Mechanisms of Lipid Droplet Formation by Conjugated Linoleic Acid (CLA) Isomers and its Effects on Cell Viability

by

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ABSTRACT

The putative peroxisome proliferator-activated receptor (PPAR) α ligand, conjugated linoleic acid (CLA) induced cytoplasmic lipid droplet (LD) formation in H4IIE rat hepatoma cells. Currently, the mechanism(s) by which CLA isomers affects hepatic LD formation is unclear. We have investigated the role of PPARα and fatty acid (FA) activation in the regulation of hepatic LD formation induced by CLA isomers [*cis*-9,*trans*-11 (c9,t11), *trans*-10,*cis*-12 (t10,c12)] and linoleic acid (LA) in an *in vitro* model of lipid accumulation. Dose response of c9,t11 and t10,c12 CLA isomers as well as LA in quiescent H4IIE cells was assessed by Oil Red O staining and subsequent quantification after 24 hours. LD formation was induced by the CLA isomers similar to LA in a dose-dependent manner. However, treatment with the acyl CoA synthetase (ACS) inhibitor, triacsin C, resulted in significantly reduced LD formation. A similar reduction in lipid accumulation was observed with the PPARα activator, Wy14643. Furthermore, CLA isomers promoted H4IIE viability at 60 μM but decreased viability at a higher dose of 180 μM.

To further understand the role of PPARα in hepatic steatosis, we studied the level and phosphorylation of PPARα in livers of male lean and *fa/fa* Zucker rats fed either a control diet or *fa/fa* Zucker rats fed a CLA isomer (0.4% wt/wt c9,t11 or 0.4% wt/wt t10,c12) diet for 8 weeks. Immunoblotting results showed that only the t10,c12 CLA isomer significantly reduced phospho-PPARα S21 compared to the lean control (ln Ctl) and it was associated with a significant increase in the phosphorylation of p38 mitogen

activated protein kinase (MAPK).These changes were not observed with the c9,t11 CLA isomer.

Taken together, we have shown that CLA isomers directly induce LD formation in quiescent H4IIEs by activation of the lipid storage pathway which was significantly reduced by triacsin C or Wy14643. Also, we demonstrate for the first time that only the t10,c12 CLA isomer significantly reduced PPARα phosphorylation while it increased p38 MAPK phosphorylation. These results indicate that the anti-steatotic effects of the t10,c12 CLA isomer is associated with changes in PPARα phosphorylation and thereby its activity in a MAPK-independent manner.

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DEDICATION

To my family

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ABBREVIATIONS

ACC	acetyl Co-A carboxylase
ACS	acyl Co-A synthetase
AF	activating factor
ACOX	acyl CoA oxidase
ADRP	adipose-differentiation related protein
ALT	alanine aminotransferase
AMPK	adenosine monophosphate-dependent kinase
ANOVA	analysis of variance
APO B	apolipoprotein B
APO E	apolipoprotein E
BCA	bicinchoninic acid
BMI	body mass index
BSA	bovine serum albumin
CPTI	carnitine-palmitoyl transferase I
CVD	cardiovascular disease
c9,t11 CLA	*cis*-9,*trans*-11 Conjugated linoleic acid
CLA	conjugated linoleic acid
CYP4A1	cytochrome P450A1
DEX	dexamethasone
DHEA	dehydroepiandrosterone

ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
FAS	fatty acid synthase
FBS	fetal bovine serum
FA	fatty acid
FFAs	free fatty acids
FRIL	freeze-fracture replica immunogold labelling
GLUT	glucose transporter
HCV	hepatitis C virus
HDL	high-density lipoprotein
Hep G2	human hepatocellular carcinoma
HRP	horseradish peroxidase
HSC	hepatic stellate cells
HSL	hormone-sensitive lipase
kDa	kilo dalton
LD	lipid droplet
LDL	low-density lipoproteins
L-FABP	liver fatty acid binding protein
LA	linoleic acid
LPL	lipoprotein lipase
MAPK	mitogen activated protein kinase
MetS	metabolic syndrome
MRS	magnetic resonance spectroscopy

MUFA	monounsaturated fatty acid
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NEFA	non-esterified fatty acid
OLETF	Otsuka Long-Evans Tokushima Fatty
PAT	perilipin/Adipose-differentiation related protein/Tail-interacting protein of 47 kDa
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PFDA	perfluorodecanoic acid
PPAR	peroxisome proliferator activated receptor
PPARα	peroxisome proliferator activated receptor α
PPARβ	peroxisome proliferator activated receptor β
PPARγ	peroxisomal proliferator activated receptor γ
PPRE	peroxisome proliferator response element
PUFA	polyunsaturated FA
PVDF	polyvinylidene difluoride
RXR	retinoid X receptor
SFA	saturated fatty acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SREBP	sterol response element binding protein
scd1	stearoyl CoA desaturase 1

t10,c12 CLA	*trans*-10,*cis*-12 conjugated linoleic acid
TBST	tris-buffered saline with Tween-20
TIP47	tail-interacting protein of 47 kDa
TG	triglyceride
T2DM	type 2 diabetes mellitus
TZD	thiazolidinedione
UFA	unsaturated fatty acid
VLDL	very low-density lipoprotein
ZDF	Zucker diabetic *fa/fa*

1. INTRODUCTION

Hepatic steatosis, as defined by accumulation of excess fat in the liver and not caused by alcohol or other known causes of liver disease, is known as non-alcoholic fatty liver disease or NAFLD (146). NAFLD is strongly linked with obesity and other metabolic changes, such as impaired glucose tolerance, insulin resistance and subsequent hyperinsulinemia, hypertension and dyslipidemia which are collectively defined as the metabolic syndrome (MetS) or Syndrome X (183). The prevalence of fatty liver and related metabolic disorders is rising rapidly worldwide.

The most common cause of fatty liver disease in Canada is obesity. In Canada, fatty liver disease is now becoming evident in children, due to an alarming increase in childhood obesity. According to data available from the Canadian Liver Foundation, it is estimated that one in ten Canadian children is overweight (1). Fatty liver disease affects 3% of normal weight children and 22-53% of obese children and can be found in children as young as four years of age. According to current statistics, more than 50% of Canadian adults are overweight. It is estimated that 75% of obese individuals are at risk of developing simple fatty liver or hepatic steatosis and up to 23% of obese individuals are at the risk of developing non-alcoholic steatohepatitis (NASH) (1).

NASH represents the more severe end of the spectrum of NAFLD. NASH differs from the simple accumulation of fat in the liver, which is a completely benign condition, as inflammation is present. The prevalence of NASH is 2-6% in the general population. Up to 20% of adults with NASH develop cirrhosis and up to 11% may experience liver-

related deaths. Many individuals develop chronic liver failure and require liver transplantation as the last resort. The first liver transplant in Canada took place in 1970 in Montreal, Quebec (3) and this procedure has saved the lives of hundreds of Canadians in the last several decades. However, in some cases, rejection of the transplanted liver by the body can lead to repeat transplants which can occur anywhere from 24 hours to many years later. Currently, hundreds of Canadians need liver transplants but available organs are limited.

To date, the underlying molecular mechanism(s) for the development of fatty liver is poorly understood. Some of the important parameters associated with the development of fatty liver include triglyceride (TG) synthesis (28), fatty acid (FA) uptake, FA synthesis and TG export in the form of very low-density lipoproteins (VLDL) (84). Imbalance in any of these normal lipid metabolism pathways can result in excess lipids in the liver. Other causes of fatty liver disease include starvation and protein malnutrition, long term use of total parenteral nutrition (a feeding procedure that involves infusing nutrients directly into the blood stream), intestinal bypass surgery for obesity and rapid weight loss (2).

A series of comparative rat liver perfusion experiments indicated that free fatty acids (FFAs) are the major source of substrate for the hepatic production of TGs (62). Excess flux of FFA into the liver contributes to liver impairment in NAFLD but the mechanism remains unclear. The main products of lipid synthesis are TGs and, although a certain amount of lipid storage may even be hepatoprotective, prolonged lipid storage can result in an activation of inflammatory reactions and loss of metabolic competency. Recently, it was discovered that cytoplasmic lipid droplets (LDs) are dynamic organelles

participating in several important metabolic reactions as well as trafficking and inter-organellar communication to distribute neutral lipids and phospholipids to various membrane-bound organelles within the cell (70, 255). However, few studies have highlighted the role of FAs on LD-associated proteins in fatty liver.

It is noteworthy to mention that exogenously supplied FAs are activated *via* acyl Co-A synthetase (ACS) to their FA acyl-CoAs to be channeled either toward lipid synthesis and storage or toward oxidation pathways. The products of ACS activity, namely, FA acyl-CoAs, can then be metabolized to form TG, phospholipids and cholesteryl esters. Muoio et al. (136) reported that triacsin C, a selective inhibitor of ACS, inhibited TG synthesis in hepatocytes from fed rats by 70% and in starved rats by 40%. Triacsin C also inhibited the synthesis of cholesteryl ester and TG in mouse peritoneal macrophages, leading to a reduction of LD formation (141).

Conjugated linoleic acid (CLA) shares a very similar backbone to oleic acid (both are 18-carbon FAs) and, therefore, it is absorbed and incorporated into lipids in a manner similar to oleic acid (12). CLA isomers have been shown to be incorporated into membrane phospholipids and alter FA homeostasis (12, 14-15, 73, 94, 195). Several studies have implicated CLA in reducing both arachidonate accumulation in phospholipids and eicosanoid production, and have suggested that the physiological effects of CLA may in part be due to competition with linoleate as substrate for Δ6 desaturation, the rate-limiting step for arachidonate formation from linoleate (14-15, 23).

CLA isomers are potent ligands of peroxisome proliferator activated receptor (PPAR)-α and increase the expression of many PPARα target genes (134, 232). In

contrast, a study conducted by Peters et al. (170) in PPARα null mice showed that genes coding liver FA oxidation and FA binding were affected by CLA, thus indicating that CLA can also operate *via* a PPARα independent mode of action. In addition to its ligand-induced activation, transcriptional activity of PPARα is regulated by phosphorylation mainly *via* extracellular signal regulated kinase (ERK)-mitogen activated protein kinase (MAPK) (49, 208). However, the isomer-specific effects of the CLA isomers [*cis*-9,*trans*-11 (c9,t11), *trans*-10,*cis*-12 (t10,c12)] on PPARα phosphorylation remain unexplored in the *fa/fa* Zucker rat.

There is evidence that a CLA mixture markedly alleviated hepatomegaly and hepatic TG accumulation (139, 150) with improved liver function [lower serum alanine aminotransferase (ALT) and alkaline phosphatase] after 8 weeks in *fa/fa* Zucker rats. A recent study from our lab has shown that the anti-steatotic effects of the t10,c12 CLA isomer in obese *fa/fa* Zucker rats were associated with a significant decrease in hepatic adipophilin level, and reduced LD size in the liver (204).

Currently, there is no medication proven to effectively treat fatty liver disease. This societal burden of steatosis warrants the need for enhanced understanding of cellular mechanisms relevant to the progression of fatty liver and has raised a lot of interest in the scientific community. Therefore, the development of efficacious therapeutic interventions to reverse or attenuate development of this disease is of primary concern. The focus of this thesis is to study the role of CLA isomers as a therapeutic treatment for fatty liver or hepatic steatosis and delineate potential mechanisms focusing on two key players of hepatic lipid metabolism, namely, ACS and PPARα.

1.1 Study Rationale

In the pathogenesis of NAFLD, accumulation of lipids in hepatocytes and reduced hepatocyte viability are strongly implicated in disease progression from the potentially reversible condition of steatosis to severe acute and chronic liver injury (184). To date, the triggers of fatty liver and the molecular mechanisms by which it relates to FA metabolism and signaling molecules are incompletely understood. Hepatic lipid overloading mainly in the form of TGs is considered a prerequisite for the development of NAFLD (113, 169). Previous evidence from our lab has shown that the t10,c12 CLA isomer reduced liver lipid content and inflammation, and improved liver function (150, 205). However, CLA isomers induced LD formation in H4IIE rat hepatoma cells (205). Currently, mechanisms by which CLA isomers affect LD formation remain unclear.

A recent study provided evidence that steatosis is associated with ACS5 up-regulation in primary human hepatocellular carcinoma (HepG2) cells and human steatotic liver (184). Another important group of molecular targets for the treatment of fatty liver and fatty liver associated metabolic abnormalities is PPARα, primarily due to its regulation of genes involved in FA oxidation (97, 170). Transcriptional activity of PPARα is regulated by phosphorylation *via* the MAPK pathway (49, 208), but the effects of CLA isomers on PPARα phosphorylation remain unexplored in the *fa/fa* Zucker rats. The cytotoxicity of CLA isomers, particulary the t10,c12 isomer, has been previously reported for rat hepatomas, however, no direct link to LD formation was shown (247). Use of rat H4IIE hepatoma cells can thus be a valuable tool in elucidating a mechanism for the direct effects of CLA isomers on hepatic LD formation and hepatocyte viability.

1.2 Statement of Hypotheses

1. CLA isomers (c9,t11 or t10,c12) induce LD formation *via* ACS which is prevented by triacsin C or Wy14643.
2. Increased LD formation by CLA isomers reduces H4IIE rat hepatoma viability.
3. The anti-steatotic effect of t10,c12 CLA isomer in obese *fa/fa* Zucker rats is associated with increased PPARα phosphorylation *via* the MAPK signal transduction pathway.

1.3 Aims of the Study

Aim 1: To study the dose response of CLA isomers (c9,t11 or t10,c12) and linoleic acid (LA) on lipid accumulation and H4IIE rat hepatoma viability.

Aim 2: To study the effect of the ACS inhibitor, triacsin C, on lipid accumulation in response to CLA treatment in H4IIE cells.

Aim 3: To determine the effect of Wy14643, a potent PPARα agonist, on H4IIE viability in response to treatment with CLA isomers or LA.

Aim 4: To determine the effects of 8 week dietary supplementation with CLA isomers on the expression and phosphorylation of PPARα in the livers of male *fa/fa* Zucker rats.

1.4 Anticipated Significance

This will be the first study to investigate the isomer-specific effects of CLA on cell viability relative to LD formation in quiescent H4IIE hepatoma cells. This study will also focus on the mechanism of LD formation and highlight the potential roles of ACS and PPARα. This will also be the first study to assess the isomer-specific effects of dietary CLA on the phosphorylation of PPARα in the livers of obese and insulin resistant *fa/fa* Zucker rats. Therefore, results from these studies will help to determine the importance of PPARα and ACS in fatty liver and/or LD formation and subsequent effects on hepatocyte viability as a direct or indirect effect of CLA isomers.

2. REVIEW OF LITERATURE

2.1 Non-Alcoholic Fatty Liver Disease (NAFLD)

Steatosis is the process of abnormal retention of lipids mainly in the form of TGs within a cell. The risk factors associated with steatosis are varied, and include diabetes mellitus (10), hypertension (22) and obesity (192) which collectively constitutes a condition known as the metabolic syndrome (MetS) or Syndrome X (183).

In the absence of alcohol consumption or any other known causes of liver disease (hepatitis B or C, toxic or autoimmune liver disease, hemochromatosis, Wilson's disease, hypobetalipoproteinemia), hepatic steatosis, as defined by accumulation of excess fat in the liver (>5-10% fat as determined by histology), is known as NAFLD (146). The Dallas Heart Study reported that liver fat of 5.6% (55.6 mg of TG/g of liver tissue) is the upper limit of normal in healthy non-obese subjects (n=345) in the absence of excessive alcohol consumption, liver disease or diabetes and with normal serum ALT levels (206)

2.1.1 Prevalence and Significance

The incidence of NAFLD and obesity are closely linked. In one study, about 75% of obese subjects were estimated to have NAFLD while 20% developed NASH, which is defined as fatty liver disease with inflammation (56). The overall morbidity risk of an individual is increased by excess accumulation of fat in adipose tissue, liver and other

organs due to increased predisposition of the individual to the development of metabolic abnormalities.

