

**DIFFERENTIAL EFFECTS OF FATTY ACIDS ON AN IN VITRO MODEL OF
HEPATOCTE STEATOSIS**

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ABSTRACT

Differential Effects of Fatty Acids on an In Vitro Model of Hepatocyte Steatosis

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Introduction: Non-alcoholic fatty liver disease (NAFLD) is characterized by excess hepatic fat accumulation in the setting of metabolic syndrome. It ranges from simple hepatic fat accumulation (steatosis), to steatosis with inflammation and fibrosis (steatohepatitis), to end-stage liver disease. Progression of NAFLD has been modeled by a 2 hit hypothesis. The first hit, characterized by triglyceride accumulation, sensitizes the hepatocyte to second hits such as inflammation. Dietary fats are an important contributor to the development of NAFLD. While saturated fatty acids (SFA) tend to promote NAFLD, poly-unsaturated fatty acids (PUFA) and ruminant *trans*-fatty acids (TFA), such as conjugated linoleic acid (CLA) may be protective. This study aims to investigate the effects of various fatty acids (FA) on hepatocyte triglyceride accumulation, inflammation and insulin sensitivity using an *in vitro* model.

Methods: AML12 hepatocytes were chronically exposed to FAs including Palmitic acid (PA), Oleic acid (OA), Docosahexanoic Acid (DHA) and CLA *t10, c12* (CLA 10,12). Hepatic triglyceride content, inflammatory, lipogenic, oxidative stress and ER stress gene expression and insulin signaling proteins were measured.

Results: AML12 cells chronically exposed to FA visibly accumulated lipid droplets. Among the various FA treatments, CLA (10,12) induced the greatest triglyceride accumulation. DHA significantly decreased the expression of the lipogenic, inflammatory, oxidative and ER stress genes independent of

effects on insulin sensitivity. Interestingly, CLA (10,12) had protective effects on gene expression similar to DHA.

Conclusion: Chronic exposure of dietary FA promotes triglyceride accumulation in this *in vitro* hepatocyte model. DHA and CLA (10,12) have beneficial effects on lipogenesis and inflammatory, oxidative stress and ER stress gene expression despite increasing triglyceride accumulation, indicating that different FA have varying effects on steatotic hepatocyte.

INTRODUCTION

Obesity, driven by an imbalance between energy intake and energy expenditure, has reached pandemic proportions, especially in developed countries like the United States (1). Excess adipose tissue increases the risk for chronic metabolic diseases such as type 2 diabetes, hypertension, and cardiovascular disease (2). In obesity, chronic exposure of tissues to elevated concentrations of lipids and other nutrients may contribute to tissue dysfunction and disease development (3). In this setting, ectopic fat accumulation in the liver results in the condition referred to as non-alcoholic fatty liver disease (NAFLD). NAFLD has become one of the most common causes of chronic liver diseases worldwide (4, 5). The prevalence of NAFLD is increasing in parallel with the ever-growing epidemic of obesity. Recent evidence suggests that NAFLD is an emerging risk factor for type 2 diabetes and CVD, although mechanisms linking these conditions are unclear (6-8).

NAFLD includes a spectrum of liver damage ranging from simple fat accumulation (steatosis) to steatosis with inflammation (Non-Alcoholic SteatoHepatitis - NASH), fibrosis, cirrhosis and hepatocellular carcinoma (4, 6, 9). A 'two-hit' model has been proposed to explain the progression of NAFLD to NASH. The primary hit involves increased triglyceride accumulation (TG) in the hepatocyte (simple steatosis), attributable to increased de novo lipogenesis, increased fatty acid (FA) substrate availability and storage, decreased FA oxidation and reduced VLDL secretion. However a second hit is often required for the development of inflammation and fibrosis, leading to NASH. Contributors to the progression include genetic factors, dietary factors and/or environmental stressors such as oxidative stress (6, 9).

The etiological factors that contribute to progressive inflammation and fibrosis in NAFLD remain poorly understood. Increased circulating FA released from obese adipose tissue in the setting of insulin resistance, can be oxidized to generate ATP, esterified into TG that are stored in the hepatocyte

or released within VLDL particles. Since the liver is not a physiological site for lipid storage, defects in any one of these pathways may lead to increased lipid accumulation and disrupt hepatic cellular function, as occurs in NAFLD (6, 9). Insulin resistance is thought to play a central role in NAFLD pathophysiology. Excess FA flux into the liver and its accumulation disrupts the pathways involved in insulin receptor translocation, leading to decreased hepatic insulin sensitivity. Insulin also fails to inhibit glucose production, lipogenesis and VLDL synthesis in a fatty liver contributing to abnormal hepatic lipid metabolism, increased lipid accumulation and disrupted hepatic cellular function. Thus insulin resistance acts as both a cause and a consequence of excess lipid accumulation in the liver (5, 6, 10). Increased circulating FAs contribute to dysregulated hepatic lipid metabolism by increasing oxidative and endoplasmic reticulum (ER) stress. Excess FA may lead to increased FA oxidation, an important source of reactive oxygen species (ROS) in the liver. Increased ROS production and oxidative stress lead to DNA damage, alterations in protein stability, lipid peroxidation and release of pro-inflammatory cytokines. Oxidative stress also impairs mitochondrial function which further leads to increased ROS production, as is seen in NAFLD/NASH patients. ER stress is also implicated in hepatic steatosis since it could dysregulate transcription factors involved in hepatic lipid metabolism. Inflammatory cells such as macrophages and Kupffer cells and inflammatory mediators such as CRP, TNF- α , IL-6 and IL-1, are believed to play a vital role in the pathogenesis of NAFLD as well. (11-13).

Although no specific guidelines exist for treatment of NAFLD, current recommendations are aimed at reducing body weight due to its strong association with obesity and metabolic syndrome. In this regard, excess calorie consumption (regardless of the macronutrient composition) is important in regulating body weight, metabolism and liver fat accumulation. Dietary FA composition also plays a vital role in hepatic lipid metabolism and therefore NAFLD pathophysiology (5).

Saturated fatty acids (SFA), which have been linked with metabolic syndrome, diabetes and CVD are now implicated in NAFLD as well (5). Studies in rodents have shown that *in vivo* exposure of hepatocytes to SFA lead to lipotoxicity, liver injury, apoptosis and steatosis. The extent of fat accumulation was comparable to that observed in livers of patients with NAFLD (14). Following a low fat diet, with less than 7% of energy from SFA, for just 4 weeks was associated with a significant decrease in liver fat in humans (15). On the other hand, n-3 polyunsaturated fatty acids (PUFA) enhance beta-oxidation, inhibit hepatic glycolysis and lipogenesis and therefore decrease liver fat (16, 17) (18). Xu et al showed that n-3 PUFA diet was effective in reducing hepatic steatosis as well as total TG and total cholesterol contents in rodent liver, compared to a SFA diet. The n-3 PUFA diet also significantly elevated hepatic antioxidant defense capacities (19). There are several clinical trials investigating the effects of n-3 PUFAs in NAFLD and NASH therapy. The trials have shown that n-3 PUFA supplementation decreases liver fat content and improves hyperlipidemia in patients with NAFLD (20-22).

