitate oxidation was assessed by conversion of palmitate to H$_2$O, as previously described [43], with minor modifications. Near confluent cells were incubated for 1 h with growth medium containing 2.0 μCi/ml [9,10-3H(N)]palmitic acid (Perkin Elmer) in 100 μM palmitate conjugated to BSA at a molar ratio of 2:1. Media was collected, protein was precipitated with 10% trichloroacetic acid, and unreacted fatty acids were removed by repeated extraction with hexane. Radiolabeled aqueous fractions were quantified by liquid scintillation counting.

Cellular lipid droplet and F-actin staining

Accumulation of cytosolic neutral lipid droplets was assessed by staining HSteC grown on glass coverslips with 0.3% Oil Red O (Sigma) in isopropanol and PBS. To visualize polymerized actin (F-actin), cells were stained with FITC-phalloidin (Molecular Probes). All coverslips were affixed to glass slides with mounting medium containing DAPI (ProLong Gold Antifade Reagent, Molecular Probes). Cells were imaged by fluorescence and light microscopy on an Olympus BX51 microscope, and images were analysed using ImageJ. Thresholds for Oil Red O and FITC-phalloidin staining were applied consistently across all experiments to eliminate false positive signals. Lipid droplet content was quantified by measurement of red (Oil Red O) and blue (DAPI) pixel densities. F-actin content was quantified by measurement of total green signal per number of nuclei per field of view. Cell lengths were measured on FITC-phalloidin stained images using ImageJ. To calculate average cell length per field of view, 10 to 25 cells per image were measured across the maximum length of the cell, as delimited by positive actin staining. Only cells with clearly visible boundaries were measured to ensure accurate length determinations.

Cellular lipid mass

Quantification of cellular lipids was performed using a modification of a previously established method [44]. Total lipids were extracted from cells with hexane/isopropanol (3:2, v:v), evaporated to dryness, and resuspended in water with 0.5% triton. Triglyceride, total cholesterol, and free cholesterol masses were determined using enzymatic colourimetric assays (Roche and WAKO), and normalized to total cellular protein. Cholesteryl ester masses were determined by subtraction of free cholesterol values from those for total cholesterol.

Immunoblotting

Whole cell lysates were prepared using RIPA buffer containing protease and phosphatase inhibitors. Lysates were resolved under reducing conditions by 10% SDS-PAGE and transferred to PVDF membranes. Phospho-eIF2α, total eIF2α and smooth muscle alpha actin (αSMA) were detected with mouse monoclonal antibodies (Cell Signaling Technology and Dako, respectively). Vimentin was detected with a rabbit polyclonal antibody (Cell Signaling Technology). GAPDH (Enzo Life Sciences) was used as a loading control. Secondary antibodies were HRP-conjugated (Santa Cruz Biotechnology). Bands were visualized by chemiluminescence, and those corresponding to eIF2α, αSMA, vimentin, and GAPDH consistently appeared at 38 kD, 42 kD, 57 kD and 36 kD, respectively. Band intensities were quantified using Quantity One (Biorad).

Statistics

With the exception of microarray analyses, all statistical analyses were performed in GraphPad Prism using Student’s t-test, or ANOVA followed by Bonferroni post hoc tests comparing all groups, as indicated. For post hoc tests following ANOVA, values with different letters are significantly different at p < 0.05, while those that share the same letter are not significantly different. Lower case, capital, and Greek letters indicate separate statistical analyses that are not comparable.

Results

Both palmitate and oleate alone, but not the combination, cause cell death in primary human activated hepatic stellate cells