Considerable variation is observed for the estimation of NAFLD prevalence depending on the methods and population studied (32). However, it is the most common cause of increased serum aminotransferase activities, markers for liver injury (33). In the population-based Dallas Heart Study, about 30-35% subjects (Caucasian, African American and Hispanic) had hepatic steatosis when liver fat was measured by ^{1}H-magnetic resonance spectroscopy (MRS) (206). Obesity and type 2 diabetes mellitus (T2DM) further increased the prevalence of NAFLD (9). Interestingly, several studies have shown that NAFLD can be used to predict cardiovascular disease (CVD) and T2DM independent of obesity (102, 212). Histological analysis of liver biopsies from morbidly obese patients undergoing bariatric surgery showed that 86% had steatosis, 24% had NASH, and 2% were cirrhotic (125). Most of the subjects (~79%) with NAFLD have normal ALT, thus suggesting a normal serum ALT activity does not exclude steatosis (24).

Simple steatosis can progress into more serious NASH with mild to moderate fibrosis (38), as characterized by lobular inflammation and hepatocellular ballooning in addition to steatosis. A biopsy may be required for diagnosis of NASH (8). Over a period of 15 years, ~5-15% of these individuals can develop severe fibrosis, of which up to ~10% will develop cirrhosis and thus have a greater risk of developing hepatocellular carcinoma (9).

2.1.2 Sources and Composition of Intrahepatic Triglyceride (TG)

Excess fat is primarily stored in adipose tissue in the form of TGs since the adipocytes are specifically designed to store energy. Obesity is associated with excess deposition of fat in the liver and is considered a major risk factor for the development of fatty liver diseases. Lipid peroxidation, cytokines and other pro-inflammatory compounds have also been implicated in steatosis and its progression to steatohepatitis (56).

Frayn et. al (58) showed that the hepatic uptake of FFAs and secretion of lipids in the form of VLDL depends on the nutritional state. In the fasting as well as in postprandial state, *de novo* hepatic lipogenesis contributes very little to the intra-hepatocellular TG in normal subjects (13). The major source of intra-hepatocellular TG during fasting is FA delivered to the liver in the form of VLDL-remnants and FFA from intravascular lipolysis in peripheral tissues (51, 69). In the postprandial state, adipose tissue lipolysis contributes the majority (~45%) of FA used for hepatic VLDL synthesis even though it is suppressed by insulin (13). However, excess amounts of both glucose and VLDL are produced by the insulin resistant fatty liver in NAFLD (191) resulting in hyperglycemia and hypertriglyceridemia (6, 197).

A recent study in normal subjects reported the dynamic role of hepatic lipid stores using ^{13}C-labeled FA in a lipid mixture (ingested along with normal breakfast) and showed that labeled FA reached peak incorporation in hepatic TG stores after 6 hours when measured with MRS (182). Also, in markedly abdominally obese subjects, splanchnic lipolysis accounts for only about 30% of hepatic FA delivery, and the main source of intrahepatocellular FA appears to originate mainly from the peripheral tissues (148).

Obesity is associated with hepatic insulin resistance and liver fat, and several studies have reported variations in liver fat content at any given body mass index (BMI) or waist circumference (101, 250). A recent study in healthy subjects overfed a fast food-based diet for 4 weeks reported ~9% weight gain, ~2-3-fold increase in liver fat content and a 4-5-fold increase in serum ALT activity (93).

Liver fat content is elevated with increased intake of fat, particularly saturated fat (174, 218), and decreased with weight loss (118, 171) with the decrease in hepatic TG being relatively faster and greater compared to other sites in the body (218). A recent study on the FA composition of intrahepatocellular TG reported increased amounts of saturated FA (SFA) and decreased amounts of unsaturated FA (UFA) in fatty livers (100). Interestingly, the main products of lipid synthesis are TGs and the FA residues in TGs are primarily composed of UFAs (178).

Recent studies have shown that fats are not the sole players in fatty liver development. Excess consumption of carbohydrates has also been found to be associated with insulin resistance, hypertriglyceridemia and increased *de novo* lipogenesis in humans (55, 77). Consumption of carbohydrates, such as fructose, stimulates hepatic *de novo* lipogenesis more than glucose in normal subjects (163). These data show that increased consumption of refined sugars, such as fructose, could play a major role in the development of fatty liver and associated MetS.

In addition to the acquired causes of NAFLD, genetic factors have also been recently suggested to contribute to NAFLD based on results from family studies (238) and studies comparing different ethnic groups (24, 172).

2.1.3 Key Players in NAFLD

Disturbances in lipid metabolism are one of the major causes of NAFLD. The adipocytes are specifically designed to store energy and accommodate excess fat as TGs in the form of large or small LDs. ACS catalyzes the activation of FAs to acyl-CoA esters, which are metabolised either toward lipid synthesis and storage (mainly as TGs) or toward oxidation pathways (131). PPARs constitute a subset of nuclear receptors that function as intracellular sensors for cholesterol metabolites, FFAs, and a range of other lipophilic molecules (66). PPARs are ligand-activated transcription factors that subsequently bind to regulatory regions in target genes, thereby modulating their expression (20, 227). PPARs have been related with the occurrence or progression of NASH (210). These receptors are attractive therapeutic targets for the treatment of disorders of lipid metabolism (20, 66).

i. Role of Lipid Droplet (LD) Formation in NAFLD

Cytoplasmic LDs were considered to be inert organelles which store neutral lipids in the form of TGs for use as an energy source. However, through proteomic studies, it is now known that LDs are metabolically active structures that play major roles in lipid transport, sorting, and signaling cascades. In particular, LDs maintain a dynamic communication with the endoplasmic reticulum (ER) and the plasma membrane *via* sphingolipid-enriched domains of the plasma membrane - the lipid rafts. These microdomains frequently harbor receptor tyrosine kinases and other signaling molecules and connect extracellular events with intracellular signaling cascades (70, 255).

Steatosis may occur in conjunction with other metabolic abnormalities such as insulin resistance in patients with chronic hepatitis C virus (HCV) infection. The pathogenic mechanisms involved in HCV-induced hepatic steatosis are mediated by the HCV core protein, whose expression is associated with LD accumulation, changes in lipogenic gene expression and/or the activity of lipogenic proteins, and effects on mitochondrial oxidative function (144). The importance of genes such as PPAR α (discussed in section 2.1.3-iii) in HCV-mediated steatosis has been elucidated in mice (144).

Although many studies have shed light on the genetics, physiology and the cellular mechanisms of fat accumulation, further understanding of LD biology could indicate potential therapeutic targets to prevent or reduce the accumulation of LDs and alleviate disease conditions such as fatty liver diseases.

Biogenesis of the LD

LD biogenesis was originally thought to occur *via* accumulation of neutral lipids within the ER membrane bilayer. Eventually, the LD matured by budding, resulting in a lipid core that was enclosed by a protein-containing phospholipid monolayer. However, a recent study by Robenek et al. (187) contradicted this notion by employing freeze-fracture replica immunogold labelling (FRIL), an electron microscopic technique, and demonstrating that LDs appear to develop externally on ER membranes at specialized sites where the lipid gets enwrapped by the ER, ultimately ending up in a phospholipid monolayer membrane. The facing leaflets of the ER membrane and droplet surface are enriched in adipophilin. This group also demonstrated that the perilipin/ADRP/TIP47 (PAT) family proteins (discussed in the following section) are not only located at the

surface of the LD but can occur throughout the LD core and are also present in the plasma membrane.

Structure and LD-associated proteins (PAT proteins)

In non-adipocytes, LDs are small, mobile and interact with other cellular organelles, such as the mitochondria and the peroxisomes (255). In contrast, adipocytes primarily contain very large and immotile LDs, suggesting that the morphological differences between LDs in adipocytes and non-adipocytes must be due to different interactions of LDs with other organelles in different cell types. Murphy et al. (138) have highlighted the complexity of LD interactions, which can be both homotypic (LD-LD) and heterotypic (LD-other organelles) in both adipocytes and non-adipocytes. Digel et al. (47) reported the heterogeneity of LDs within a single cell with a focus on microscopy, especially live cell imaging. LDs are also heterogeneous in size, location and protein content. The proteins that coat LDs change during LD biogenesis and are dependent upon the cell's metabolic state (52).

The PAT family proteins are involved in the accretion and mobilization of lipids (216). In mammals, five members of the PAT protein family have been identified, and these include perilipin, ADRP or adipophilin, tail-interacting protein of 47 kDa (TIP47), S3-12 and OXPAT/myocardial LD protein/lipid-storage droplet protein 5 (18, 52, 114, 167, 241). All PAT proteins bind intracellular LDs, either constitutively or in response to metabolic stimuli, such as increased lipid flux into or out of LDs. PAT proteins are structurally related and regulate the access of lipases to the lipid esters present in the LD core (114). Lipolysis in adipocytes is stimulated by the translocation of hormone-sensitive lipase (HSL) from the cytosol to the surface of the intracellular LD, which is

coated with perilipin (115). PAT proteins are important in the regulation of cellular lipid metabolism both in mammals and in model organisms (18).

When energy is required, the stored TGs in LDs are hydrolyzed *via* activation of lipolytic pathways that govern the release of stored FAs from the adipocyte TG pool which constitutes the major energy reserve in animals. The released FAs are then transported by serum albumin to various tissues to provide energy.

Very little is understood about how mammals package fat within cells. PAT proteins play important roles in packaging fat into LDs and regulate the coordination of lipid storage and utilization. The two major PAT proteins (perilipin and adipophilin) constitutively associate with LDs and play important roles in sustained fat storage and regulation of lipolysis (241). However, during rapid fat storage, nascent LDs are coated with other PAT family proteins such as TIP47, S3-12 and OXPAT/MLDP/PAT-1 (241).

FA pathways in the liver

FAs in the liver can enter a catabolic or a biosynthetic pathway by being directed for oxidation, TG storage, phospholipid synthesis, ketone body formation, or they can be secreted from the liver in the form of VLDL. Under hyperinsulinemic and normoglycemic conditions, insulin decreases apolipoprotein B (apoB)-100 and VLDL-TG production in normal subjects (90, 121) by direct suppression of apoB-100 synthesis and VLDL assembly. In addition, insulin also suppresses the hepatic uptake of FFA *via* its antilipolytic effect (68, 78, 112).

ii. Acyl-CoA synthetase (ACS)

Enhanced lipogenesis appears as a major abnormality of hepatic fatty metabolism in subjects with NAFLD. Thus, decreasing hepatic lipogenesis may help to reduce hepatic TG synthesis and content in such patients (50).

ACSs activate FAs for intracellular metabolism and are involved in the regulation of FA uptake. The intracellular utilization of long chain FAs may be subdivided into three steps:

a. uptake across the plasma membrane

b. activation by esterification with coenzyme A, and

c. subsequent metabolism.

ACS enzymes are essential for *de novo* lipid synthesis, FA catabolism and membrane remodeling. The length of the carbon chain of the FA species defines the substrate specificity for the five different ACS enzymes (which were characterized on this basis). Mammalian ACSs activate FAs with usual chain lengths of 12 to 20 carbon atoms.

The ACS isoforms differ in their subcellular location. Using non-cross-reacting ACS1, ACS4, and ACS5 peptide antibodies, Lewin et al. (111) showed that ACS4 was the only ACS isoform present in peroxisomes and in mitochondria-associated membrane fractions isolated from livers of gemfibrozil-treated rats and associated with TG synthesis. ACS1 was present in ER fractions and ACS5 was present in mitochondrial fractions.

A recent study provided evidence that hepatocyte steatosis is associated with ACS5 up-regulation in primary human hepatocytes, HepG2 cells and human steatotic

liver and the authors proposed that ACS5 could play an important role in fatty liver-related disorders (184). The predominant pathways for FAs include their storage, membrane biosynthesis or conversion to energy. ACS is essential for both oxidation and esterification of FAs. However, the molecular mechanism of FA activation and subsequent channeling toward one particular metabolic pathway is not well understood.

Vanden Heuvel et al. (225) showed that ACS activity was reduced significantly by perfluorodecanoic acid (PFDA), a potent peroxisome proliferator, in rat liver mitochondria and microsomes. The inhibition of ACS by PFDA was similar in liver mitochondria and microsome preparations. PPARα activators such as fibrates have been reported to induce ACS mRNA levels in liver or in adipose tissue which was associated with a concomitant increase in FA uptake (127). These data suggest that the hypolipidemic effect of peroxisome proliferators may be potentially mediated by the ACS enzyme resulting in increased FA oxidation and reduced TGs.

iii. Peroxisome Proliferator Activated Receptors (PPARs)

PPARs belong to the nuclear receptor superfamily and have been identified in a wide range of species, and include numerous cellular receptors for nutrients and steroids (224). PPARs are activated by a variety of ligands that regulate the transcription of genes involved in a number of biological processes, including lipid and glucose metabolism, and overall energy homeostasis (26-27, 57, 224). Some of the endogenous ligands of PPARs are FAs and eicosanoids (36, 67). PPAR agonists have been widely used for the treatment of dyslipidemia (fibrates) and insulin resistance (thiazolidinedione or TZDs)

(57). Therefore, PPARs serve as an important group of molecular targets for the treatment of fatty liver and fatty liver associated metabolic abnormalities.

PPARs play an important role in the regulation of energy metabolism (124) and share a common molecular structure and mechanism of action (46). Following their activation by specific ligands, PPARs heterodimerize with the nuclear receptor retinoid X receptor (RXR) and subsequently bind to specific DNA response elements called peroxisome proliferator response elements (PPRE) located in the promoter or in intronic regions of PPAR target genes (75, 79, 124, 142). Upon ligand binding to PPARs, co-activators are recruited and co-repressors are dissociated. This leads to chromatin remodeling which permits the initiation of gene transcription (20, 203).

The three PPAR isoforms identified so far include PPARα, PPARβ/δ and PPARγ (46, 248). Each PPAR isoform governs the expression of specific target genes in a tissue-specific manner that ultimately displays ligand specificity.

PPARα

PPARα is highly expressed in metabolically active tissues including liver, muscle and brown adipose tissue. In the liver, PPARα plays a pivotal role in numerous processes including gluconeogenesis and lipid metabolism (124). PPARα is an important regulator of hepatic nutrient metabolism including FA oxidation (peroxisomal and mitochondrial), FA uptake, amino acid metabolism, glycerol metabolism, and lipoprotein assembly and transport (96, 124, 165). An anti-obesity role for PPARα is well supported by several studies in rodents administered synthetic PPARα agonists (71, 123, 226).

PPARα mediates the effects of hypolipidemic fibrate drugs, which decrease plasma TGs and increase plasma HDL concentrations (59-60). PPARα activation up-

regulates genes involved in FA oxidation pathways and increases hepatic uptake and esterification of FFAs (59). Murakami et al. (137) indicated a protective effect of PPARα agonism against abnormal lipid metabolism in the liver of obese Zucker *fa/fa* rats. Thus PPARα is a key regulator of lipid homeostasis in hepatocytes and is a target for not only the hypolipidemic drugs but also FAs (240).

PPARα can exist in a phosphorylated form, and its phosphorylation is increased by ciprofibrate in Fao cells (106). However, relatively little is known about the specific signaling pathways through which it operates. In addition to its ligand-induced activation, PPARα is regulated by ubiquitination (176) and phosphorylation *via* ERK-MAPK, protein kinase A (PKA) and protein kinase C (PKC) (49).