Trans- fatty acids (TFA) which are unsaturated fatty acids mostly derived from industrial partial hydrogenation of vegetable oils, may adversely affect lipid and glucose metabolism (23). However, there is evidence for beneficial effects of conjugated linoleic acid (CLA), a naturally formed TFA in ruminant animals, on NAFLD. Thus there is growing interest in CLA due to its potential antiobesogenic, anticarcinogenic, antiatherogenic and immune enhancing properties. CLA exists as many different isomers; 16 have been identified so far (24-26). Some studies show that CLA increases liver fat accumulation and promotes hepatic steatosis (27) while others show protective effects of CLA on NAFLD (23, 28, 29).

Overall, dietary FAs have differential effects on liver fat accumulation.

The mechanisms by which various FA aid the development and progression of hepatic steatosis remain to be clarified. We hypothesized that DHA, a n-3 PUFA improves the metabolic profile of hepatocytes in the setting of steatosis while CLA (10,12), a TFA worsens it. Using an *in-vitro* model of chronic FA exposure we show that DHA and CLA (10,12) have beneficial metabolic effects on AML12 cells despite leading to increased TG accumulation.

MATERIALS AND METHODS

Cells and Reagents

AML12 mouse hepatocyte cells were obtained from ATCC. Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo-Fisher Scientific, Waltham, MA. Palmitic acid (C16:0 - PA), oleic acid (18:1 ω -9 - OA), docosahexanoic acid (22:6 ω -3 - DHA) and conjugated linoleic acid (*c9 t11* - CLA (9,11) and *t10 c12* - CLA (10,12)) were from Sigma-Aldrich, St. Louis, MO. Akt and p-Akt antibodies were obtained from Cell Signaling Technology, Inc, Danvers, MA.

Fatty acid Treatments

Cells were seeded at density 40,000 cells/well in 6 well plates and cultured in DMEM-Ham F-12 medium supplemented with 10% FBS, 5 μ g/ml ITS, dexamethasone (1×10^{-7} M), penicillin (100 unit/ml) and streptomycin (100 μ g/ml) for 48 hours. PA, OA, DHA and CLA were prepared by conjugation with bovine serum albumin as described previously (30). Cells at 50% confluency were serum starved for 24 hours and followed by exposure to various FA alone or in combination at 250 μ M concentration. Media and FA were replaced every day and cells were harvested on day 5 for analysis.

Oil-Red-O staining

To assess intracellular neutral lipid, FA treated cells were fixed using 10% formalin and stained using Oil-Red-O (Sigma Aldrich, St. Louis, MO) for 15 minutes at room temperature followed by counter staining with hematoxylin (Vector Laboratories, Burlingame, CA) for 30 seconds. Stained cells were rinsed with 60% isopropanol and water and differences in lipid accumulation were visualized identified using microscopy.

Cell Triglyceride Content analysis

AML12 cells were harvested using phosphate buffer (containing 1% cholic acid and 0.1% triton X-100). Protein extraction was performed from cell homogenates in buffer containing protease and phosphatase inhibitors according to standard techniques. Protein was quantified using a protein assay kit (Thermo-Fisher Scientific) and cellular TG content was analyzed using a colorimetric TG assay kit (Stanbio Laboratory, Boerne, TX).

Real time PCR

Total RNA was isolated from harvested cells using mRNA isolation kit (QIAGEN, Valencia, CA). After spectroscopic quantification, 2 μ g of RNA was reverse-transcribed. cDNA thus obtained was analyzed for gene expression with PCR primers and fluorescein amidite (FAM) probes (Life Technologies, Carlsbad, CA) in the ABI 7500 instrument. The relative amount of target gene was calculated using the $\Delta\Delta$ Ct formula and *Gapdh* as the housekeeping gene.

Western Blot Analysis

On the day of harvest, cells were stimulated with 100nmol of insulin for 15 mins. Equal amounts of total cellular proteins were separated by SDS-PAGE. Phosphorylation status of akt was analyzed by western blot using standard protocols and appropriate antibodies.

Statistical Analysis:

Data was analyzed using GraphPad Prism 3 (GraphPad Software Inc., La Jolla, CA) and are presented as means and standard errors of at least 3 independent experiments performed in triplicate. ANOVA with Bonferroni post hoc testing was used to detect differences among groups. $P < 0.05$ was considered statistically significant.

RESULTS

An in vitro model of hepatic steatosis is established using AML12 hepatocytes

AML12 cells are widely used as an *in vitro* hepatocyte model, as they display morphological and gene expression patterns characteristic of differentiated hepatocytes (31). However, most studies *in vitro* have employed short-term exposure to FA of 6 - 24h (32-34). To mimic conditions of chronic FA exposure as occurs in, *in vivo*, AML12 cells were treated with continued exposure to FA for 72 – 96 h. Oil-Red-O staining revealed that lipid droplet accumulation occurred with exposure to all FA (Fig 1A panels i-vi). Thus we were able to mimic an *in vivo* model of hepatic steatosis by chronic exposure to FA.

Effects of chronic DHA exposure on AML12 cells, *in vitro*

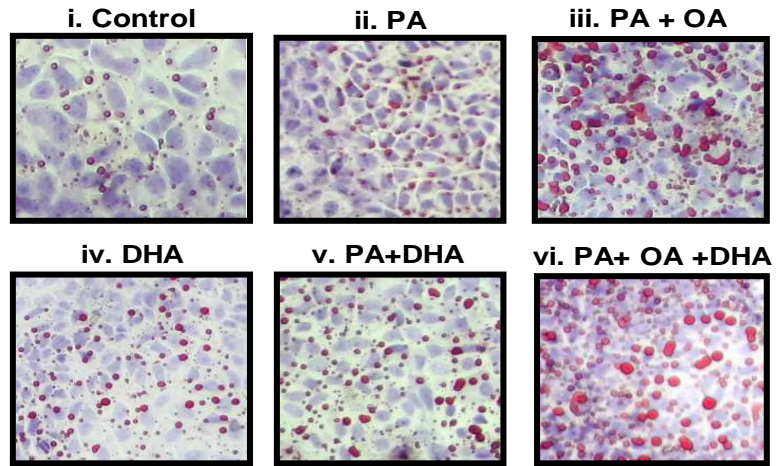
Chronic exposure to DHA decreases expression of lipogenic genes

Since increased hepatocyte TG accumulation is a characteristic feature of NAFLD (6), we quantified intracellular TG to determine the effect of prolonged exposure of various FA on TG accumulation. PA in the presence of OA increased TG accumulation significantly, when compared to control ($p < 0.001$) and just PA ($p < 0.01$). DHA also significantly increased TG accumulation individually and in the presence of PA \pm OA ($p < 0.001$ vs control, $p < 0.01$ vs PA) (Fig 1B). We also performed dose response studies using PA (250 - 500 μ M), DHA (50 – 1000 μ M) and combinations of PA, OA and DHA (total concentration 500 – 1500 μ M). DHA at concentrations greater than 500 μ M and PA at

concentrations greater than 250 μ M proved to be toxic to AML12 cells, assessed based on total protein isolated (fig not shown).