The fatty acid and lipid overload conditions that induce cytotoxicity in hepatoma cell lines are generally well defined [38, 39]. However, this is not the case for hepatic stellate
cells, despite their abundance in liver tissue and known contribution to the progression of NAFLD. To address this issue, we measured cell proliferation and cell death in primary human activated hepatic stellate cells (HSteC) compared to HepG2 cells during exposure to excess fatty acids. Both cell types were incubated for up to 48 h with palmitate or oleate, or a 2:3 combination of palmitate plus oleate (PA+OA), complexed to BSA and prepared to total final concentrations of 1.0 mM in growth media. Interestingly, both HSteC and HepG2 cells remained proliferative during exposure to the combination of palmitate plus oleate, despite the high total concentration of fatty acids (Fig. 1A, B). HSteC, in particular, exhibited enhanced proliferation in palmitate plus oleate conditions – an observation which neared statistical significance (p = 0.09) when compared to BSA alone. In contrast, HSteC population doublings were decreased to the same extent after 48 h exposure to either palmitate or oleate alone (Fig. 1A). HepG2 cell proliferation was inhibited only by exposure to palmitate (Fig 1B). Consistent with these cell proliferation data, flow cytometric analyses of propidium iodide and annexin V staining showed that HSteC underwent lipotoxic cell death to approximately the same extent in the presence of either palmitate or oleate alone, while HepG2 cells were
only affected by palmitate (Fig. 1C, D). Apoptosis was significantly increased at this time point only in HSteC exposed to high oleate (Fig. 1C). The cell viability data for HSteC were particularly interesting since we found that the initial uptake of $^3$H-palmitate was actually higher than that of $^3$H-oleate (2.9 ± 0.1 versus 2.3 ± 0.1 nmol/mg cell protein/min, p < 0.02) in these cells. No significant cell death was observed in response to fatty acid excess in either cell type at 24 h.

Lipid metabolic pathways are differentially active in HSteC compared to HepG2 cells

We next took an unbiased approach to identify genes and pathways that are differentially expressed between HSteC and HepG2 cells, and that may underlie the divergent lipotoxic responses to palmitate and oleate in these liver cell types. Using mRNA microarray expression analyses we identified a list of 6786 transcripts (approximately 23.5% of genes represented on the microarray) that were differentially expressed at greater than 2-fold, p < 0.01. Of this list, 3387 were downregulated and 3399 were upregulated in HSteC compared to HepG2 cells. As expected based on the known functions of activated hepatic stellate cells versus hepatocytes, the top 20 upregulated genes included several related to cell polarity and regulation of extracellular matrix (e.g. PRR5L, STUB1, POLR2L, BMP2), while the top 20 downregulated genes included several known hepatocyte products (e.g. APOH, AFP, APOB, ALB) (Table 1). Also consistent with the cell types compared, the 20 gene ontology (GO) categories with the highest gene representation included groups related to regulation of cell proliferation, migration and extracellular matrix (8 of 20 GO categories), and to regulation of lipid and lipoprotein metabolism (4 of 20 GO categories) (Table 1). Interestingly, the top 20 KEGG pathways included 10 gene groups related to intermediary glucose and lipid metabolism (Table 1), suggesting that despite potentially existing within the same diseased tissue, and being exposed to the same lipid sources, fuel substrates may be handled quite differently in activated hepatic stellate cells compared to hepatoma cells.

This possibility was first confirmed by measuring relative mRNA contents for key enzymes of fatty acid $\beta$-oxidation (CPT1, ACOX1) and triglyceride synthesis (LPIN1, DGAT1, DGAT2) which were identified on the 2-fold differentially expressed gene list, followed by functional measurements of lipid metabolism. We found that although CPT1A was decreased in HSteC compared to HepG2 cells, this was likely compensated for by increased expression of the alternate isoform CPT1C (Fig. 2A). However, ACOX1, the enzyme responsible for the rate-limiting initial reaction of $\beta$-oxidation, was 10-fold down-regulated in HSteC (Fig. 2A),
which corresponded to a 45% reduction in fatty acid oxidation compared to HepG2 cells (Fig. 2B). We further found that \( \text{LPIN1} \), which catalyses the penultimate reaction of triglyceride synthesis, was increased in HSteC. But, both \( \text{DGAT} \) isoforms were greatly decreased suggesting a limited capacity for triglyceride synthesis in activated hepatic stellate cells (Fig. 2A). Measurements of cellular neutral lipid droplet and lipid mass accumulation in HSteC and HepG2 under lipotoxic conditions supported this possibility. Oil Red O staining of HSteC exposed to 1.0 mM fatty acids for 6 h revealed significant accumulation of neutral lipid droplets, particularly in the presence of oleate (Fig. 3A, B) as has been observed previously in
other cell types. Lipid mass measurements were consistent with our histochemical staining, indicating that HSteC exposed to oleate accumulated significantly more triglyceride than those exposed to palmitate (Fig. 3C). This was not the case in HepG2 cells which accumulated similar amounts of triglyceride in the presence of either fatty acid, and accumulated 5- to 10-fold more triglyceride than HSteC (Fig. 3C). No significant changes in cholesteryl ester or free cholesterol content were observed in either cell type after 6 h exposure to excess palmitate or oleate (Fig. 3D, E).