Diradourian et al. (49) showed that anisomycin, a p38 activator, induced a dose-dependent phosphorylation of PPARα and a 50% inhibition of its transcriptional activity in COS-7 cells. Anisomycin-induced p38 phosphorylation decreased both endogenous and PPARα ligand-enhanced hepatic carnitine-palmitoyl transferase I (CPTI) and acyl CoA oxidase (ACOX) gene expression in H4IIE hepatoma cells (49). Paumelle et al. (168) reported that simvastatin inhibited PKCα inactivation of PPARα transrepression activity by inhibition of PPARα phosphorylation. However, dehydroepiandrosterone (DHEA) regulates PPARα action by inducing PPARα mRNA and protein levels as well as increasing PPARα transcriptional activity through decreased receptor phosphorylation at serines in the activating factor (AF) 1 region (208). They demonstrated that the serines at positions 12 and 21 were rapidly dephosphorylated upon treatment of HepG2 with DHEA and nafenopin. Mutation of serines at position 6, 12, and 21 to an uncharged

alanine residue significantly increased transcriptional activity, whereas mutation to negatively charged aspartate residues (representing phosphorylation) reduced it.

PPARβ/δ

While PPARα and PPARγ have been extensively studied over many years, much less is known about the function of the PPARβ/δ isotype. Studies with genetically modified PPARβ/δ mice have illustrated the importance of this nuclear receptor in white adipose tissue and skeletal muscle, two organs that have a key role in glucose homeostasis (134, 211, 230-231). It was shown that activation of PPARβ/δ in adipose tissue protects against adiposity and hyperlipidemia by inducing FA catabolism (231).

PPARβ/δ is expressed ubiquitiously and has been implicated in a variety of cellular processes ranging from regulation of FA oxidation and inflammation to wound healing in skin (117). However, within the liver, PPARβ/δ has been found to be differentially expressed in different cell types with the highest levels reportedly found in hepatic endothelial cells (81).

PPARγ

PPARγ serves as the molecular target for an important class of anti-diabetic drugs, the TZDs, and is activated by polyunsaturated FAs (PUFAs) and FA-derived molecules. PPARγ expression is highest in adipose tissues where it plays an important role in the process of cell differentiation and acquisition of the adipocyte phenotype (103, 189). Most of the target genes of PPARγ are involved in adipogenesis and lipogenesis, including FA binding protein 4, glucose transporter (GLUT)-4, glycerol 3-phosphate dehydrogenase, lipoprotein lipase (LPL) and glycerol kinase (109). PPARγ activation by TZDs effectively lowers plasma glucose levels by promoting insulin responsiveness in

tissues, thereby stimulating glucose uptake. In rodents, TZDs also reduce plasma FFA concentrations (152, 209).

Several studies have shown that the expression of lipogenic and adipogenic genes such as sterol regulatory element binding protein (SREBP), ADRP and PPARγ are strongly up-regulated in steatotic livers (133, 194). High fat diets up-regulated PPARγ in the hepatic tissue of PPARα$^{-/-}$ mice and increased the expression of adipocyte markers, which may be a contributing factor to the development of fatty liver (166).

2.2 Conjugated Linoleic Acid (CLA)

2.2.1 Source and Synthesis

CLA consists of several conjugated and stereoisomeric variations of LA (*cis*, *cis*-Δ9,12-octadecadienic acid) with a unique spatial structure and distinct biological functions. The c9,t11 and t10,c12 isomers are the most well-studied CLA isomers because of their unique physiological effects (157).

Source

CLA can be found naturally in ruminant products, such as milk, cheese and beef (72, 164, 196), and exists predominantly (80-90%) as c9,t11 isomer, which is also known as rumenic acid (119, 129, 164).

Some of the dietary supplements contain a 50:50 mixture of the c9,t11- and t10,c12-CLA isomers and are now readily available in the market (105). However, specific benefits for humans appear to be relatively small and conflicting. Therefore, further investigations are still required to confirm the presence or absence of any detrimental effects in humans.

Synthesis

Kay et al. (92) have shown that the endogenous synthesis is responsible for more than 91% of the c9,t11-CLA isomer found in milk fat of cows fed fresh pasture. In ruminants, CLA is either absorbed or further metabolized to vaccenic acid (*trans*-11-octadecenoic acid) (95) in the gut, which can be again converted by the enzyme Δ9 desaturase back to the c9,t11-CLA isoform (157, 175).

CLA can also be chemically synthesized by several methods (21, 44-45). CLA food supplements are currently synthesized by alkaline isomerization of LA enriched vegetable oils such as safflower and sunflower oil and are mostly available in a 1:1 ratio of c9,t11- and t10,c12-CLA isomeric mixture (129, 214).

2.2.2 Physiological Effects

A mixture of CLA isomers was shown to prevent cancer in an experiment with fried ground beef conducted in mice that were chemically treated to induce epidermal neoplasia (72). This initial discovery of the anti-carcinogenic property of the isomeric mixtures of CLA ignited a considerable amount of research (4, 157)**.**

Several experiments have reported that CLA isomers or mixtures have beneficial effects against obesity, diabetes, insulin resistance, hepatic steatosis, atherosclerosis and cancer *in vivo* and *in vitro*. Variations on some of these effects have also been reported between species, and also between the c9,t11 and t10,c12 CLA isomers with the t10,c12 CLA isomer apparently responsible for changes in body composition and adipocyte morphology (34, 39, 54, 76, 157, 162).

A brief synopsis of some of the physiological effects of CLA is provided below with a specific focus on NAFLD.

i. Obesity and Insulin-Resistance

Park et al. (161) first observed the delipidative effects of a CLA mixture in the ICR line of mice where a CLA-supplemented diet (5.0% corn oil plus 0.5% CLA) produced about a 60% decrease in body fat after four to five weeks of feeding. Several studies have reported the body fat lowering effect of the t10,c12 CLA isomer in several species, including mice, rats, chickens, pigs and humans (82, 158). Dietary CLA mixtures have been reported to reduce adiposity in Sprague-Dawley and Zucker rats (11, 200, 247). However, the opposite effect was observed in obese Zucker rats fed 0.5% dietary CLA (200). Several studies in swine have reported that CLA mixtures decreased fat deposition and increased lean tissue (155-156, 215, 237). The t10,c12 CLA isomer significantly reduced adipose tissue mass but did not significantly increase fat accumulation in liver or muscle, potentially confirming observations in earlier experiments that CLA increases energy expenditure in mice (43, 91, 235-236).

The *fa/fa* Zucker rat has a mutation in the leptin receptor and is considered a model for the metabolic syndrome since it is morbidly obese as well as insulin resistant. Noto et al. (149) observed an improvement in oral glucose tolerance and higher adipose GLUT-4 mRNA levels when 6 week old *fa/fa* Zucker rats were fed a 50:50 mixture of CLA for 8 weeks, but there was no decrease in body weight. Studies involving this animal model have shown the isomer-specific effects of CLA with the t10,c12 CLA isomer being responsible for inducing weight loss, improving muscle glucose transport

and decreasing fasting glucose and insulin levels (80). However, no changes in body weight, adiposity, muscle glucose or insulin levels were observed with the c9,t11 CLA isomer (80). Noto et al. (151) observed significantly larger adipocytes in the *fa/fa* control rats than those fed the t10,c12 CLA isomer suggesting an improvement in adipocyte function or an improved ability to mobilize stored lipids.

The effect of CLA on body composition changes in humans remains unclear. Most of the research in humans has shown that CLA isomers do not have a significant effect on body weight (105, 122, 214). A CLA mixture reduced body fat mass but had no effect on BMI in a randomized double-blind trial using 60 overweight or obese people (19). The same study also reported that no additional effect on body fat mass is achieved with doses > 3.4 g CLA per day. Similar effects were observed in other human trials (186, 202, 217).

Krieder et al. (104) evaluated the effects of supplementation with a CLA mixture on body composition in 24 resistance trained males for 28 days. No significant differences were reported in this short term study, although trends for improved strength and performance were reported for the CLA group. Interestingly, the loss of body fat induced by CLA mixture is 40-50% greater in mice fed diets containing 0.5-1% CLA as compared to that observed in humans (214). These differences may be attributed to the duration, dose of CLA, and age of species. Generally, studies in mice are conducted with a much higher dose than humans, and mostly involve growing animals. Further investigation in humans with higher dose and longer duration will be needed to understand the role of CLA in humans (143).

ii. Diabetes

A CLA mixture was shown to improve hyperinsulinemia and normalize glucose tolerance in Zucker diabetic *fa/fa* (ZDF) rats (83), a finding that was later confirmed by Ryder et al. (190) who reported improved insulin-stimulated glucose tolerance and glycogen synthase activity in the soleus muscle of ZDF rats. They also showed that these effects were predominantly exerted by the t10,c12 CLA isomer (190). Several studies have also confirmed these results in *fa/fa* Zucker rats and suggested that the t10,c12 CLA isomer exerts its anti-diabetogenic effects through a reduction in oxidative stress and muscle lipid levels (80, 213).

Nagao et al. (140) showed that severe hyperinsulinemia in ZDF rats was attenuated with a CLA mixture and that this response was associated with an enhanced level of plasma adiponectin and improved insulin sensitivity. In contrast, several studies have shown that t10,c12 CLA supplementation leads to insulin resistance in mice (34, 42, 188, 223) and humans (185-186), which may be attributed to a decrease in plasma leptin levels (230) or an increase in TG levels in the muscle (214). These results show opposite effects of CLA in mice and humans compared to rats, thereby emphasizing the importance of species-specific differences associated with the supplementation of CLA in diabetes.

iii. Atherosclerosis

Lee et al. (107) found that a CLA mixture (0.5 g CLA/rabbit per day) had anti-atherosclerotic effects in rabbits based on a decrease in total and LDL cholesterol and TGs, as well as decreased atherosclerotic plaque in the aorta. Similar results were

reported in hamsters with diet-induced hypercholesterolemia where a CLA mixture reduced total plasma cholesterol (147, 239). However, one study in hamsters fed a diet supplemented with either both isomers (c9,t11 and t10,c12 CLA) or pure c9,t11 CLA found a reduction in plasma lipids with the mixed isomers only (65). In contrast, a dietary CLA mixture was reported to increase aortic fatty streak formation in C57BL/6mice (135).

In humans, no differences were observed in platelet aggregation, thrombosis, plasma cholesterol, LDLs or TGs with a CLA mixture intervention (16-17). Interestingly, a study showed that both the CLA isomers (c9,t11 or t10,c12) have an effect on human platelet aggregation and suggested that CLA isomers have anti-thrombotic properties (221). Subsequently, Toomey et al. (220) showed that a dietary CLA mixture prevented the development of lesions and caused their regression in apolipoprotein E (apoE) null mice with pre-established atherosclerotic lesions.

iv. Cancer

Ha et al. (72) have contributed immensely on the anti-carcinogenic effects of isomeric mixtures of CLA with their experiments in a mouse model treated with 7,12-dimethylbenz[α]anthracene to induce epidermal neoplasia. They also showed that CLA mixtures inhibited mutagenesis in bacteria. The group later reported that CLA mixtures had antioxidant properties and inhibited tumorigenesis in the forestomach of mice (73).

The c9,t11-CLA isomer was predominantly involved in the anti-carcinogenic effect, since it was the only isomer present in liver and mammary tumor extracts (87). Several studies have indicated that CLA may exert its anti-carcinogenic effects by

regulating the cell cycle (25, 88, 120) and apoptotic pathway (89, 153-154, 159-160). In contrast, some experiments did not observe any anti-carcinogenic effect (173, 242). As such, the use of CLA in cancer therapy should be further investigated.

v. NAFLD

CLA supplementation produces markedly different responses in the liver of rats and mice. In mice, the t10,c12-CLA isomer predominantly exerts steatotic effects and increases the mass of the liver up to four times (15, 34, 40, 94, 223). A study in mice attributed this to an increase in liver TG, cholesterol, cholesterol esters and FFAs (94). In contrast, CLA mixtures have been shown to decrease hepatic TG accumulation in the ZDF rat (234), Zucker *fa/fa* rat (139, 150) and Otsuka Long-Evans Tokushima Fatty (OLETF) rat (181) models. The t10,c12 CLA isomer has been shown to decrease liver TG in OLETF rats (229) and *fa/fa* Zucker rats (205). This effect was not observed with the c9,t11 CLA isomer (195).

2.2.3 Potential Mechanisms by which CLA affects NAFLD

To date, the triggers of fatty liver or hepatic steatosis and the mechanisms by which it relates to excessive lipid accumulation the liver are incompletely understood. The molecular mechanism underlying the development of steatosis and progression to steatohepatitis has not been delineated. Likewise, the factors underlying the effect of CLA isomers on NAFLD are unclear.

LD Formation

Recently, LDs have emerged as important intracellular organelles in lipid homeostasis and are important for FA and sterol biosynthesis, FA activation and lipolysis and contribute to the pathophysiology of metabolic diseases, particularly in fatty livers. (61).

A few recent studies have highlighted the role of LD-associated proteins in fatty liver using *in vitro* and *in vivo* models (74, 169, 205). Chung et al. (31) showed that LD-associated proteins play an important role in adipocyte TG metabolism and demonstrated that the t10,c12 CLA-mediated reduction of human adipocyte TG content is associated with the differential localization and expression of ADRP and perilipin A protein.

When energy is required, the stored TGs in LDs are hydrolyzed *via* activation of lipolytic pathways that govern the release of stored FAs from the adipocyte TG pool which constitutes the major energy reserve in animals. The released FAs are then transported by serum albumin to various tissues to provide energy.

A CLA mixture markedly alleviated hepatomegaly and hepatic TG accumulation (139, 150) with improved liver function (lower serum ALT and alkaline phosphatase) and favorable modification of the serum lipoprotein profile (reduced VLDL and LDL and elevated HDL) after 8 weeks in *fa/fa* Zucker rats. Liver FA binding protein (L-FABP) and ACOX, markers of FA transport and oxidation, respectively, were expressed at higher levels with the supplementation of 1.5% CLA mixture in *fa/fa* Zucker rats (150).

A recent study from our lab has shown that the anti-steatotic effects of t10,c12 CLA isomer in obese *fa/fa* Zucker rats were associated with a significant decrease in hepatic adipophilin, but not perilipin levels, and reduced LD size in the liver (205). The

same study (205) also demonstrated that treatment of H4IIE rat hepatoma cells with CLA isomers (c9,t11 or t10,c12) neither prevented nor reversed, but rather induced cytoplasmic LD formation in these cells. Currently, mechanisms by which CLA isomers affect hepatic steatosis are unclear. CLA may be working either independently or dependently on PPARα or by regulating other molecules. H4IIE cells can thus be a valuable tool in elucidating a mechanism for the effects of CLA on fatty liver.

Genes involved in FA Synthesis and Oxidation

A CLA mixture was observed to increase both liver FA synthesis and oxidation in rats (15, 134, 170)). These observations were also confirmed at both the gene and protein level in two lines of mice (ICR and C57BL/6J) (207). However, the degree of increase differed between lines for some enzymes involved in these two processes. For FA synthesis, they reported an increase in activity and mRNA levels of acetyl Co-A carboxylase (ACC), FA synthase (FAS), and malic enzyme in CLA-treated mice (207). However, an increase in ACC mRNA, but not FAS, was reported by Tsuboyama-Kasaoka et al. (222-223). For FA oxidation, an increase in the activity of mitochondrial and peroxisomal palmitoyl-CoA oxidation, CPTI, peroxisomal ACOX, 3-hydroxyacyl-CoA dehydrogenase, and an increase in mRNA levels of CPTI and II, ACOX and bifunctional enzyme was reported in CLA-treated mice (207). Several studies have also reported an increase in CPTI activity, with a greater effect being exerted by the t10,c12 CLA isomer (41, 128). An increase in ACOX activity and gene expression and CPTI activity coupled with an increased rate of carnitine-dependent palmitate oxidation was reported in mice supplemented with the t10,c12 CLA isomer. In addition, an increase in

the expression of CPTI in the liver (100%) and muscle (200%) with an almost doubled expression of CPTII was observed (41).