We then evaluated effects of DHA on lipogenic gene expression. DHA treatment significantly decreased expression of *Dgat2* in AML12 cells individually ($p < 0.05$ vs control, PA, PA and OA) and in the presence of PA \pm OA ($p < 0.05$ vs control, PA, PA and OA) (Fig 2A). DHA also significantly decreased *Apob* expression in the presence of PA ($p < 0.05$ vs control, $p < 0.05$ vs PA) (Fig 2B), suggesting a decrease in VLDL synthesis. In the presence of PA \pm OA, DHA significantly decreased the expression of *Fas* ($p < 0.01$ vs control, $p < 0.01$ vs PA, $p < 0.05$ vs PA + OA – in the presence of PA \pm OA) and *Acc*, ($p < 0.01$ vs control and PA – individually; $p < 0.01$ vs control, PA and PA + OA – in the presence of PA, $p < 0.01$ vs control – in the presence of PA + OA) the two rate limiting enzymes in the *de novo* synthesis of FA in hepatocytes (Fig. 2C). DHA also decreased *Scd1* expression in the presence of PA \pm OA ($p < 0.01$ vs control and PA, $p < 0.5$ vs PA + OA – individually; $p < 0.01$ vs control and PA \pm OA – in the presence of PA, $p < 0.01$ vs control – in the presence of PA \pm OA) (Fig. 2C). This decrease was more than the reduction induced by just OA. There were no differences in the expression of *Cpt1a* involved in the β -oxidation of FA, among the various FA treatments (Fig 2D). We also did not detect any differences in the expression of *G6p*, a gene coding for an essential enzyme in hepatic gluconeogenesis (figure not shown).

A. Steatosis



B. Triglyceride accumulation

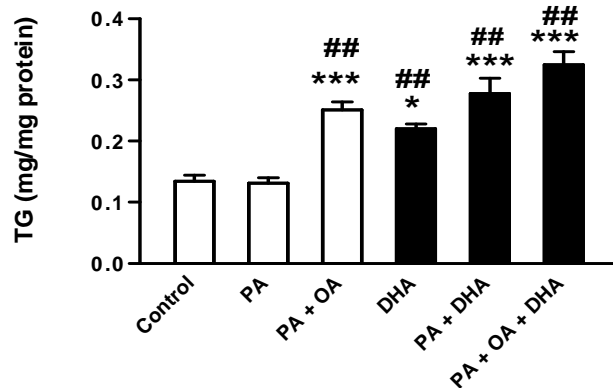
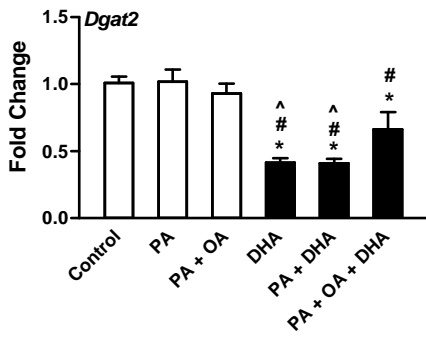


Fig. 1. An-vitro model of hepatic steatosis: A. Representative images from AML12 cells treated with 250 μ M palmitic acid (PA), oleic acid (OA) \pm docosahexanoic acid (DHA) for 72 – 96h showing steatosis. AML12 cells were fixed and stained using Oil-Red-O and hematoxylin.

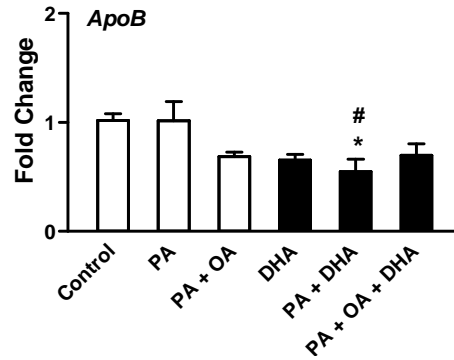
B. Cellular triglyceride content of AML12 cells treated with various fatty acids analyzed using colorimetric assay.

* $p < 0.05$ vs control; *** $p < 0.001$ vs control; ## $p < 0.01$ vs PA

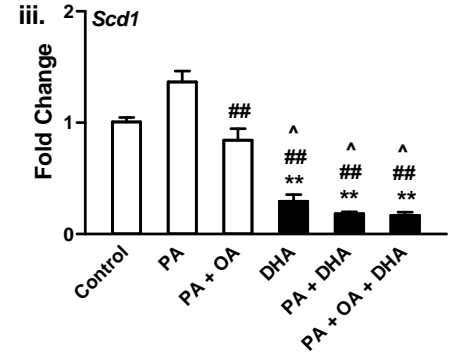
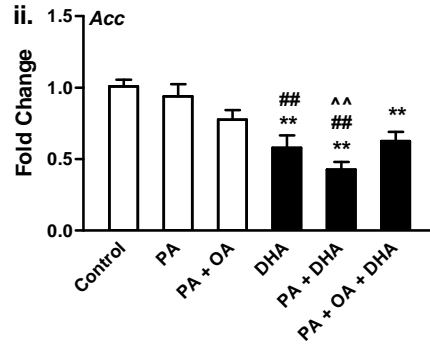
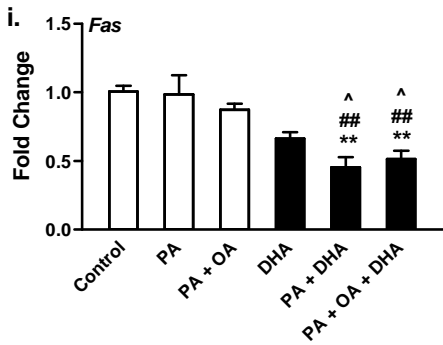
A. Triglyceride synthesis gene



B. VLDL Synthesis gene



C. Fatty acid Synthesis genes



D. Fatty acid Oxidation gene

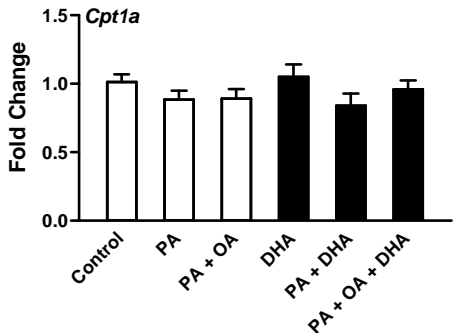


Fig 2. Chronic DHA exposure beneficially affects lipid synthesis: AML12 cells were treated with 250 μ M palmitic acid (PA), oleic acid \pm docosahexanoic acid (DHA) for 72 – 96h. Total RNA was isolated and gene expression was determined **A.** Triglyceride synthetic gene *Dgat2*. **B.** VLDL synthetic gene *ApoB*. **C.** Fatty acid synthetic genes *Fas*, *Acc*, *Scd1*. **D.** Fatty acid oxidation gene *Cpt1a*.