Palmitate, but not oleate, induces ER stress preceding cell death in HSteC

We and others have previously shown that ER stress contributes to cell death in hepatocytes exposed to high concentrations (> 0.5 mM) of saturated fatty acids [45, 46]. But this is not the case for oleate which, even at high concentrations (> 0.5 mM), can promote efficient incorporation of fatty acids into triglyceride-rich lipoproteins (in part through increased MTP expression) for export by hepatocytes and hepatocyte-derived cell lines, thereby channeling excess lipid away from toxic pathways [47-49]. Since HSteC, unlike HepG2 cells, cannot assemble and secrete triglyceride-rich lipoproteins, we postulated that both palmitate and oleate would induce ER stress in HSteC, as a result of increased lipid synthetic burden at the ER. Interestingly, this did not appear to be the case. We first confirmed that the classical inducer of ER stress, tunicamycin, resulted in rapid, robust, and sustained phosphorylation of eIF2α in HSteC, suggesting that these cells have a functional
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Fig. 4. Changes in intracellular signaling pathways in response to fatty acids in HSteC. (A) Cells were incubated for 6 h with fatty acid-supplemented medium and sampled for analysis. Values are means ± SEM, n = 4-5. (B) Palmitate (PA) rapidly induces eIF2α phosphorylation in HepG2 cells (Fig. 4A), as shown by Western blotting. (C) Oleate (OA) does not induce eIF2α phosphorylation in HepG2 cells (Fig. 4A). (D) Palmitate (PA) and oleate (OA) induce apoptosis in HSteC, with the combination of PA and OA resulting in the highest level of apoptosis. (E) TXNIP expression was increased in response to the combination of PA and OA, as shown by Western blotting. Representative blots are shown. All data are means ± SEM, for n = 4-5. Statistically significant differences were determined by ANOVA followed by post hoc tests comparing all groups. Values with different letters are significantly different at p < 0.05, while those that share the same letter are not significantly different. Lower case and capital letters indicate separate statistical analyses that are not comparable.

Fig. 5. Changes in cytoskeleton components and morphology of HSteC upon exposure to palmitate or oleate. (A) Cells grown on glass coverslips were incubated for 6 h with growth media containing palmitate (PA), oleate (OA), or a 2:3 combination of palmitate plus oleate (PA+OA), complexed to BSA. Total concentrations of fatty acids were 1.0 mM for all treatments. Medium supplemented with BSA alone was used as the control. Cells were fixed using paraformaldehyde, lipid droplets were visualized using Oil Red O (ORO) to stain neutral lipids (red), and polymerized actin (F-actin) was visualized using FITC-phalloidin (green). Nuclei were counterstained with DAPI (blue). Representative micrographs are shown. White arrows indicate lipid droplet-rich cells with less polarized morphology. White outline indicates an enlarged inset of a lipid droplet rich region. Scale bar represents 100 μm. (B) F-actin content was quantified by measuring FITC signal relative to the number of nuclei per field of view using ImageJ software. (C) Average cell length per field of view was measured using Image J. (D, E) Cells were incubated for 6 h (D) or 16 h (E) with growth media containing fatty acids as described for A. Smooth muscle alpha actin (αSMA) and vimentin in whole cell lysates were detected by immunoblotting. Bands were quantified by densitometry and normalized to GAPDH. Representative blots are shown. All data are means ± SEM, for n = 4-5. Statistically significant differences were determined by ANOVA followed by post hoc tests comparing all groups. Values with different letters are significantly different at p < 0.05, while those that share the same letter are not significantly different. Lower case and capital letters indicate separate statistical analyses that are not comparable.

unfolded protein response (UPR) (Fig. 4A). We also confirmed, as has been shown by others [47], that palmitate rapidly induces eIF2α phosphorylation in HepG2 cells (Fig. 4A). However, only exposure to palmitate resulted in phosphorylation of eIF2α in HSteC (Fig. 4B, C). In order to determine a potential mechanism for fatty acid induced cell death in HSteC, we examined aspects of signalling downstream of eIF2α phosphorylation that are known to be linked to the ER stress response, as opposed to the unfolded protein response (UPR). Palmitate stimulated transient expression of the apoptosis inducer, CHOP, in HSteC (Fig. 4D). In contrast, exposure to oleate decreased CHOP expression, and increased expression of TXNIP (Fig. 4E). TXNIP (thioredoxin-interacting protein) can be induced by activation of either PERK or IRE1 during ER stress, and can mediate inflammation and/or cell death under these conditions [50]. Interestingly, TXNIP was also induced in response to the combination of palmitate plus oleate (Fig. 4E), conditions which did not result in cell death (Fig. 1A, C).
Oleate, but not palmitate, decreases cytoskeleton proteins preceding cell death in HSteC