A recent study reported that CLA is metabolized by hepatocytes at a higher rate than LA and that CLA is a poorer substrate for cellular and VLDL-TG synthesis (177). These data suggest that increased FA oxidation with consequent decreased FA availability for TG synthesis is a potential mechanism by which CLA can reduce TG levels in rat liver (177).

ACS

Other investigators have reported that triacsin C, a selective inhibitor of ACS, inhibited TG synthesis by 70% in hepatocytes from fed rats and by 40% in starved rats and suggested greater inhibition of *de novo* TG synthesis than FA reacylation (136). Triacsin C also inhibited the synthesis of cholesteryl ester and TG in mouse peritoneal macrophages, leading to a reduction of LD formation (141). Matsuda et al. (130) demonstrated that triacsin C has anti-atherogenic properties in LDL receptor-knockout ($LDLR^{-/-}$) mice fed a high cholesterol diet. Incubation with 5 µM triacsin C completely abolished LD formation in anti-Fas-treated apoptotic human HuT 78 cells, with concomitant reversion of TG, CE and phospholipids to control levels (86). Therefore, ACS is an important enzyme in the regulation of TG levels and subsequent LD formation, and represents a potential mechanism by which CLA isomers may affect hepatic lipid metabolism.

PPARα

CLA isomers increase the expression of ACOX, cytochrome P450A1 (CYP4A1: ω-hydroxylation of FAs) and L-FABP, all known target genes of PPARα (134, 150, 232).

In contrast, a study conducted by Peters et al. (170) in PPARα null mice showed that genes coding liver FA oxidation and FA binding were affected by CLA, thus indicating a PPARα independent mode of action by CLA. Isomer-specific effects of CLA were also reported with t10,c12-CLA causing a decrease in PPARα expression and an increase in ACO (but not CYP4A1), and with c9,t11-CLA causing an increase in PPARα expression (232).

Since PPARα is the main PPAR isoform expressed in liver and it has been shown to regulate the expression of genes involved in hepatic oxidation (97, 170), it would be interesting to study the effects of CLA on this transcription factor in the process of LD formation resulting in lipid accumulation.

Desaturase index

The ratio of SFAs and MUFAs is one of the determining factors in understanding disease conditions related to abnormal lipid metabolism such as hepatic steatosis. Several experiments have confirmed a shift in the ratio of SFA:MUFA *in vivo* and *in vitro*, particularly palmitate:palmitoleate (16:0/16:1) and stearate:oleate (18:0/18:1) with t10,c12 CLA supplementation (23, 29, 53-54, 64, 108, 195). These results implicate an important role of scd1, the enzyme that catalyzes the biosynthesis of MUFAs (91, 108). In contrast, a recent study in mice fed a diet supplemented with 0.5 g/100 g of CLA mixture for 4 days found no change on the SFA:MUFA ratio (245), suggesting that the change may be due to downstream, as opposed to direct effects of CLA. Another study demonstrated the scd1 independent delipidative effects of t10,c12-CLA isomer using scd1 null mice, which displayed reduced hepatomegaly with the supplementation of this isomer (91). The t10,c12-CLA isomer was also associated with an increase in 18:1 n-9

and a decrease in 18:2 n-6 which may be attributed to changes in the activity of desaturase enzymes such as scd1 and thereby alterating the FA profiles (94).

2.3 Effects of Lipid Accumulation on Cell Viability

The concentration-dependent accumulation of lipid as well as mitochondrial damage are regarded as two early events for hepatotoxicity (198). Culturing of trophoblasts (isolated from normal term human placentas) in 0.25 mM non-esterified FA (NEFA) for 24 hours up-regulated FA esterification processes, inhibited FA oxidation, inhibited glycerol release (a marker of lipolysis) and promoted ADRP and LD formation, all consistent with the up-regulation of FA storage and buffering capacity. However, there was no effect on cell viability, apoptosis or hormone secretion. HepG2 cells displayed elevated oxidative stress, loss of mitochondrial function and loss of viability when challenged with pro-oxidants such as ethanol or PUFAs such as arachidonic acid (243). Exposure of murine or human hepatocytes to MUFAs resulted in lipid accumulation without changes in cell viability. In contrast, incubating cells with SFAs significantly decreased cell viability and increased caspase activation and apoptosis, with only minor LD accumulation.

To date, one study has determined the dose-dependent cytotoxicty of mixed CLA or CLA isomers in rat hepatic stellate cells (HSC-T6), incubated for 24 hours with 10-180 µM of t10,c12 , c9,t11 or a mixed form (c9,t11:t10,c12; 41%:44%) of CLA (251). The results from this study showed that the c9,t11 CLA isomer exhibited the most intense cytotoxic effect as the survival rate of the HSCs was reduced to 60% with 80 µM of c9,t11 CLA treatment, while cell survival was only slightly affected by the CLA mixture.

The degree of DNA fragmentation was most severely affected in HSCs treated with 80 µM of c9,t11 CLA. Other investigators reported that after 48 hours supplementation with 50 µM t10, c12 CLA isomer or 50 µM of c9, t11 CLA isomer, mouse mammary tumor cell viability was significantly reduced by 80% and 20%, respectively (98). Although a certain amount of lipid storage may even be hepatoprotective, prolonged excess lipid storage can result in an activation of inflammatory reactions and loss of metabolic competency (7). The cytotoxicity of CLA isomers, the t10,c12 isomer in particular, has been previously reported for rat hepatomas, however, no direct link to LD formation was shown (247).

2.4 Treatment of NAFLD

There is no one treatment available for NAFLD, however, PPARγ agonists (TZDs) have been reported to decrease liver fat content and increase hepatic insulin sensitivity in patients with T2DM. Metformin, which activates AMP-dependent kinase (AMPK), on the other hand, has been reported to only ameliorate hepatic insulin sensitivity but may not change liver fat content (219, 249).

2.5 Summary of Current State of Knowledge

The t10,c12 CLA isomer appears to have an indirect effect on reducing hepatic steatosis in *fa/fa* Zucker rats while both CLA isomers appear to promote LD formation in H4IIE cell cultures. The mechanism for this difference is currently unknown. Some evidence indicates that triacsin C and Wy14643 reduce TG accumulation in lipid loaded

hepatocytes, implicating the importance of ACS and PPARα in LD formation. CLA may be increasing FA uptake and directing the pool of activated FA acyl CoAs toward a lipid storage pathway *via* the activation of the ACS in H4IIE cells. On the other hand, the effect of CLA isomers on H4IIE viability relative to LD formation remains to be explored.

In addition to its ligand-induced activation, transcriptional activity of PPARα is regulated by phosphorylation mainly *via* ERK-MAPK (49, 208). However, the isomer-specific effects of the c9,t11 or t10,c12 CLA isomers on PPARα phosphorylation remain unexplored.

3. MATERIALS AND METHODS

3.1 Rat H4IIE Hepatocytes

3.1.1 Cell Culture

Rat H4IIE hepatoma cells (American Type Culture Collection, CRL 1548) were maintained as previously described (252). Cells were grown to 60-70% confluency on a 12-, 24- or a 96-well plate in alpha-Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS). Quiescence was achieved by incubation in serum-free media for 72 hours.

3.1.2 Cell Treatments

The inhibitors or activators were typically added 10 minutes prior to the addition of the FAs directly into the cell culture media in order to allow enough time for the activation of specific transcription factors or signal transduction pathways (253-254). The chemicals are listed in Table 1. The optimal concentration of the chemicals was determined following a dose-response and a time-course study for up to 24 hours.

3.1.3 Cell Lysing

The treatments were terminated by aspirating the media from the wells and washing the cells twice with 2 mL of 1× Phosphate Buffered Saline (PBS) (Thermo Scientific, Rockford, IL). Two hundred µL of 2× sample buffer (0.5 M Tris-HCl pH 6.8; 10% SDS; Glycerol) was added to the cells, which were allowed to lyse by incubating at 37 °C for 5 min. The cells and the solubilised material were pipetted into separate

microfuge tubes and sonicated to shear the DNA. The samples were then stored at -80^{o}C for further analysis.

3.1.4 Oil Red O Staining

Lipid accumulation in H4IIE cells was quantified by using Oil Red O staining as previously described (132). Cells were fixed with 10% phosphate-buffered formalin for 1 hour and washed with 60% isopropanol. The cells were allowed to completely dry before staining with Oil Red O for 10 minutes. The Oil Red O stain was then washed under tap water followed by elution of the stain using 100% 2-propanol for 10 minutes.

The optical density (OD) of the eluate was measured at 500 nm using a FLUOstar Omega plate reader and analyzed with Omega Data Analysis Software version 1.00 (BMG Labtech, Durham, NC) in order to quantify the degree of staining and, therefore, the amount of lipid present in the cells.

3.1.5 Cell Viability Assay

Cell viability in H4IIE cells was assessed using the cell counting kit-8 (cck-8) assay that utilizes the tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]. WST-8 is reduced by dehydrogenases in cells to give a yellow-colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. The cck-8 assay is much more sensitive and has many advantages over the conventional MTT, XTT, MTS or WST-1 assays. It consists of a ready-to-use solution and does not require any organic solvent or isotope. There is also no need for any harvesting, washing or

solubilization step and, therefore, experimental error is greatly reduced.

H4IIE rat hepatoma cells were seeded in 96-well plates (5,000 cells/well) and grown to 60-70% confluency in α-MEM supplemented with 10% FBS. Quiescence was achieved by incubation in serum-free media for 72 hours.

Required materials

- Ready to use cck-8 solution
- FLUOstar Omega plate reader (450 nm filter)
- 96-well plate
- CO_2 incubator

Cells were treated in triplicate and then incubated for another 24 hours at 37 °C. Ten µl of the cck-8 solution was added to each well of the plate. After 4 hours of incubation at 37 °C in a CO_2 incubator, absorbance was measured using a FLUOstar Omega plate reader at 450 nm.

3.2 Liver Tissue

Livers from seven-week old male lean and *fa/fa* Zucker rats fed either a control diet (the ln CTL and fa CTL groups, respectively) or a diet containing 0.4% wt/wt *cis*-9, *trans*-11 (fa 9,11 group) or 0.4% wt/wt *trans*-10,*cis*-12 (fa 10,12 group) in free fatty acid (FFA) form for 8 weeks (kindly provided by Danielle Stringer) were excised, rinsed in phosphate-buffered saline (PBS) pH 7.4, weighed, and snap-frozen in liquid nitrogen before being stored at -80°C for further studies.

3.2.1 Protein Extraction from the Hepatic Tissue of Zucker Rats

For the quantification of proteins present in the hepatic tissue of the Zucker rats, livers were homogenised in a detergent-containing solution in order to release the various proteins. The homogenate was then centrifuged to remove insoluble materials and make the proteins available for analysis by immunoblotting.

Required reagents

- 3× Sample Buffer: 3% SDS, 30% glycerol, 0.2 M Tris-HCl pH 6.8, ddH_2O)
- Liquid nitrogen (N_2)

Hepatic tissue was weighed (40-50 mg) and quickly covered with liquid N_2 in a mortar and allowed to freeze. It was ground into a fine powder with a pestle and 3× sample buffer was added to the powder which was then stirred into a uniform paste and allowed to sit for a minimum of 15 minutes to permit lysis of the cells. The suspension was pipetted into a 1.5 ml microfuge tube and centrifuged (Eppendorf Centrifuge 5804, Hamburg, Germany) at 15,294 g for 5 minutes. After centrifugation, the supernatant was removed and put into another tube and sonicated (Sonic Dismembrator, Model 100, Thermo Fisher Scientific Inc.) for 15 seconds to shear the DNA, thereby decreasing the viscosity of the sample. The samples were then quantified or stored at -80^{o}C for further analysis.

3.3 Quantification of Protein Samples

Following sonication, protein concentration of the Zucker rat hepatic tissue and the H4IIE cell lysates was determined by using the Bicinchoninic acid (BCA) protein

assay (Pierce Rockford, IL) to enable equal protein loading onto the SDS-polyacrylamide gels. The reaction between the BCA reagents and the proteins produces a colored product and the intensity can be measured by spectrometric analysis at 550 nm. The measured intensity is directly proportional to the amount of protein in each sample. The results from each assay are compared to a standard calibration curve and the quantity of protein is calculated.

Required reagents

- Pierce protein assay reagent A (Cat #23223, Thermo Scientific, Rockford, IL): sodium bicarbonate, BCA and sodium tartrate in 0.2 M sodium hydroxide
- Pierce protein assay reagent B (Cat #23224, Thermo Scientific, Rockford, IL): cupric sulphate
- Protein standard: 2 mg/mL bovine serum albumin standard (Cat # 23209, Thermo Scientific, Rockford, IL) diluted with 3× sample buffer for tissue samples or with 2× sample buffer for H4IIE cell lysates to produce 6 standards (0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mg/mL)
- 2× sample buffer (0.5 M Tris HCl pH 6.8; 10% SDS; Glycerol (Cat # BP229-1, Fisher))
- 3× Sample Buffer (3% SDS, 30% Glycerol, 0.2 M Tris-HCl pH 6.8, ddH_2O)

Ten microlitres of each of the standards and the protein samples were pipetted, in triplicate, into the wells of a 96-well microplate (Cat #167008, Nunclon, Roskilde, Denmark). A ratio of 221 μL of reagent A to 4 μL of reagent B was mixed in a volume to allow 200 μL to be pipetted into each well. The plate was incubated at 37^{o}C for 30 minutes and then read at 550 nm on a microplate reader (Thermomax, Molecular Devices

Corp, Sunnyvale, CA). Softmax Pro Software (Version 2.34, Molecular Devices Corp, Sunnyvale, CA) was used to determine the final protein concentration in each sample.

3.4 Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

After quantification of the protein extracts from the liver tissue or the H4IIE cell lysates, SDS-PAGE was used to separate the various proteins according to their molecular mass. Samples were loaded into the wells of the gel and a current was applied. The current draws the denatured and negatively charged proteins though the gel depending on the molecular mass.

Required reagents

- 20% acrylamide
- 10% SDS
- 1.5 M Tris-HCl pH 8.8
- 10% ammonium persulfate
- N, N, N', N'-Tetramethylethylenediamine (TEMED) (Cat # 805615, MP Biomedicals, Solon, OH)
- ddH_2O
- 0.5 M Tris-HCl pH 6.8
- H_2O-saturated butanol
- 10% Bromophenol blue
- 2-mercaptoethanol (Cat # 6010, Omnipur, U.S.A)

- SDS-PAGE electrode buffer: 0.125 M Tris, 0.96 M glycine, 0.5% SDS

One or 1.5 mm spaced glass plates were inserted into a sandwich clamp assembly, placed into a casting stand and the appropriate separating gel percent was poured between the glass plates. Using a pasteur pipette, a small amount of H_2O-saturated butanol was gently added over the top of the separating gel to make the surface of the gel smooth and even. The gel was left to polymerize for approximately 30 min, then the H_2O-saturated butanol was discarded and the gel was rinsed with ddH_2O. The stacking gel was subsequently poured on top of the separating gel. A 10 or 15 well comb was inserted and the gel was left to polymerize for 30 minutes. When the stacking gel had polymerized, the sandwich clamp assembly was transferred to the electrophoresis apparatus and the SDS-PAGE buffer was poured into the middle of the electrophoresis apparatus as well as into the buffer tank.