* $p < 0.05$ vs control; ** $p < 0.01$ vs control; # $p < 0.05$ vs PA; ## $p < 0.01$ vs PA; ^ $p < 0.05$ vs PA + OA; ^^ $p < 0.01$ vs PA + OA

DHA markedly decreases inflammatory gene expression induced by PA and OA

Progression of simple steatosis to steatohepatitis is characterized by increased inflammation (12). To evaluate the effects of various FA on inflammation, we measured expression of inflammatory genes. PA + OA significantly increased expression of the inflammatory gene *Saa1* when compared to control ($p < 0.05$) (Fig 3A panel i). DHA strikingly reduced *Saa1* expression in cells exposed alone ($p < 0.001$ vs PA, $p < 0.05$ vs PA + OA) or in combination with PA \pm OA ($p < 0.001$ vs PA, $p < 0.05$ vs PA + OA – in the presence of PA; $p < 0.05$ vs PA + OA – in the presence of PA + OA). Expression of *Ccl2* showed a similar pattern but did not reach statistical significance (Fig 3A panel ii).

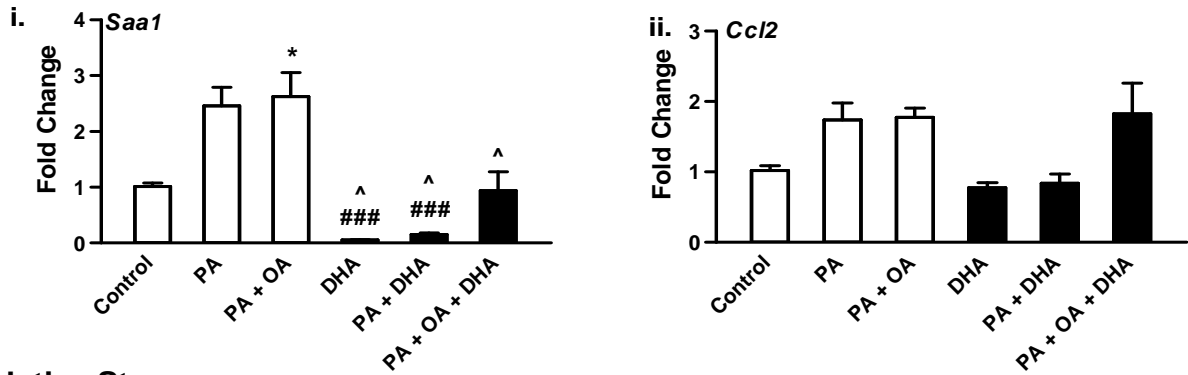
DHA decreases oxidative stress and ER stress gene expression

Increased ROS generation and ER stress have been implicated as contributors to progressive hepatic inflammation and fibrosis (35). Although expression of *Nox2* did not increase over control levels with exposure to PA \pm OA, DHA significantly reduced its expression by itself ($p < 0.05$ vs PA, $p < 0.001$ vs PA + OA) or in conjunction with PA ($p < 0.05$ vs P, $p < 0.001$ vs PA + OA) (Fig 3B). Expression of *Nox4* did not differ significantly between the FA treatments. Similarly expression of the ER stress gene, *Xbp1* was also decreased in AML12 cells by DHA alone ($p < 0.05$ vs control, $p < 0.01$ vs PA, $p < 0.05$ vs PA + OA) or in combination with PA ($p < 0.05$ vs control, $p < 0.01$ vs PA, $p < 0.05$ vs PA + OA) (Fig 3C).

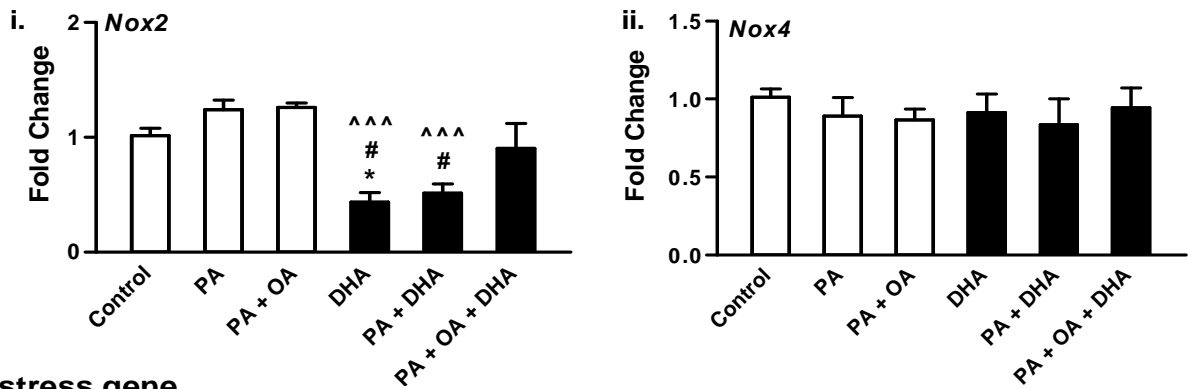
Chronic exposure to DHA does not alter insulin signaling in AML12 cells

Increased circulating FA worsen insulin resistance by disrupting insulin signaling in the setting of obesity and type 2 diabetes (10). We next evaluated the effect of the various FA on hepatic insulin signaling by measuring the levels of p-akt. There were no differences in akt phosphorylation with any of the FA treatments in our model, although we did observe a trend towards decreased phosphorylation with the PA \pm OA exposure (Fig. 4A and 4B).

A. Inflammatory genes



B. Oxidative Stress genes



C. ER stress gene

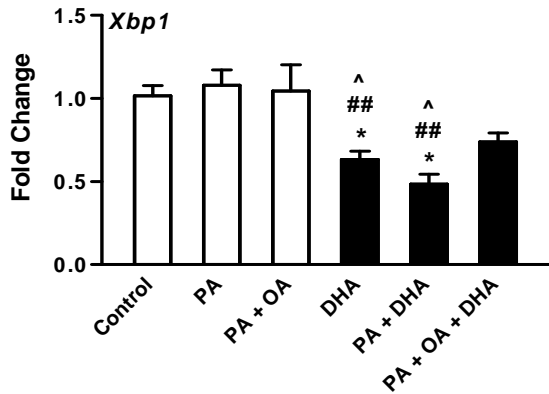
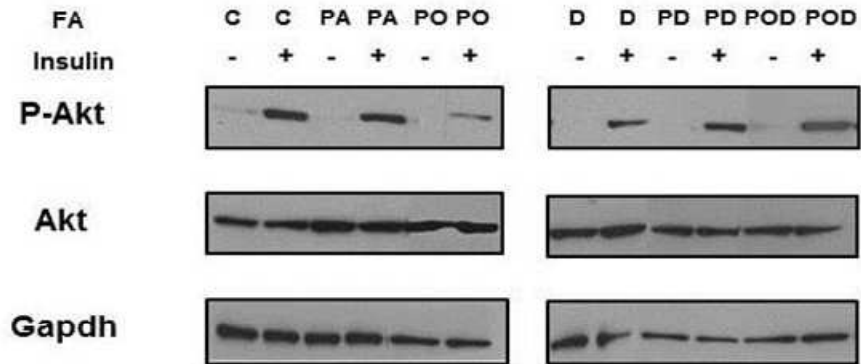


Fig 3. Chronic DHA exposure markedly suppresses expression of inflammatory, oxidative stress and ER stress genes: AML12 cells were treated with 250 μ M palmitic acid (PA), oleic acid \pm docosahexanoic acid (DHA) for 72 – 96 h. Total RNA was isolated and gene expression was determined **A.** Inflammatory genes *Saa1*, *Ccl2*. **B.** Oxidative stress genes *Nox 2*, *Nox 4*. **C.** ER stress gene *Xbp1*.