Given the much greater triglyceride accumulation in HSteC exposed to oleate compared to those exposed to palmitate (Fig. 3A-C), and the fact that actin stress fiber formation is an early response to lipotoxic conditions in fibroblast cell types [51], we next proposed that the oleate-induced accumulation of cytosolic lipid droplets might lead to cell death by disrupting the cytoskeleton of this highly polarized, migratory cell type. In HSteC exposed to palmitate for 6 h, we observed a significant increase in polymerized actin (F-actin) (Fig. 5A, B), a lipotoxic response which we previously observed in CHO fibroblasts [51]. This increased stress fiber formation was not evident in HSteC exposed to oleate (Fig. 5A, B). In fact, prominent F-actin fibres appeared to be diminished in cell regions with an abundance of lipid droplets (Fig. 5A, OA inset), and highly polarized morphology decreased (as indicated by decreased average cell lengths) in HSteC upon exposure to oleate (Fig. 5A, C). Cellular protein contents of the cytoskeletal components smooth muscle alpha actin (αSMA) and vimentin, which are established markers of HSteC activation [28, 29], were unchanged after 6 h of exposure to either palmitate or oleate (Fig. 5D). However, after 16 h of exposure to oleate, αSMA and vimentin were decreased by 48% and 38% respectively (Fig. 5E), suggesting that excess oleate, but not palmitate disrupts the cytoskeleton of activated HSteC preceding cell death.

Discussion

As NAFLD progresses to end-stage liver diseases, including fibrosis, cirrhosis and hepatocellular carcinoma, fibrotic activated stellate cells and epithelial cancerous cells can become abundant, changing the cellular composition of this organ. Yet despite chronic exposure of the liver as a whole, at all stages of NAFLD, to high concentrations of fatty acids, the responses of activated hepatic stellate cells to lipid overload are not well characterized. Also, direct comparisons of lipid metabolic and lipotoxic response pathways between activated hepatic stellate cells and hepatocellular carcinoma cells have not been reported. Based on the location of stellate cells, between hepatocytes or hepatocellular carcinoma cells, and endothelial cells [30, 36], we postulated that they would be exposed to lipotoxic conditions during all stages of NAFLD, but may respond differently than hepatocytes or hepatocyte-derived cancer cells. We discovered that both primary human activated hepatic stellate cells (HSteC) and HepG2 hepatoma cells remained proliferative during exposure to a combination of high palmitate plus high oleate, reflective of the general saturated versus unsaturated fatty

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**Fig. 6.** Working model for the acute lipotoxic effects of palmitate and oleate in HSteC versus HepG2 cells. (A) In HSteC, both high oleate alone and high palmitate alone cause cell death, but do so through different mechanisms. Palmitate, but not oleate, induces ER stress and actin stress fiber formation (green lines). In contrast, oleate, but not palmitate, dramatically increases cytosolic lipid droplets (CLD, red circles), and decreases cytoskeleton proteins and cell polarity preceding cell death. (B) In HepG2 cells, only high palmitate induces sufficient ER stress to cause cell death. In contrast, high oleate increases CLD and promotes lipoprotein (Lp) assembly and secretion, thereby channeling excess fatty acids away from pathways that lead to cell death.
acid composition of western diets. HSteC in particular, exhibited a trend toward increased proliferation under these conditions, which is intriguing given the key role of this cell type in NAFLD progression. We further found that HSteC, in contrast to HepG2 hepatoma cells, undergo lipotoxicity in response to high concentrations of either palmitate or oleate alone, and that HSteC stress responses preceding cell death are different for palmitate compared to oleate (Fig. 6).