The concentration of protein determined from the BCA protein assay was used to calculate the appropriate volume of each sample. Each protein sample was mixed 1:1 with a solution of bromophenol blue (to help visualize the dye front) and 2-mercaptoethanol (to denature the proteins). The samples were heated in a 90^{o}C water bath for 5 minutes to further denature the proteins. Protein samples (10-15 µg) were separated on SDS-polyacrylamide gels. The gel percentage depended on the size of the desired protein with smaller proteins (< 30 kDa) run on a 15% gel, 30-100 kDa proteins run on 7.5% or 10% gels and proteins >100 kDa were run on a 5% gel. The gel was run at 20 mA constant current per gel for 70-90 minutes. To determine the molecular mass, the Bench Mark^{TM} Prestained Protein Ladder (BioRad) was used.

3.5 Gel Transfer

Following SDS-PAGE, the proteins in the gel were electrophoretically transferred to a 6.8 cm ×8 cm polyvinylidene difluoride (PVDF) membrane.

Required reagents

Transfer Buffer

- 20% methanol, 0.25 mM Tris, 130 mM glycine, ddH_2O
- 5× Tris-buffered saline in Tween-20 (TBST): 0.1 M Tris-HCl pH 7.4, 0.25% Tween-20
- 1× TBST: 1 part 5× TBST and 4 parts ddH_2O
- Methanol (Fisher Scientific, Fair Lawn, NJ)

The glass plates from the SDS-PAGE were removed and the stacking gel was discarded. The PVDF membrane was first equilibrated in the transfer buffer for 5 minutes and then placed on top of the gel while immersed in transfer buffer. The membrane was covered with blotting paper on both sides. Any bubbles were pressed out and the components were placed inside a transfer cassette in the transfer buffer solution. The transfer buffer solution was poured into the tank after addition of an ice pack and a stir bar and placed on a magnetic stirrer on medium speed. Electrode wires were attached to the electrode module and current applied at 100 volts (60 minutes for a 1.0 mm thick gel or 75 minutes for 1.5 mm thick gel). Membranes with the transferred proteins were removed and placed in a container with 1× TBST covering the membrane and stored at 4^oC.

3.6 Immunoblotting

Immunoblotting was used to identify the proteins of interest using a method adapted from Gallagher et al (63). This procedure uses an antibody to detect and quantify a single protein after electrophoretic transfer of a protein mixture to a PVDF membrane.

Required reagents

- 1× TBST
- 3% BSA in TBST (Bovine serum albumin fraction V, Cat #10735080001, Roche Diagnostics, Mannheim, Germany)
- 1% BSA in TBST
- Lumigen PS-3 detection reagent (Ref # RPN2132 V1 + V2, Lumigen PS3, GE Health, Piscataway, NJ)
- Primary (1^o) antibody
- Horseradish peroxidase (HRP)-linked secondary (2^o) antibody

The membranes were agitated on an orbital shaker, blocked with 3% BSA-TBST (bovine serum albumin in Tris-buffer saline with Tween-20) for 1 hour followed by 1 hour incubation with the 1^o antibody at room temperature (RT). Subsequently, the 1^o antibody was removed and the membrane was washed for a minimum of 20 minutes in 1× TBST, with the TBST refreshed each time. The membrane was then incubated with HRP-conjugated 2^o antibody for 1 hour and the washing step was repeated as previously described for the 1^o antibody. All 2^o antibodies were used at a dilution of 1:10,000 in PBS containing 1% BSA. The 1^o and the 2^o antibodies are listed in Table 2 and Table 3, respectively. Afterwards, the membrane was dipped in ECL plus Western blotting detection system (GE Health Care) and the relative intensity was captured by exposing

the membrane to an X-ray film (Kodak Scientific Imaging). The band of interest on the film was later quantified using Quantity One software (version 4.5.0) on a GS-800 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA).

Optimisation of the 1^{o} antibody

One of the most common problems in immunoblotting is determining the correct dilution of the 1^{o} antibody. Therefore, it is imperative to optimise the 1^{o} antibody concentrations which may require contacting the antibody manufacturing companies and/or other researchers who have made use of the antibodies in their publications.

All of the 1^{o} antibodies were used at a 1:1000 dilution in this study. However, the main difference was between the PPARα and the other antibodies. A much better result was achieved with 2 hours of blocking for all the PPARα antibodies. In addition, the non-phospho-PPARα antibody required an overnight incubation while the phospho-PPARα antibodies gave a good signal with the usual 1 hour incubation.

3.7 Stripping of PVDF Membranes

The PVDF membrane was stripped and washed so that it could be re-probed with a new antibody and/or a loading control.

Required reagents

Stripping buffer

- 10% SDS; 0.5 M Tris-HCl pH 6.8; ddH_2O
- 2-mercaptoethanol
- 1× TBST
- 10% Bleach

In order to strip the PVDF membrane, it was covered with 25 mL of stripping buffer per membrane and 80 μL of 2-mercaptoethanol was added for every 10 mL of the stripping buffer. This procedure was carried out in a fume-hood. The container was completely sealed and placed in a larger container which was again sealed in order to reduce the strong smell of the reagents. The container was agitated overnight at RT following which the solution was poured down the sink in the fume hood. The membrane was placed in a new container and covered with 1× TBST and washed by agitation for 5 minutes. This process was repeated until no odour was detected. The old containers were rinsed with 10% bleach and left in the fume hood until the odour dissipated.

3.8 Data Analysis

Statistical analysis was performed using one way Analysis of Variance (ANOVA) with Statistical Analysis Software (SAS Version 9.1.3, SAS Institute Inc., Cary, NC.) for Western blot data using a mixed-model analysis with a random intercept for the repetitions and by estimate statements for individual comparisons of treatments versus the control. For all other *in vitro* data, statistical analysis was performed using one way ANOVA with Origin. Significant differences among treatment group means were determined with Tukey's test. Differences were considered statistically significant at $p<0.05$ and are indicated by different lowercase letters, n=3. Data are expressed as mean ± standard error of the mean (SEM).

Table 1. List of Chemicals

Chemical	**Functions**	**Working Conc.**	**Vehicle**	**Company**	**Catalog #**
Dexamethasone	synthetic glucocorticoid	0.25 μM	Water	Sigma	D4902
Wy14643	PPARα agonist	250 μM	DMSO	Tokyo Kasei Kogyo	FII02
Triacsin C	ACS Inhibitor	4 μM	DMSO	Biomol International	EI218
c9,t11 CLA	*cis*-9,*trans*-11 CLA isomer	60 μM	Ethanol	Cayman Chemical Co.	90140
t10,c12 CLA	*trans*-10,*cis*-12 CLA isomer	60 μM	Ethanol	Cayman Chemical Co.	90145
Linoleic acid	PPARα agonist	60 μM	Ethanol	Cayman Chemical Co.	90150

Table 2. List of Primary Antibodies

Primary Antibody	**Size (kDa)**	**Secondary**	**Company**	**Catalog #**
PPARα (H-98)	52	Rabbit	Santa Cruz	9000
Phospho PPARα (Ser 12)	52	Rabbit	Abcam	3484
Phospho PPARα (Ser 21)	52	Rabbit	Abcam	3485
Phospho MAPK p44/42 (Thr 202/Tyr 204)	44, 42	Rabbit	Cell Signaling	9101
MAPK p44/42	44, 42	Rabbit	Cell Signaling	9102
Phospho MAPK p38	43	Rabbit	Cell Signaling	9211
MAPK p38	43	Rabbit	Cell Signaling	9212
scd1 (E-15)	37	Goat	Santa Cruz	14720
eEF2	95	Rabbit	Cell Signaling	2332

Table 3. List of Secondary Antibodies

Secondary Antibody	**Working Dilution**	**Company**	**Catalog #**
HRP Rabbit: Blotting grade goat anti-rabbit IgG (H + L)(Human IgG adsorbed) HRP	1:10000	BioRad	170-6515
HRP Goat: Peroxidase-conjugated chrom-pure Goat IgG	1:10000	Jackson Immunoresearch Laboratories	005-030-003

4. RESULTS

4.1 Dose response of CLA isomers and linoleic acid on LD formation in rat hepatoma cells

Rat H4IIE hepatoma cells were grown to 60-70% confluency on a 12- or 24-well plate in alpha-Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS). Quiescence was achieved by incubation in serum-free media for 72 hours. Quiescent H4IIE cells were treated with different concentrations (60 µM, 120 µM and 180 µM) of *cis*-9,*trans*-11 (c9,t11) or *trans*-10,*cis*-12 (t10,c12) CLA isomers or linoleic acid (LA). Cells without any of the above treatments were included as controls. The cells were incubated for 24 hours at 37 °C. In order to assess lipid accumulation, the cells were stained with Oil Red O to visualize LDs and counter-stained with hematoxylin to assess the histological appearance after treatment with respective CLA isomer or LA. Compared to the untreated controls, treatment of H4IIE cells with individual CLA isomers or LA resulted in significant LD formation in a dose-dependent manner (Figures 1-6). However, there was no significant difference in lipid accumulation at higher concentrations, i.e. between 120 µM and 180 µM of c9,t11 CLA (Figure 2) or t10,c12 CLA (Figure 4) or LA (Figure 6) which neither increased nor decreased relative to 60 µM but remained at a plateau phase. Interestingly, even though treatment with 180 µM of LA resulted in a higher LD concentration, fewer cells were observed in comparison with the control or lower concentrations of LA (Figure 6). A possible explanation is that most of the cells treated with 180 µM LA were stained more due to a much larger number of LDs

in these cells. Alternatively, cell death led to LD deposition onto the plate and this material was stained with the Oil Red O.

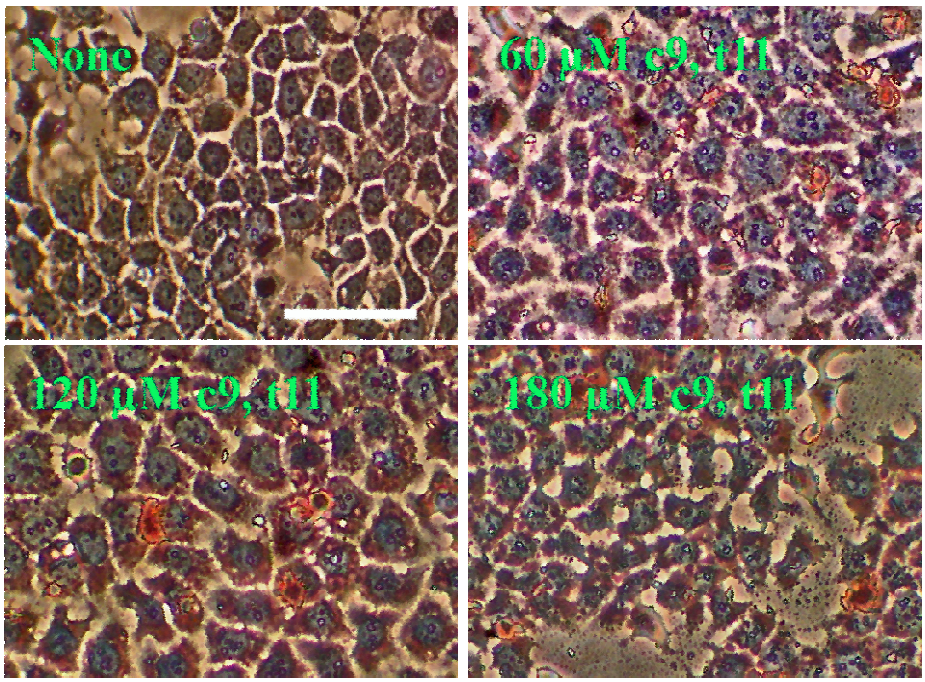


Figure 1. Dose response of *cis*-9,*trans*-11 (c9,t11) CLA isomer on lipid accumulation in quiescent H4IIE cells.

H4IIE rat hepatoma cells were treated with 60 µM, 120 µM and 180 µM c9,t11 CLA for 24 hours to assess the direct effects of this isomer on the cytoplasmic LD formation. Lipid accumulation was determined by staining cells with Oil Red O and counter-stained with hematoxylin. LDs are stained red while the nuclei appear blue. Treatments were conducted in triplicate and the pictures are representative of three independent experiments. Scale bar represents 10 µm and is applicable to all panels.

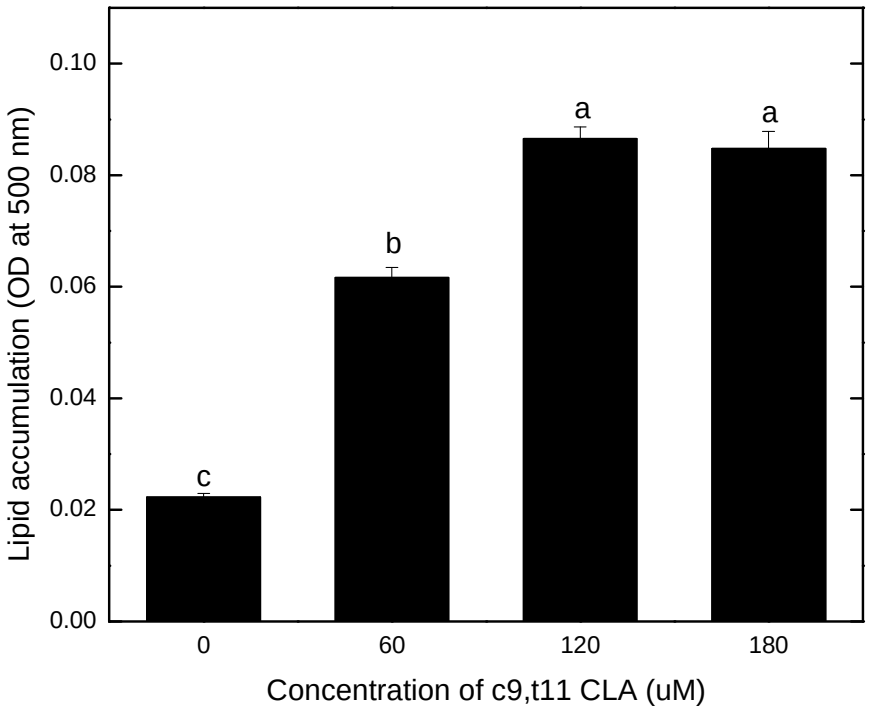


Figure 2. Quantification of lipid accumulation in quiescent H4IIE cells treated with increasing concentrations of *cis*-9,*trans*-11 (c9,t11) CLA isomer.

H4IIE rat hepatoma cells were treated with 60 µM, 120 µM and 180 µM c9,t11 CLA for 24 hours to assess the direct effects of this isomer on the cytoplasmic LD formation. Lipid accumulation was quantified after 24 hours by eluting the Oil Red O stain in 2-propanol and measuring the absorbance of the eluate at 500 nm. Data are presented as adjusted means ± SEM of three independent experiments performed in triplicate. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

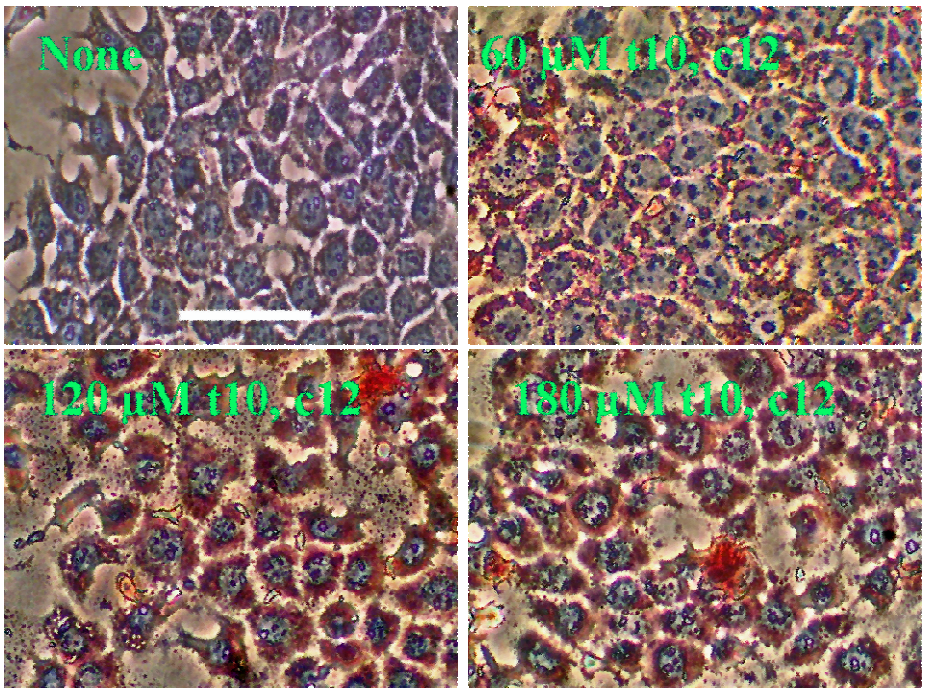


Figure 3. Dose response of *trans*-10,*cis*-12 (t10,c12) CLA isomer on lipid accumulation in quiescent H4IIE cells.