* $p < 0.05$ vs Control; # $p < 0.05$ vs PA; ## $p < 0.01$ vs PA; ### $p < 0.001$ vs PA; ^ $p < 0.05$ vs PA + OA; ^^ $p < 0.05$ vs PA + OA

A. Western Blot analysis of Akt and P-Akt



B. Quantitation of phosphorylated Akt/Akt

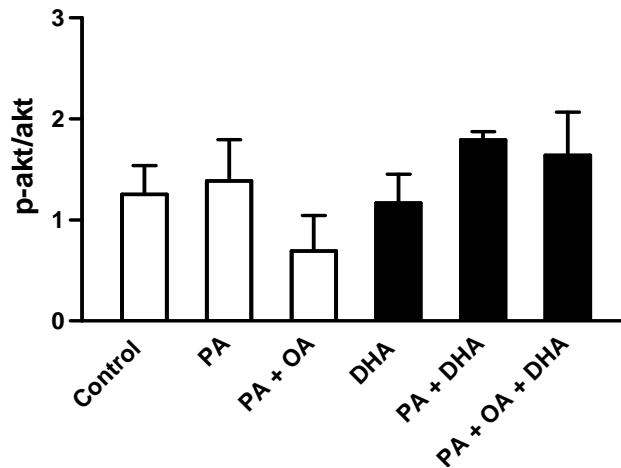


Fig 4. Chronic DHA exposure does not alter akt phosphorylation: AML12 cells were treated with 250 μ M palmitic acid (PA), Oleic acid \pm docosahexanoic acid (DHA) for 72 – 96 h. 16 hours before harvest, cells were starved of serum and insulin. On the day of harvest, cells were treated with or without 100nmol insulin for 15 mins and harvested for total protein extraction. **A.** Western blot analysis of phosphorylated akt, akt and Gapdh

B. Quantitation of western blot as band density of p-akt/akt.

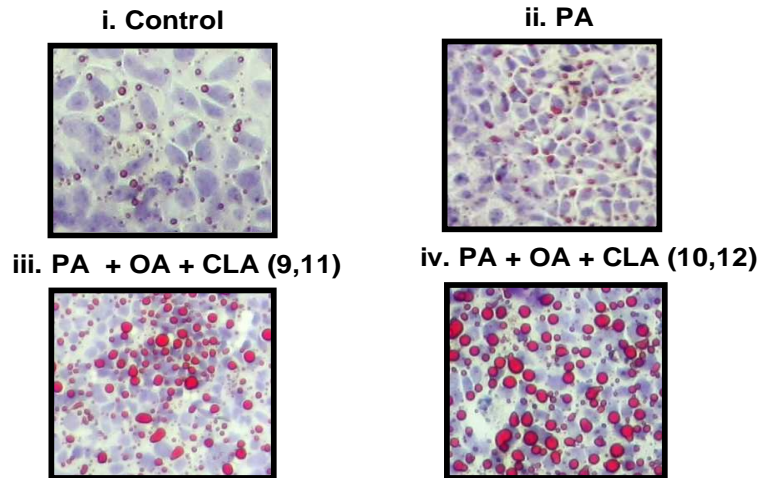
Effects of chronic CLA (10,12) exposure on AML12 cells

CLA (10,12) decreases lipogenic gene expression when exposed to AML12 cells

Similar to DHA, chronic exposure of CLA (10,12) to AML12 cells, significantly increased TG accumulation as observed by Oil-Red-O (Fig 5A panels i - iv) and colorimetric assay ($p < 0.05$ vs control) (Fig. 5B).

Dgat2 and *ApoB* expression was significantly different in AML12 cells exposed to CLA (10,12) in the presence of PA + OA ($p < 0.001$ vs control and PA + OA \pm CLA (9,11)) (Fig. 6A and 6B). CLA (10,12) also significantly decreased expression of lipogenic genes *Fas* ($p < 0.05$ vs control, $p < 0.001$ vs PA + OA; $p < 0.01$ vs PA + OA + CLA (9,11)), *Acc* ($p < 0.001$ vs control and PA + OA, $p < 0.01$ vs PA + OA + CLA (9,11)) and *Scd1* ($p < 0.05$ vs control), suggesting that CLA (10,12) decreases FA and TG synthesis (Fig 6C panels i - iii). No significant difference was observed in *Cpt1a* (Fig 6D) and *G6p* expression (figure not shown).

A. Steatosis



B. Triglyceride accumulation

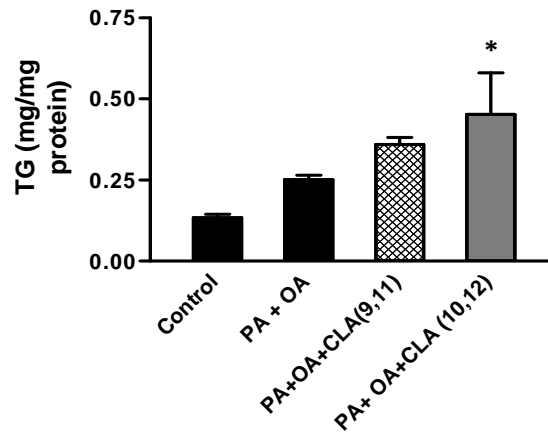


Fig. 5. Intracellular triglyceride accumulation: **A.** Representative images from AML12 cells treated with 250 μ M palmitic acid (PA), oleic acid (OA) \pm conjugated linoleic acid (CLA (9,11) or CLA (10,12) for 72 – 96h showing steatosis. AML12 cells were fixed and stained using Oil-Red-O. **B.** Cellular triglyceride content of AML12 cells treated with various fatty acids analyzed using colorimetric assay.