Our comparisons of HSteC and HepG2 cells suggest that they handle lipid quite differently, which is consistent with the known divergent behaviours of these cell types. Both gene ontology and metabolic pathway analyses of mRNA microarray data revealed highly significant differences in gene groups involved in intermediary glucose and lipid metabolism, and in the regulation of lipid and lipoprotein metabolism. Subsequent functional analyses of lipid metabolism were consistent with our gene expression data, indicating that primary activated HSteC have limited capacities for β-oxidation, and for triglyceride synthesis and storage of excess fatty acids. Interestingly, these characteristics conferred increased susceptibility to oleate- but not palmitate-induced cell death, compared to HepG2 cells (Fig. 6). Although the process of hepatic stellate cell activation involves downregulation of retinyl palmitate synthetic activity [35], it is possible that these cells retain some capacity to metabolize palmitate through this pathway. This would allow activated HSteC an additional route for the clearance of excess palmitate, possibly preventing additional toxicity of this fatty acid in comparison to HepG2 cells despite their reduced capacity for palmitate oxidation.

The induction of ER stress that we observed in activated HSteC upon exposure to high palmitate is consistent with saturated fatty acid-induced lipotoxic responses observed in many other cell types, including hepatocytes and hepatoma cell lines [45, 46]. High oleate can also induce ER stress and the UPR in hepatocytes [52]. But, as we observed here and in our previous studies [45], oleate does not cause sufficient cell stress to induce cell death in cultured hepatocytes and hepatoma cells [13, 17, 18, 53-57]. Remarkably, this does not appear to be the case for activated HSteC.

The observation of oleate-induced death in HSteC is intriguing, as other fibroblast cell types are known to be relatively resistant to oleate [58-60]. However, HSteC have an extended, polarized morphology and are highly migratory [61] – characteristics which are not likely compatible with abundant cytosolic lipid droplets. In fact, lipid droplet accumulation is known to significantly impair the migration of mesangial cells [62], the mesenchymal cells of the kidney which contribute to extracellular matrix deposition and glomerulosclerosis during diabetic nephropathy [63]. Our observations of disrupted actin stress fiber formation, decreased cell length, and decreased cytoskeletal proteins in HSteC exposed to high oleate, are consistent with the possibility that excessive lipid droplet accumulation is part of the mechanism of lipotoxic cell death in these cells (Fig. 6A). It remains to be determined whether HSteC exposed to high oleate undergo anoikis (cell detachment-mediated apoptosis) as a result of lipid droplet interference with cytoskeleton function, but our observation of increased apoptotic HSteC under this condition supports this possibility.

Exposure to conditions that included high oleate, either alone or in combination with palmitate, induced TXNIP (thioredoxin-interacting protein) expression in HSteC. Upregulation of this factor has been shown to mediate programmed cell death in response to ER stress in pancreatic β-cells, through initiation of the NLRP3 inflammasome [64, 65]. However, this appears not to be the case in activated HSteC, since TXNIP was induced to the same extent upon exposure to high fatty acid conditions which resulted in cell death (oleate alone), or in cell survival and sustained proliferation (oleate plus palmitate). In agreement with the work of Chen et al. [66], these data do not implicate TXNIP in lipotoxic cell death. However, they may have significance for understanding NAFLD progression in vivo. HSteC proliferation, and the inflammatory responses that they promote, contribute extensively to the progression of NAFLD [67]. Since the combination of palmitate plus oleate we used represents the fatty acid composition of western diets, HSteC proliferation and increased expression of an inflammasome activator (TXNIP) in response to these conditions is plausible. Interestingly, palmitate plus oleate has also recently been shown to promote
inflammatory cytokine expression in HepG2 cells by interfering with FGF21 signalling [68]. Whether TXNIP participates in this process may warrant further investigation [69].

The recently recognized potential for effective nutritional therapies for NAFLD is encouraging [70]. It is tempting to speculate, based on the differential effects of palmitate and oleate we observed in these distinct liver-derived cell types, that dietary fatty acid composition could be optimized to limit disease progression, even at later stages of NAFLD when the tissue composition of the liver may be shifted towards fibrotic, or even cancerous cell types. In support of this general concept, studies of mice with carbon tetrachloride-induced liver injury have shown decreased fibrosis in animals maintained on a high oleate diet [71]. The response of activated hepatic stellate cells and hepatocellular carcinomas in vivo to diets with altered fatty acid compositions during the progression of NAFLD warrants further investigation.

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Disclosure Statement

The authors have no competing financial interests to declare.

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