H4IIE rat hepatoma cells were treated with 60 µM, 120 µM and 180 µM t10,c12 CLA for 24 hours to assess the direct effects of this isomer on the cytoplasmic LD formation. Lipid accumulation was determined by staining cells with Oil Red O and counter-stained with hematoxylin. LDs are stained red while the nuclei appear blue. Treatments were conducted in triplicate and the pictures are representative of three independent experiments. Scale bar represents 10 µm and is applicable to all panels.

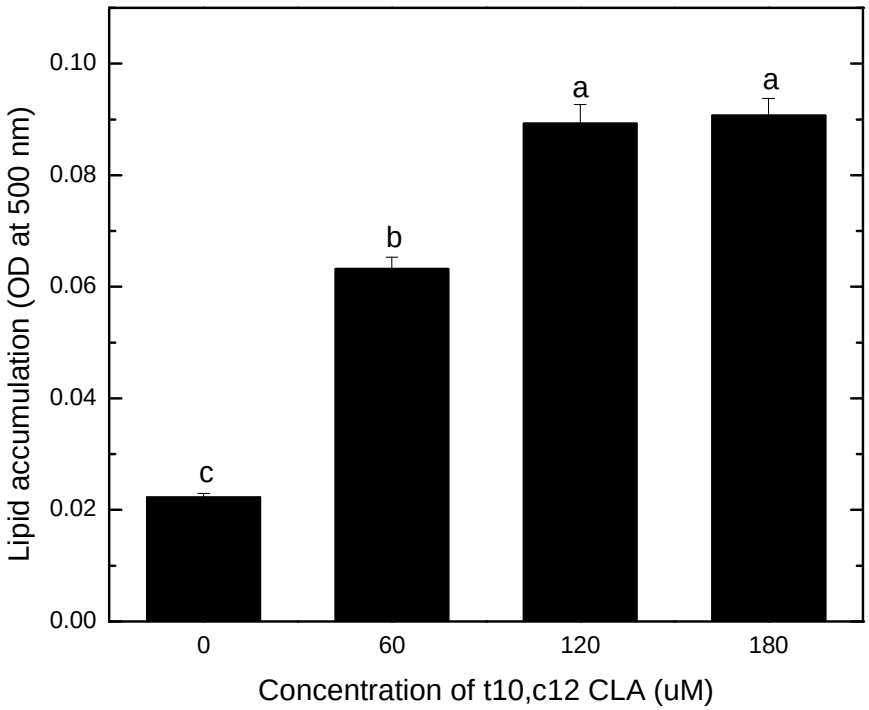


Figure 4. Quantification of lipid accumulation in quiescent H4IIE cells treated with increasing concentrations of *trans*-10,*cis*-12 (t10,c12) CLA isomer.

H4IIE rat hepatoma cells were treated with 60 µM, 120 µM and 180 µM t10,c12 CLA for 24 hours to assess the direct effects of this isomer on the cytoplasmic LD formation. Lipid accumulation was quantified after 24 hours by eluting the Oil Red O stain in 2-propanol and measuring the absorbance of the eluate at 500 nm. Data are presented as adjusted means ± SEM of three independent experiments performed in triplicate. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

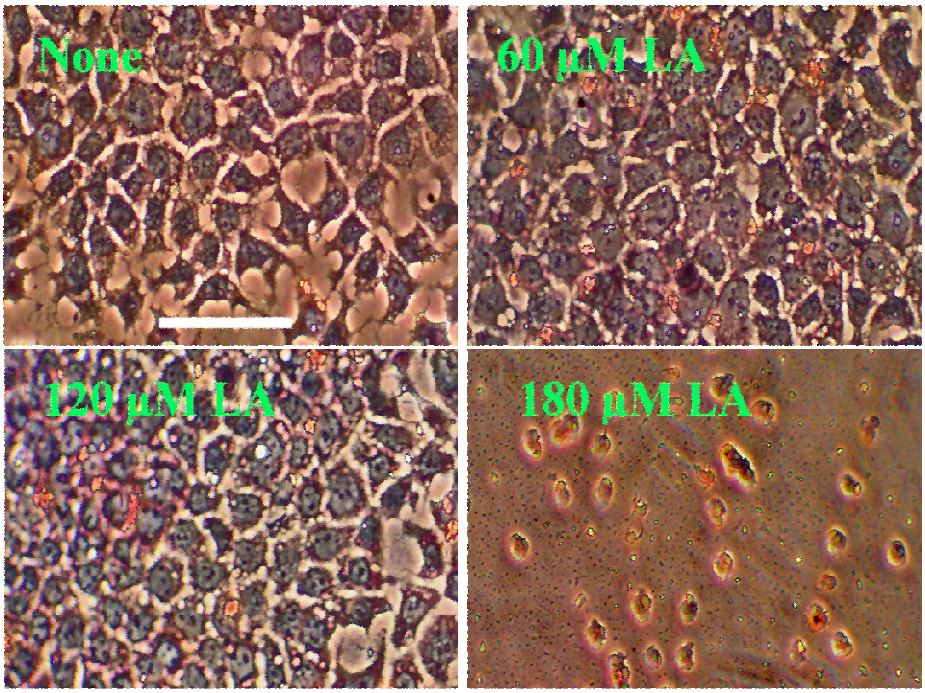


Figure 5. Dose response of linoleic acid on lipid accumulation in quiescent H4IIE cells.

H4IIE rat hepatoma cells were treated with 60 µM, 120 µM and 180 µM LA for 24 hours to assess the direct effects of LA on the cytoplasmic LD formation. Lipid accumulation was determined by staining cells with Oil Red O and counter-stained with hematoxylin. LDs are stained red while the nuclei appear blue. Treatments were conducted in triplicate and the pictures are representative of three independent experiments. Scale bar represents 10 µm and is applicable to all panels.

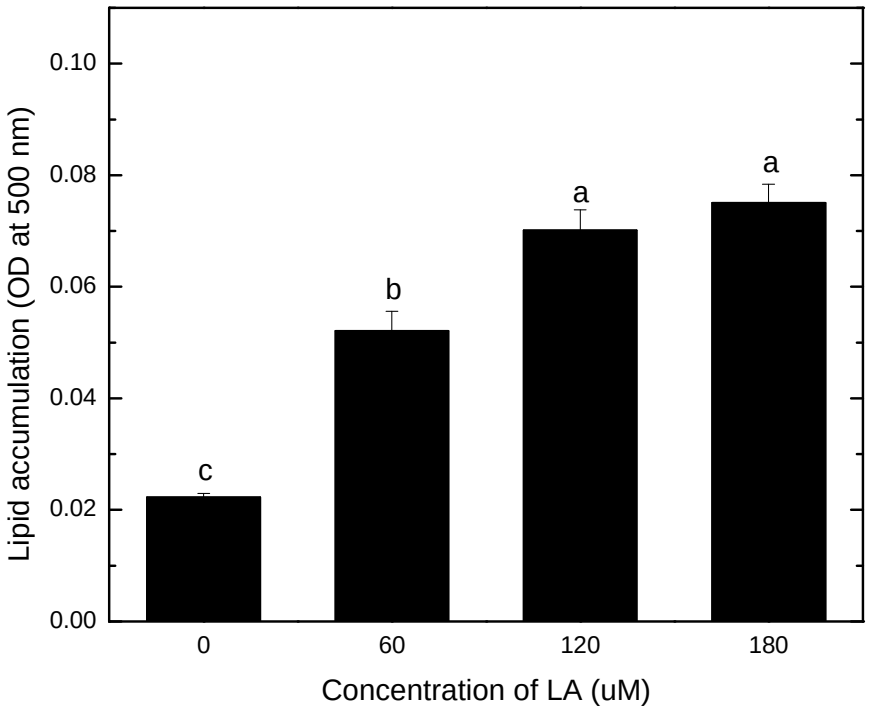


Figure 6. Quantification of lipid accumulation in quiescent H4IIE cells treated with increasing concentrations of linoleic acid.

H4IIE rat hepatoma cells were treated with 60 µM, 120 µM and 180 µM LA for 24 hours to assess the direct effects of LA on the cytoplasmic LD formation. Lipid accumulation was quantified after 24 hours by eluting the Oil Red O stain in 2-propanol and measuring the absorbance of the eluate at 500 nm. Data are presented as adjusted means ± SEM of three independent experiments performed in triplicate. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

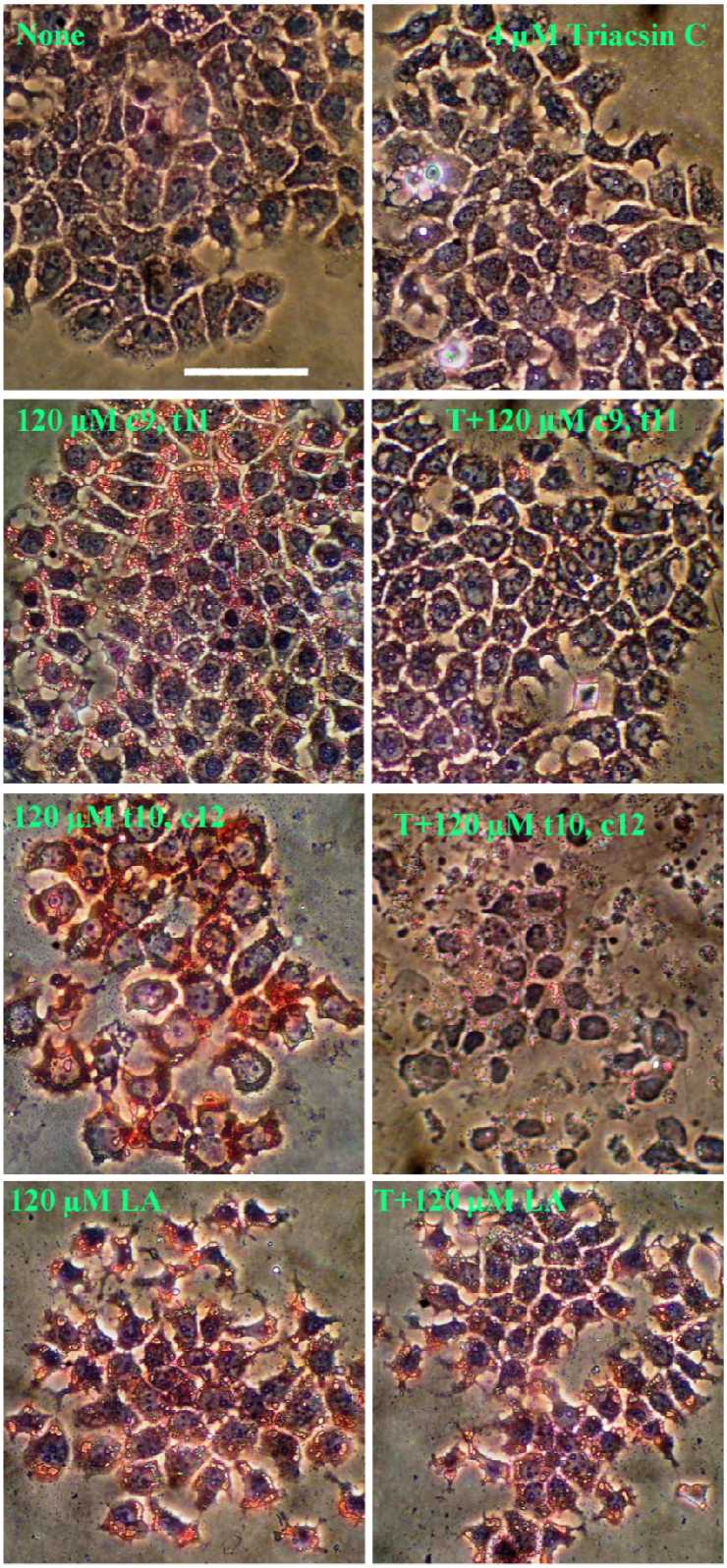
4.2 Triacsin C effectively reduces LD formation in H4IIE cells

Exogenously supplied FAs are activated *via* acyl co-A synthetase (ACS) to FA acyl-CoAs to be channeled either toward lipid synthesis and storage or toward oxidation pathways. The FA acyl-CoAs can then be metabolized to form triglycerides (TGs), phospholipids (PL) and cholesteryl esters (CE). If there is an excess supply of exogenous FAs, they can be stored as TGs in LDs. Therefore, we investigated the effect of inhibiting this first essential step in the metabolism of long-chain FA metabolism in the hepatocytes. Quiescent H4IIE cells were pre-treated with 4 µM of the ACS inhibitor, triacsin C, for 10 minutes. Cells were then treated with 60 µM of the individual CLA isomers (c9,t11 or t10,c12) or LA for 24 hours. Cells without any of the above treatments were included as controls. After 24 hours incubation, the cells were stained with Oil Red O to visualize LDs and counter-stained with hematoxylin to assess histological appearance. Compared to the untreated controls, treatment of H4IIE cells with individual CLA isomers resulted in significant LD formation. Interestingly, pre-treatment of H4IIE cells with triacsin C before the supplementation with individual CLA isomers or LA significantly reduced LD formation (Figures 7-8).

These results indicate that LD formation by CLA isomers or LA is regulated at the initial level of TG synthesis and suggest that ACS plays an important role in the regulation of CLA isomer- or LA-induced LD formation in H4IIE hepatocytes.

Figure 7. Effect of triacsin C on lipid accumulation induced by *cis*-9,*trans*-11 (c9,t11) and *trans*-10,*cis*-12 (t10,c12) CLA isomers and linoleic acid in quiescent H4IIE cell.

Quiescent H4IIE rat hepatoma cells were pre-treated with 4 µM triacsin C (T) or vehicle for ten minutes prior to the addition of 120 µM of c9,t11 CLA, 120 µM t10,c12 CLA or 120 µM LA. Cells were incubated for another 24 hours to assess the effects of triacsin C on cytoplasmic LD formation in response to the respective FAs. Lipid accumulation was determined by staining cells with Oil Red O and counter-stained with hematoxylin. LDs are stained red while the nuclei appear blue. Treatments were conducted in triplicate and the pictures are representative of three independent experiments. Scale bar represents 10 µm and is applicable to all panels.