* $p < 0.05$ vs control; # $p < 0.05$ vs PA

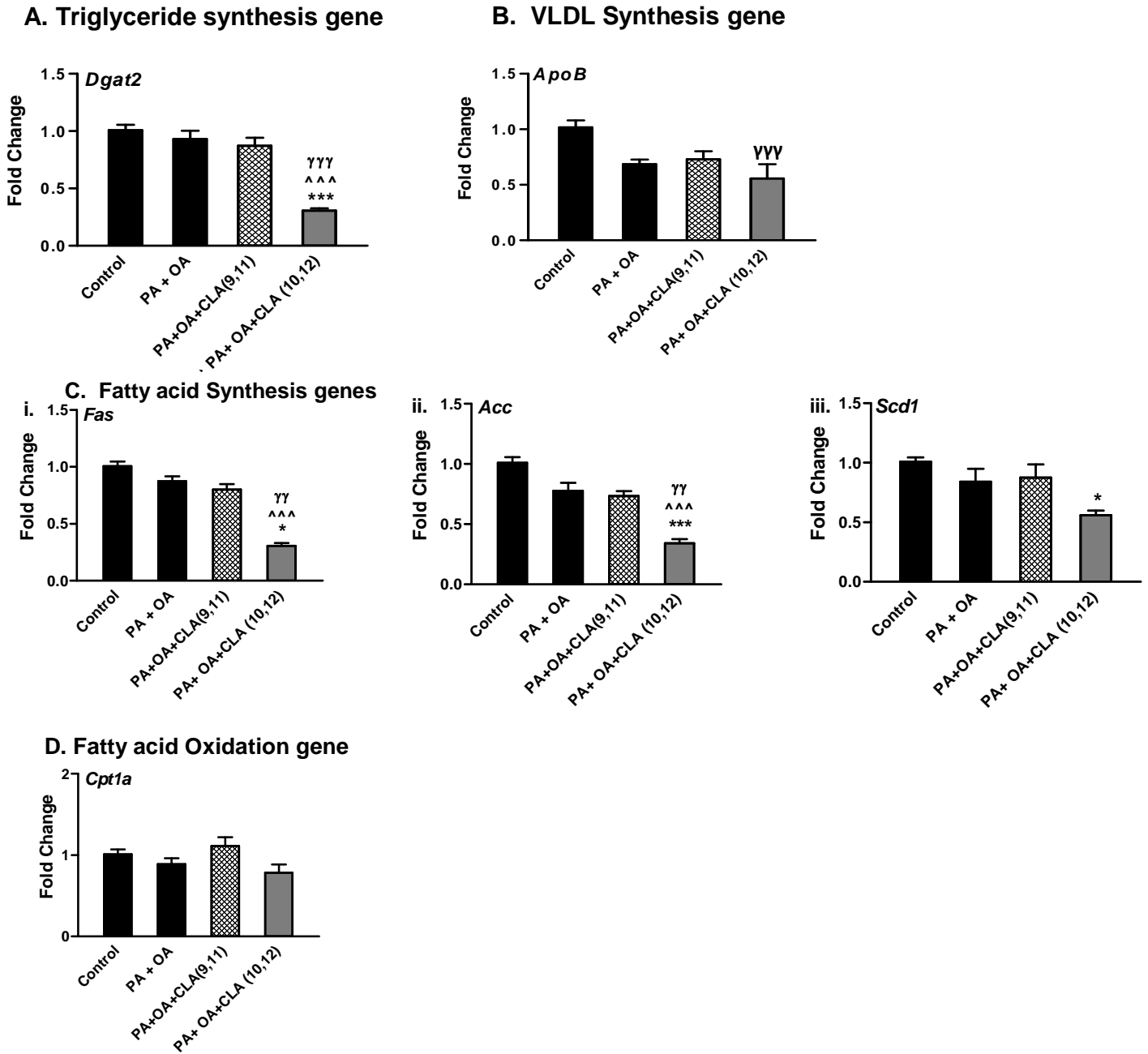


Fig 6. Chronic CLA (10,12) exposure beneficially affects lipid synthesis: AML12 cells were treated with 250 μ M palmitic acid (PA), oleic acid \pm conjugated Linoleic acid – CLA (9,11) or CLA (10,12) for 72 – 96 h. Total RNA was isolated and gene expression was determined **A.** Triglyceride synthetic gene *Dgat2*. **B.** VLDL synthetic gene *ApoB*. **C.** Fatty acid synthetic genes *Fas*, *Acc*, *Scd1*. **D.** Fatty acid oxidation gene *Cpt1a*.

* $p < 0.05$ vs control; *** $p < 0.001$ vs Control; ^^ $p < 0.001$ vs PA + OA; $\gamma\gamma$ $p < 0.01$ vs PA + OA + CLA (9,11); $\gamma\gamma\gamma$ $p < 0.001$ vs PA + OA + CLA (9,11)

CLA (10,12) decreases PA, OA and CLA (9,11) induced increase in inflammatory gene expression

CLA (9,11) in the presence of PA + OA, significantly increased the expression of both *Saa1* and *Ccl2* ($p < 0.05$ vs control). In contrast, CLA (10,12) strikingly decreased *Saa1* and *Ccl2* expression in the presence of PA + OA ($p < 0.01$ vs PA+ OA and $p < 0.01$ vs PA + OA + CLA (9,11) respectively) (Fig. 7A, panels i and ii).

CLA (10,12) decreases oxidative and ER stress gene expression

CLA (10,12) significantly decreased *Nox2* ($p < 0.05$ vs PA + OA, $p < 0.01$ vs PA + OA + CLA(9,11)) (Fig. 7B panel i) and *Xbp1* ($p < 0.001$ vs control and PA + OA) expression when exposed in combination with PA + OA (Fig. 7C). However, *Nox4* expression was not significantly different among the various FA treatments (Fig. 7B panel ii).

Chronic CLA (10,12) does not affect insulin signaling in AML12 cells

We evaluated the effects of CLA on insulin signaling in AML12 hepatocytes by measuring p-akt levels. There were no significant differences in akt phosphorylation among the various FA treatments. However, CLA (9,11) showed a trend towards increased insulin sensitivity in the presence of PA while CLA (10,12) showed a trend of decreased insulin sensitivity (Fig. 8A and 8B).