None
4 µM Triacsin C
120 µM c9, t11
T+120 µM c9, t11
120 µM t10, c12
T+120 µM t10, c12
120 µM LA
T+120 µM LA

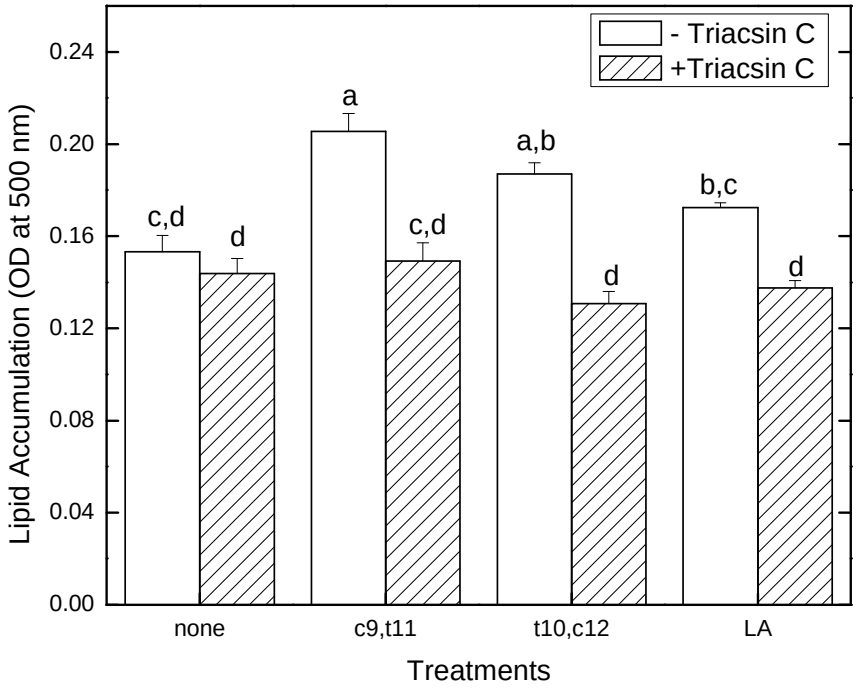


Figure 8. Quantification of lipid accumulation in quiescent H4IIE cells treated with triacsin C on lipid accumulation induced by *cis*-9,*trans*-11 (c9,t11) and *trans*-10,*cis*-12 (t10,c12) CLA isomers and linoleic acid.

Quiescent H4IIE rat hepatoma cells were pre-treated with 4 µM triacsin C or vehicle for ten minutes prior to the addition of 120 µM of c9,t11 CLA, 120 µM t10,c12 CLA or 120 µM LA. Lipid accumulation was quantified after 24 hours by eluting the Oil Red O stain with 2-propanol and measuring the absorbance of the eluate at 500 nm. Data are presented as adjusted means ± SEM of three independent experiments performed in triplicate. Differences between treatments are considered significant at $p<0.05$ and are indicated by different lowercase letters.

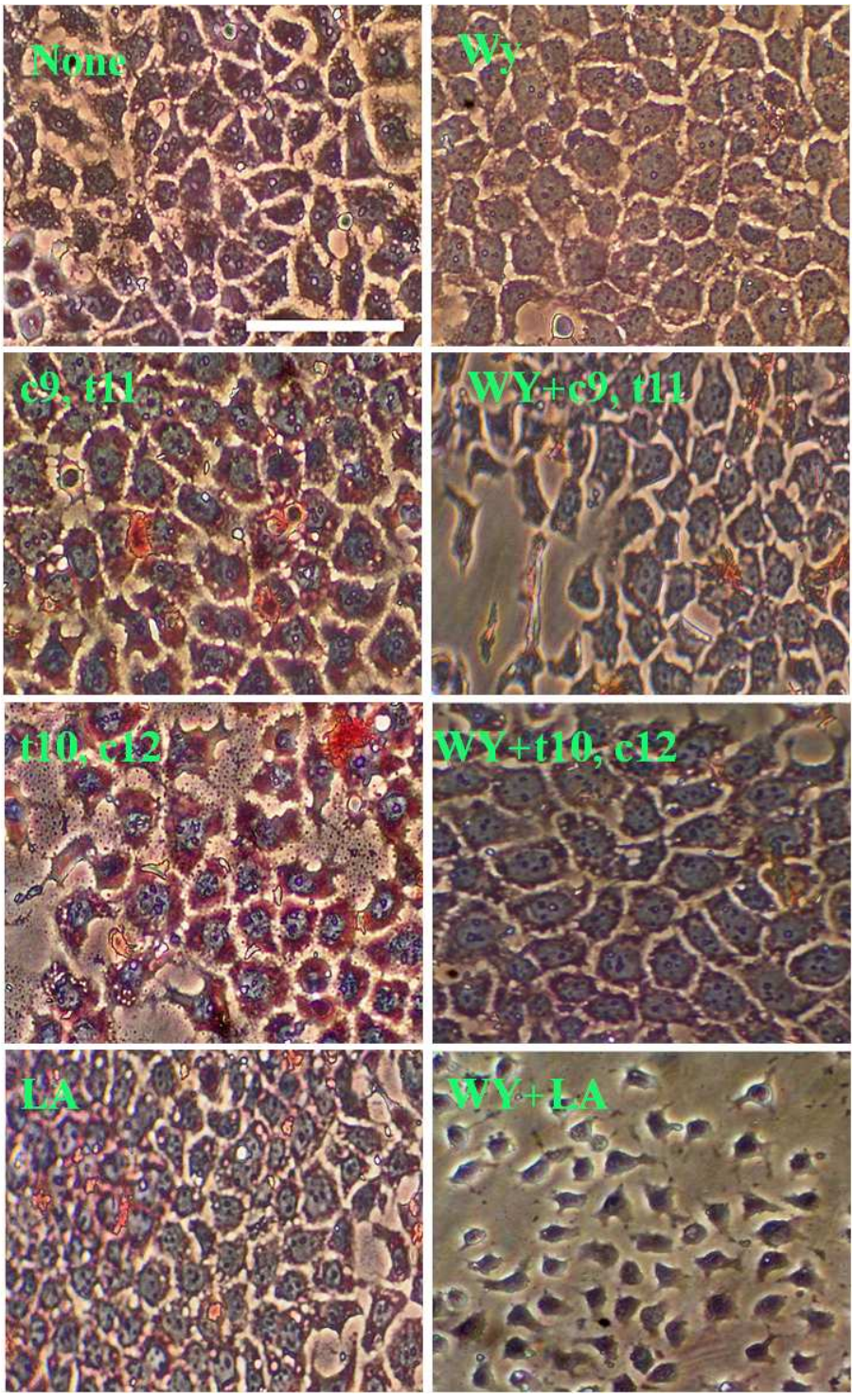
4.3 Wy14643 reduces lipid accumulation in CLA isomer as well as linoleic acid treated cells

Fibrates are well known hypolipidemic drugs that operate *via* the nuclear transcription factor PPARα. H4IIE cells specifically express the PPARα isoform and it acts as a lipid sensor in the cells. Therefore, we investigated the direct effects of PPARα activation on CLA- and LA-induced LD formation in H4IIE cells using the potent PPARα activator, Wy14643. Quiescent H4IIE cells were pre-treated with 250 µM of Wy14643 for ten minutes. Cells were then treated with 120 µM of the individual CLA isomers (c9,t11 or t10,c12) or LA for 24 hours. Cells without any of the above treatments were included as controls. After 24 hours incubation, the cells were stained with Oil Red O to visualize LDs and counter-stained with hematoxylin to assess the histological appearance. Compared to the untreated controls, treatment of H4IIE cells with individual CLA isomers or LA resulted in significant LD formation. Treatment with Wy14643 prior to individual CLA isomer supplementation significantly reduced LD formation (Figures 9-10). However, H4IIE cells pre-treated with Wy14643 prior to LA supplementation exhibited no difference to the untreated controls.

These results indicate that PPARα activation plays an important role in the reduction of CLA isomer- and LA-induced LD formation in H4IIE hepatocytes.

Figure 9. Effect of Wy14643 on lipid accumulation induced by *cis*-9,*trans*-11 (c9,t11) and *trans*-10,*cis*-12 (t10,c12) CLA isomers and linoleic acid in quiescent H4IIE cells.

Quiescent H4IIE rat hepatoma cells were pre-treated with 250 µM Wy14643 or vehicle for ten minutes prior to the addition of 120 µM of c9,t11 CLA, 120 µM t10,c12 CLA and 120 µM LA. Cells were incubated for another 24 hours to assess the effects of Wy14643 (Wy) on cytoplasmic LD formation in response to the respective FAs. Lipid accumulation was determined by staining cells with Oil Red O and counter-stained with hematoxylin. LDs are stained red while the nuclei appear blue. Treatments were conducted in triplicate and the pictures are representative of three independent experiments. Scale bar represents 10 µm and is applicable to all panels.


None
Wy
c9, t11
WY+c9, t11
t10, c12
WY+t10, c12
LA
WY+LA

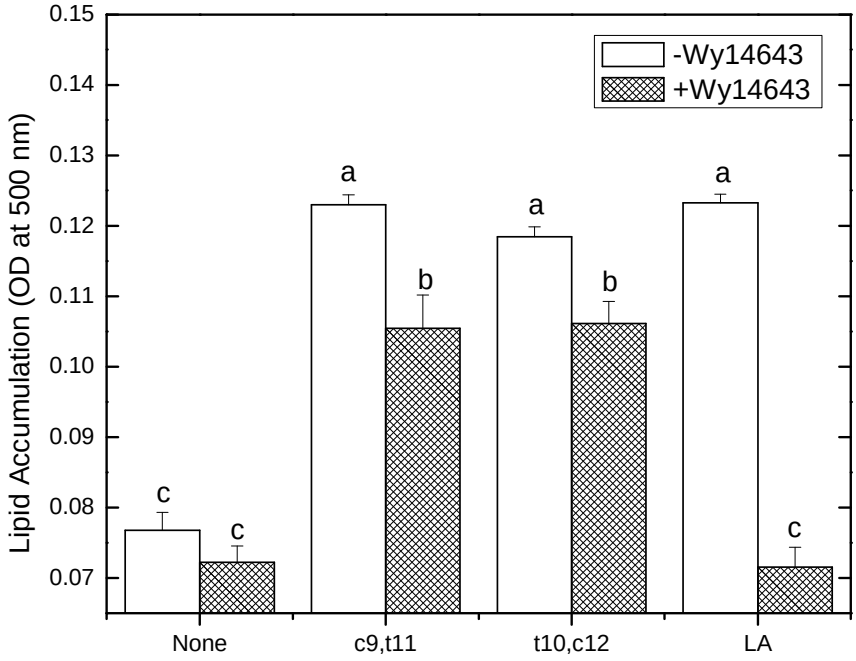


Figure 10. Quantification of lipid accumulation in quiescent H4IIE cells treated with Wy14643 on lipid accumulation induced by *cis*-9,*trans*-11 (c9,t11) and *trans*-10,*cis*-12 (t10,c12) CLA isomers and linoleic acid.

Quiescent H4IIE rat hepatoma cells were pre-treated with 250 µM Wy14643 or vehicle for ten minutes prior to the addition of 120 µM of c9,t11 CLA, 120 µM t10,c12 CLA and 120 µM LA. Lipid accumulation was quantified by eluting the Oil Red O stain with 2-propanol and measuring the absorbance of the eluate at 500 nm. Data are presented as adjusted means ± SEM of three independent experiments performed in triplicate. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

4.4 CLA isomers promote cell viability at low concentration

To evaluate the effect of lipid accumulation on the viability of H4IIE rat hepatomas, quiescent H4IIE cells were treated with different concentrations (0 µM, 60 µM, 120 µM and 180 µM) of CLA isomers (c9,t11 or t10,c12) or LA for 24 hours at 37 °C. Cells without any of the above treatments were included as controls. After incubating for 24 h, cell viability was measured with the cck-8 assay. As shown in Figure 11 and 12, cell viability was significantly increased at the 60 µM concentration of both CLA isomers (compared with the untreated controls, $p < 0.05$). However, a much higher concentration of c9,t11 CLA isomer (180 µM) significantly decreased cell viability compared with controls as well as with the 60 µM c9,t11 CLA group, ($p < 0.05$). A similar increase in viability was observed for cells treated with the t10,c12 CLA isomer at 60 µM, compared to 0, 120 and 180 µM but none of the treatments decreased viability compared to the untreated control (Figure 12). There was no statistically significant effect of the 60 µM LA treatment on cell viability, however, treatment with LA at 180 µM resulted in a significant decrease in H4IIE viability compared with the untreated control ($p < 0.05$, Figure 13).

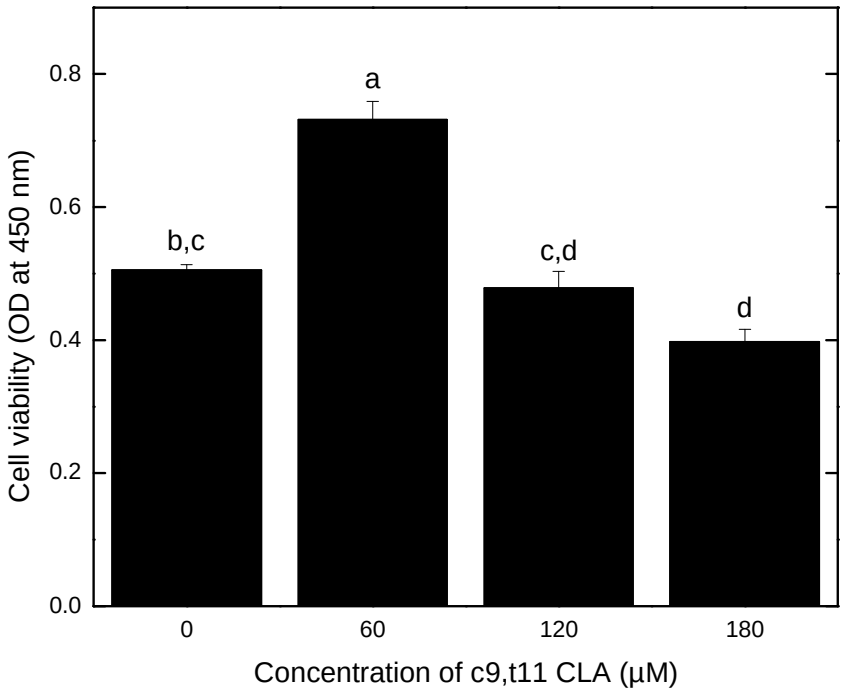


Figure 11. Effects of *cis*-9,*trans*-11 (c9,t11) CLA isomer on quiescent H4IIE cell viability.

Quiescent H4IIE rat hepatoma cells were treated with 60 µM, 120 µM and 180 µM of c9,t11 CLA isomer in separate wells of a 96-well plate. Cells were incubated for 24 hours at 37 °C. Cell viability was assessed by using the cck-8 kit. The absorbance was measured at 450 nm using a microplate reader. Data are presented as adjusted means ± SEM of three independent experiments performed in triplicate. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

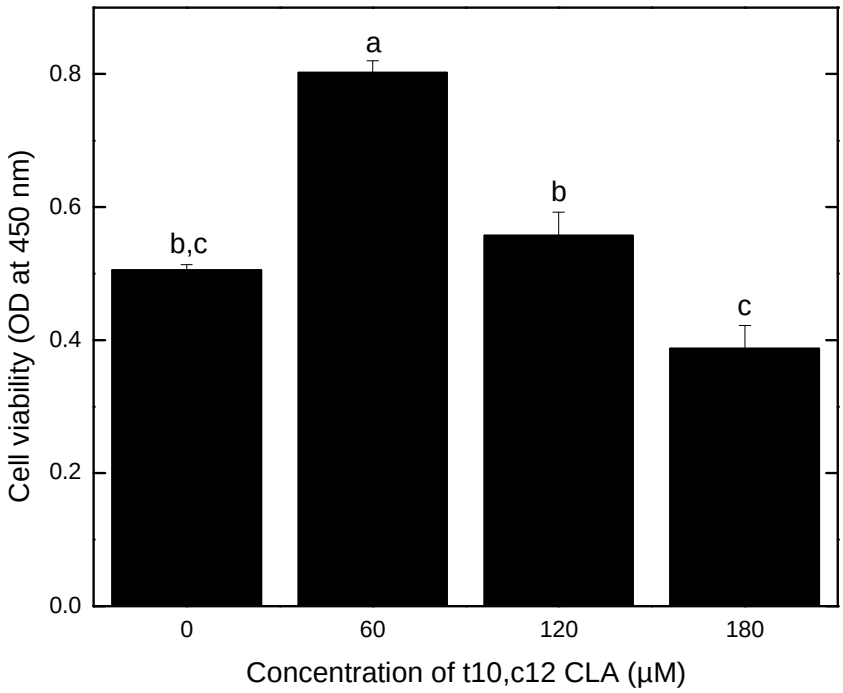


Figure 12. Effects of *trans*-10,*cis*-12 (t10,c12) CLA isomer on quiescent H4IIE cell viability.