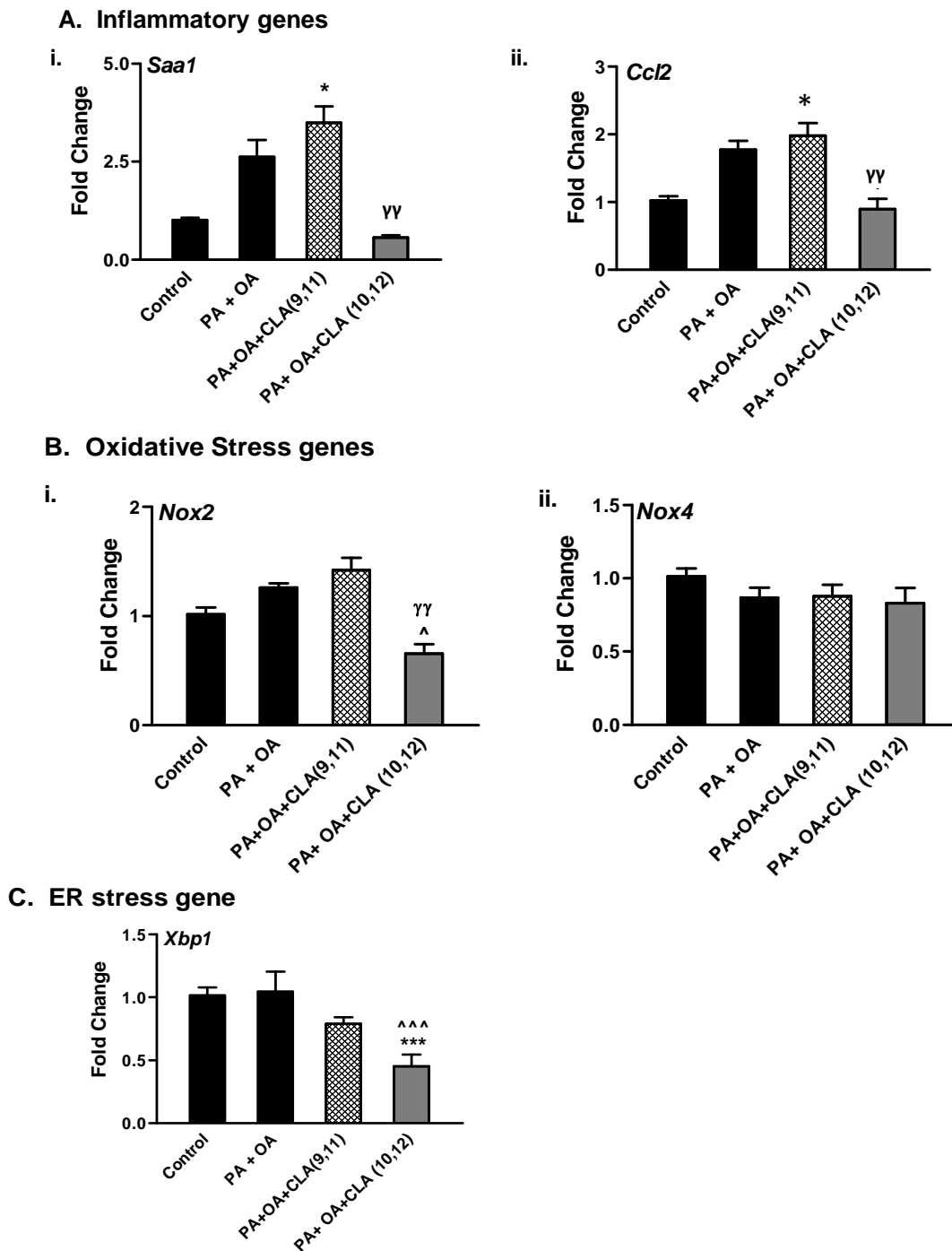
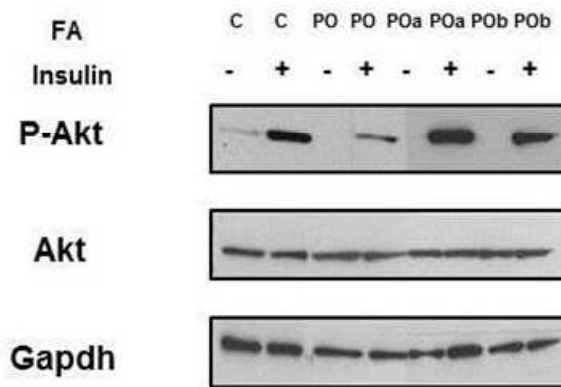


Fig 7. Chronic CLA (10,12) exposure markedly suppresses expression of inflammatory, oxidative stress and ER stress genes: AML12 cells were treated with 250 μ M palmitic acid (PA), oleic acid (OA) \pm conjugated linoleic acid – CLA (9,11) or CLA (10,12) for 72 – 96 h. Total RNA was isolated and gene expression was determined **A.** Inflammatory genes *Saa1*, *Ccl2*. **B.** Oxidative stress genes *Nox 2*, *Nox 4*. **C.** ER stress gene *Xbp1*.

* $p < 0.05$ vs Control; ^ $p < 0.05$ vs PA + OA; ^^ $p < 0.05$ vs PA + OA
 γ $p < 0.05$ vs PA + OA + CLA (9,11); $\gamma\gamma$ $p < 0.01$ vs PA + OA + CLA (9,11)

A. Western Blot analysis of Akt and P-Akt



B. Quantitation of phosphorylated Akt/Akt

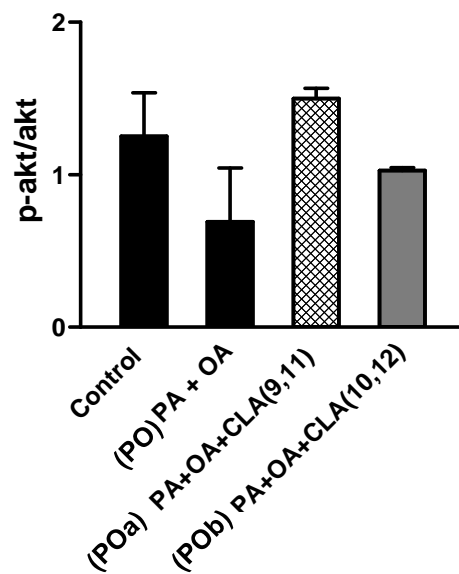


Fig 8. Chronic CLA (10,12) does not alter akt phosphorylation: AML12 cells were treated with 250 μ M palmitic acid (PA), oleic acid (OA) \pm conjugated linoleic acid – CLA (9,11) or CLA (10,12) for 72 – 96 h. On the day of harvest, cells were treated with or without 100nmol insulin for 15 mins and harvested for total protein extraction. **A.** Western blot analysis of phosphorylated Akt, akt and Gapdh **B.** Quantitation of western blot as band density of p-akt/akt.

DISCUSSION

In this study we compare and contrast the effects of various FA on metabolic consequences that occur with chronic FA exposure in an *in vitro* model system. Herein, we demonstrate that chronic exposure AML12 cells to FA results in fat droplet accumulation over a period of 72 – 96 h. We also show that despite increased TG accumulation, chronic exposure to two different types of FA, namely DHA and CLA (10,12) resulted in an improved metabolic profile in AML 12 treatments cells with decreased expression of genes involved in inflammation, ER and oxidative stress.

Understanding the downstream consequences of hepatocyte lipid accumulation in NAFLD is vital to dissecting the underpinnings of chronic liver disease. The cumulative and differential effects of various FA on liver cells have not been compared before in a single study. Thus far, most published studies have employed short-term FA exposure in hepatocyte culture models. The importance and novelty of our model is that, AML12 hepatocytes exposed to various FA chronically for 72 – 96 h (alone or in combination) developed steatosis, which can be used to study the molecular mechanisms of NAFLD. Using this model, we show that DHA, a n-3 PUFA and CLA (10,12), a ruminant TFA had protective effects on AML12 cells despite leading to an increased TG accumulation.

Recent data demonstrate that NAFLD is closely associated with visceral adiposity, dyslipidemia and insulin resistance and has been described as the hepatic component of metabolic syndrome (36). Several recent large scale observational studies have shown that obesity is an independent risk factor of NAFLD (37-39). Dietary habits may promote steatohepatitis directly by increasing hepatic TG accumulation (first hit) and/or promoting inflammatory activity (second hit). Since there is no effective treatment available, lifestyle modifications that include dietary changes is vital for NAFLD management (5, 40). In addition to total dietary fat content, specific types of dietary fat play an important role in NAFLD pathophysiology. Rodent studies have shown that iso-caloric high fat diets lead to an increased

hepatic lipid accumulation, compared with a control low-fat diet (5). Dietary intake of NASH patients have also been found to be richer in SFA and poorer in PUFA (41)

In our study, PA the SFA at 250 μ M concentration, did not appear to have a significant effect on TG accumulation, lipogenesis, inflammation and insulin signaling. However, in combination with OA, the MUFA, PA significantly increased the expression of inflammatory genes. PA + OA also increased TG accumulation and oxidative and ER stress gene expression. Previous studies have also shown that OA is more steatogenic than PA (42). Ruddock et al showed that PA decreased insulin sensitivity *in vitro* in a dose dependent manner (1 - 1000 μ M). Significant decreases in insulin sensitivity were observed at concentrations \geq 250 μ M (43). The negative effects observed by other studies may be due to higher doses of PA utilized. However our dose response studies have shown that concentrations of FA greater than 750 μ M are toxic to hepatocytes and hence we chose a concentration of 250 μ M per FA.

n-3 PUFA have been shown to inhibit de-novo lipogenesis, reduce inflammation and hepatic fat content (44, 45). It is known that DHA downregulates the expression of lipogenic genes and suppresses hepatic proinflammatory gene expression including TNF α , IL-1 β and IL-6 by DHA (46-48). In our study, we show that DHA reduced the expression of FA synthetic, inflammatory, oxidative and ER stress genes when exposed to hepatocytes by itself and along with PA \pm OA which promotes steatosis. DHA treatment also induced a significant decrease in expression of genes associated with VLDL synthesis and TG synthesis. DHA supplementation in hypercholesterolemic rabbits is associated with hepatic TG accumulation and increased mRNA levels of lipoprotein hepatic receptors (LDL and VLDL receptors) (49). It is possible that the concentration of DHA used in our study is not high enough to increase the expression of lipoprotein receptors and thereby decrease hepatic TG accumulation. Also, DHA did not have a significant effect on insulin sensitivity in our *in vitro* model. This can potentially be explained by the observation that n-3 PUFA inhibits insulin's inductive effects on hepatic *de novo*

lipogenesis and glycogenesis by antagonizing insulin regulation of the Akt pathway. The underlying mechanisms for these effects are not yet fully defined (50). Taken together, these results suggest that DHA has protective effects on AML12 cells exposed to FAs, despite not decreasing hepatic lipid accumulation. Moreover, DHA appears to negate the toxic effects of PA and OA on AML12 cells.

CLA has attracted considerable attention because of its potential beneficial effects in attenuating atherosclerosis, alleviating diabetes and reducing body fat in animal models. However, the literature is inconsistent regarding the effects of CLA on steatosis. Some animal studies show that CLA could reduce adiposity, improve glucose tolerance and insulin sensitivity (28, 51), while others show that it promotes hepatic steatosis despite reducing adiposity (52). This inconsistency could be due to varying CLA doses, isomers (and their combinations), duration of treatment and animal species used in the different studies. The isomers *cis 9, trans 11* and *trans 10, cis 12* are of particular interest due to their bioactive properties. The differential effects of these isoforms on liver cells have not been clearly elucidated. Our study shows that CLA (10,12) compared to CLA (9,11) significantly decreased expression of FA synthetic genes despite increasing hepatic TG accumulation when exposed to AML12 cells along with PA and OA. These results are consistent with previous rodent studies that showed that CLA (10,12) was protective against hepatic injury (28, 53). However unlike previous studies that showed increased inflammatory gene expression, CLA (10,12) treatment in our study decreased expression of *Saa1* (54). It is important to note here that despite causing an increase in TG accumulation, CLA (10,12) has beneficial effects on metabolic characteristics of AML12 cells, which is strikingly similar to the observed effects of DHA. Further research comparing the effects of DHA and CLA (10,12) is needed to explain this phenomenon.

Limitations

The main limitation of this study is its *in vitro* study design. Although it is challenging to extrapolate results to mice or humans, an *in vitro* system provides the ideal conditions to elucidate the molecular mechanisms involved in the development and progression of a disease, as it greatly simplifies the system under study. It also helps to formulate hypotheses that can be studied and tested in animal and human studies. By measuring gene expression and not the actual protein content (or activity), we may have overlooked the downstream effects of the various FA on protein synthesis and function.

Future Research Avenues

Future research should focus on delineating the effects of DHA and CLA (10,12) on AM12 cells. *In vitro* studies to investigate the differential effects of FA on factors downstream to gene expression including translation and protein synthesis/activity are also required. Co-culture studies of various FA treated hepatocytes and macrophages or Kupfer cells are important to elucidate the beneficial effects of DHA and/or CLA (10,12) and determine if the protective effects are still observed in the presence of cytokines. Also, it would be interesting to see if DHA or CLA (10,12) can potentially reverse the adverse effects of PA and/or OA on hepatocytes.

We show that DHA and CLA (10,12) beneficially modulate the metabolic profile of AML12 cells despite increasing TG accumulation. Additional studies are necessary to analyze the nature of lipid droplets within hepatocytes exposed to various FAs.

The same research, when extended to animal studies might further validate the protective effects of DHA and CLA (10,12) on hepatocytes. Experiments using *ob/ob* mouse vs lean mouse would be helpful in comparing and confirming the effects of the various FA on insulin signaling, adiposity, liver fat accumulation and inflammation. The tendency of DHA and CLA (10,12) to induce beneficial effects despite increasing hepatic TG accumulation warrants further investigation. Feeding LDLr^{-/-} mice diets

rich in the different FAs might be helpful in this investigation as it has been shown that feeding high fat diet to LDL *r*^{-/-} induces steatosis and inflammation (55).

CONCLUSION

In conclusion, AML12 cells exposed to various FA chronically for 72 – 96 h are an *in vitro* model of hepatic steatosis. PA and CLA (9,11) increases inflammation and TG accumulation in the hepatocyte. DHA beneficially modulates lipogenesis, inflammatory, ER and oxidative stress genes in the presence of PA, a SFA and increases TG accumulation. Similarly CLA (10,12) appears to protect hepatocytes against steatotic injury despite increasing intracellular TG accumulation. This indicates that not all lipid accumulation in the hepatocyte is harmful. Different FAs have varying effects on the steatotic hepatocyte. Hence, the type of fat is more important than total fat content in our diets. Developing a nuanced understanding of the molecular mechanisms underlying NAFLD development and progression will lead to effective dietary management for this increasingly prevalent disease, which to date has no proven treatment to prevent or reverse its course.

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