Quiescent H4IIE rat hepatoma cells were treated with 60 µM, 120 µM and 180 µM of t10,c12 CLA isomer in separate wells of a 96-well plate. Cells were incubated for 24 hours at 37 °C. Cell viability was assessed by using the cck-8 kit. The absorbance was measured at 450 nm using a microplate reader. Data are presented as adjusted means ± SEM of three independent experiments performed in triplicate. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

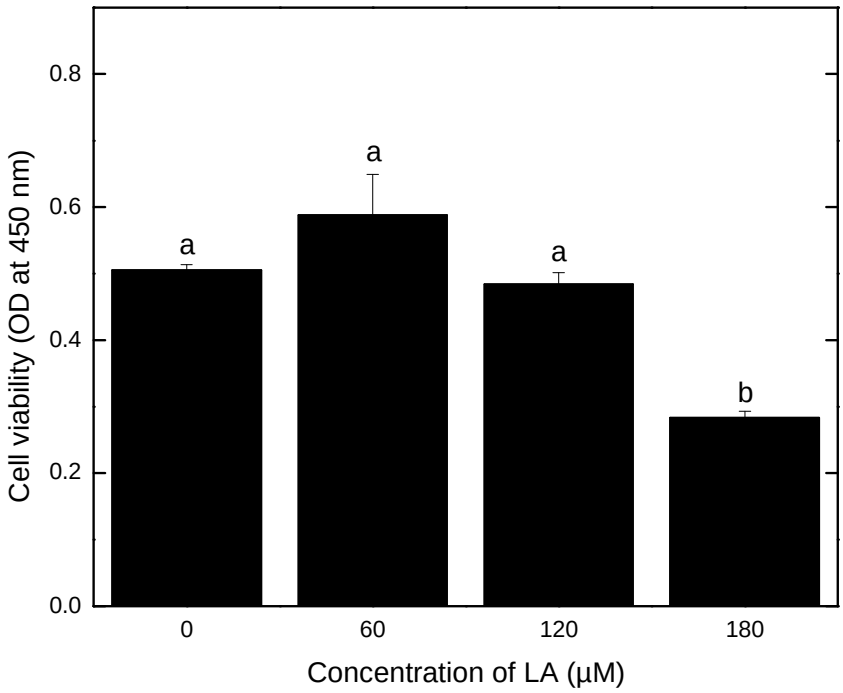


Figure 13. Effects of linoleic acid on quiescent H4IIE cell viability.

Quiescent H4IIE rat hepatoma cells were treated with 60 µM, 120 µM and 180 µM LA in separate wells of a 96-well plate. Cells were incubated for 24 hours at 37 °C. Cell viability was assessed by using the cck-8 kit. The absorbance was measured at 450 nm using a microplate reader. Data are presented as adjusted means ± SEM of three independent experiments performed in triplicate. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

4.5 Wy14643 reduces cell viability in the presence of CLA isomers or linoleic acid

As shown previously, compared to the untreated control cells, treatment with 60 µM of the individual CLA isomers significantly increased cell viability, but there was no effect of 60 µM LA treatment. Interestingly, combined treatment with a PPARα activator, Wy14643, resulted in decreased cell viability when compared to the cells treated with 60 µM concentration of the respective FAs alone. However, Wy14643 alone without any of the FA treatments had no effect on cell viability (Figure 14) indicating that PPARα activation under conditions of lipid accumulation results in reduced cell viability.

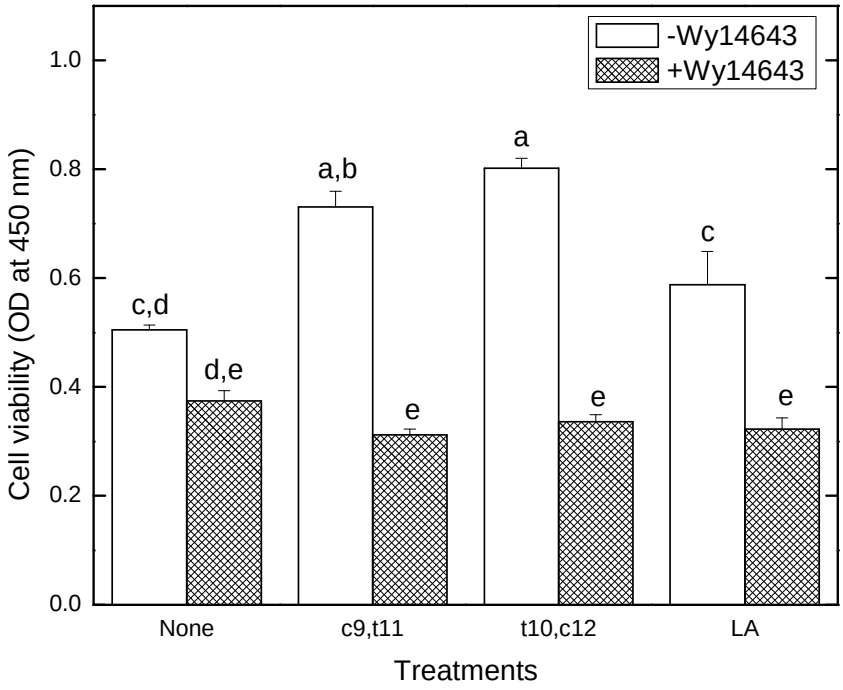


Figure 14. Effect of Wy14643 and fatty acid supplementation on quiescent H4IIE cell viability.

Quiescent H4IIE rat hepatoma cells were treated with 250 µM Wy14643 or vehicle for ten minutes prior to the addition of 60 µM of c9,t11 CLA, 60 µM t10,c12 CLA or 60 µM LA in separate wells of a 96-well plate. Cells were incubated for another 24 hours at 37°C. Cell viability was assessed by using the cck-8 kit. The absorbance was measured at 450 nm using a microplate reader. Data are presented as adjusted means ± SEM of three independent experiments performed in triplicate. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

4.6 Role of dietary CLA isomers on expression and phosphorylation of PPARα in *fa/fa* Zucker rats

The reduction in LD formation in Wy14643-treated H4IIE cells suggests that PPARα may effectively modulate FA metabolism under conditions with elevated FAs such as obesity and steatosis. Therefore, we investigated the effect of dietary CLA isomers on PPARα expression and phosphorylation in the livers of 7 week old male *fa/fa* and lean Zucker rats fed either a control or a CLA isomer (0.4% wt/wt c9,t11 or 0.4% wt/wt t10,c12) diet for 8 weeks (kindly provided by Danielle Stringer).

Hepatic tissues from these animals were used for immunoblotting. Our results showed that the *fa/fa* rats fed t10,c12 CLA isomer (fa 10,12) had a significantly lower level of phospho-PPARα S21 compared to the lean control (ln Ctl) (Figure 16). However, there was no significant difference in the phosphorylation of PPARα at the two serine positions (S12 and S21) between the *fa/fa* control (fa Ctl) and the CLA-fed *fa/fa* rats (Figures 15-16). There was no significant effect of dietary CLA on PPARα levels (Figure 17). Also, no significant changes were detected in the levels of stearoyl co-A desaturase1 (scd1) in the fa groups as compared to the ln Ctl (Figure 20). In contrast, there was a trend towards an increase in the phosphorylation of p44/42 MAPK in the fa Ctl and the fa 10,12 groups compared to the ln Ctl group which was not observed with the c9,t11 diet (Figure 18). The t10,c12 CLA isomer significantly increased the phosphorylation of p38 MAPK in the *fa/fa* rats (fa 10,12 group) compared to ln Ctl (Figure 19).

These results suggest that only the t10,c12 CLA isomer affects the phosphorylation of PPARα, specifically through a decrease in its phosphorylation at Ser

21, while the c9,t11 isomer had no significant effect on the phosphorylation at either of the serines (S12 as well as S21). Interestingly, the decrease in PPARα phosphorylation by the t10,c12 CLA isomer was associated with activation of the p38 MAPK pathway. This indicates that the t10,c12 CLA isomer affects PPARα phosphorylation and thereby its transcriptional activity in a MAPK-independent manner. It is interesting to note that the phosphorylation levels of PPARα S21 (Figure 16) and p38 MAPK (Figure 19) are reversed suggesting that the two signalling pathways may be inversely regulated in the Zucker rats.

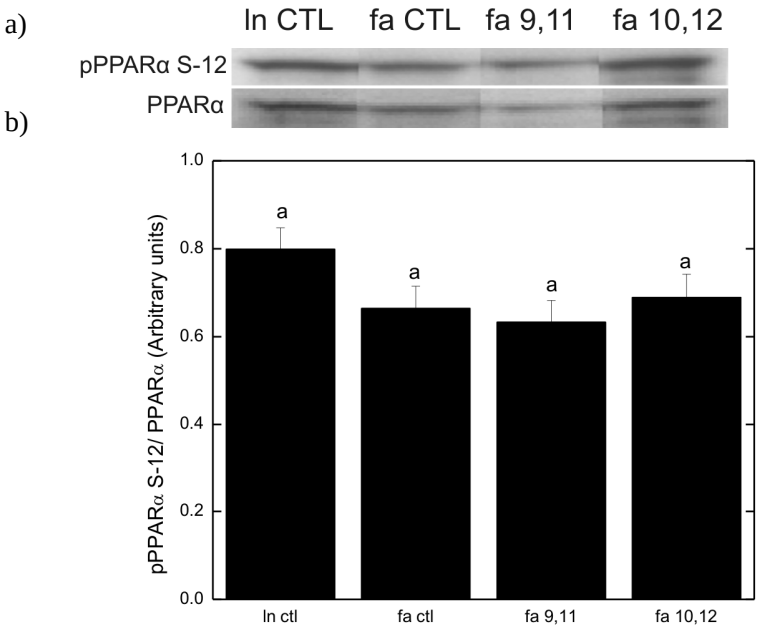
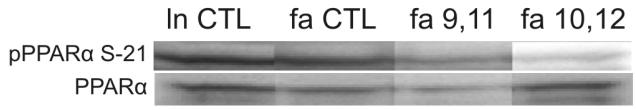


Figure 15: Phospho-peroxisome proliferator activated receptor (PPAR) *α* S-12 levels in livers of lean and *fa/fa* Zucker rats.

a) Hepatic protein extracts from lean Zucker rats (ln ctl), *fa/fa* Zucker rats (fa ctl), *fa/fa* Zucker rats fed *cis*-9,*trans*-11 CLA (fa 9,11) and *fa/fa* Zucker rats fed *trans*-10,*cis*-12 CLA (fa 10,12) for 8 weeks were analyzed by immunoblotting using antibodies that recognize phospho-PPARα S-12 and PPARα. A representative blot is shown.

b) Corresponding densitometric analysis of the bands (panel a) in arbitrary units. Experimental data were analyzed with SAS software (version 9.1.3), using a mixed model analysis with a random intercept for the repetitions. Data are presented as adjusted means ± SEM (n=6). Contrast statements were used to make individual comparisons of treatments versus control. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

a)



b)

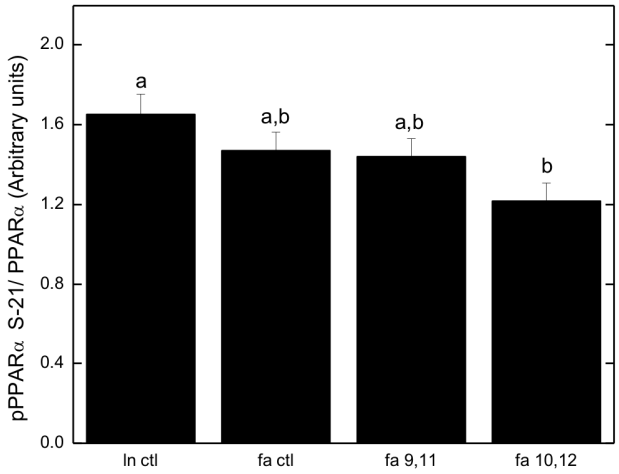
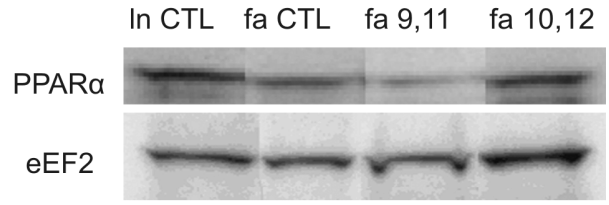


Figure 16: Phospho-peroxisome proliferator activated receptor (PPAR) α S-21 levels in livers of lean and *fa/fa* Zucker rats.

a) Hepatic protein extracts from lean Zucker rats (ln ctl), *fa/fa* Zucker rats (fa ctl), *fa/fa* Zucker rats fed *cis*-9,*trans*-11 CLA (fa 9,11) and *fa/fa* Zucker rats fed *trans*-10,*cis*-12 CLA (fa 10,12) for 8 weeks were analyzed by immunoblotting using antibodies that recognize phospho-PPARα S-21 and PPARα.. A representative blot is shown.

b) Corresponding densitometric analysis of the bands (panel a) in arbitrary units. Experimental data were analyzed with SAS software (version 9.1.3), using a mixed model analysis with a random intercept for the repetitions. Data are presented as adjusted means ± SEM (n=6). Contrast statements were used to make individual comparisons of treatments versus control. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

a)



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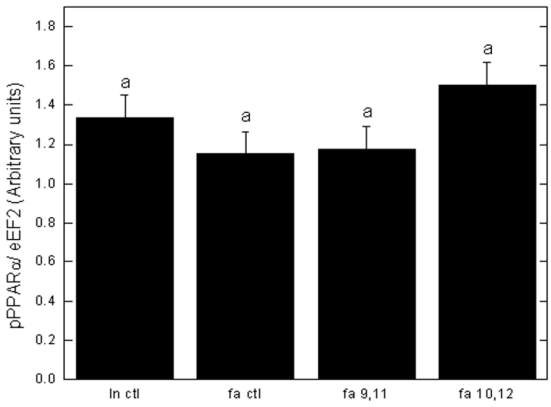


Figure 17: Peroxisome proliferator activated receptor (PPAR) α levels in livers of lean and *fa/fa* Zucker rats.

a) Hepatic protein extracts from lean Zucker rats (ln ctl), *fa/fa* Zucker rats (fa ctl), *fa/fa* Zucker rats fed *cis*-9,*trans*-11 CLA (fa 9,11) and *fa/fa* Zucker rats fed *trans*-10,*cis*-12 CLA (fa 10,12) for 8 weeks were analyzed by immunoblotting using an antibody that recognizes PPARα. A representative blot is shown.

b) Corresponding densitometric analysis of the bands (panel a) in arbitrary units. Experimental data were analyzed with SAS software (version 9.1.3), using a mixed model analysis with a random intercept for the repetitions. Data are presented as adjusted means ± SEM (n=6). Contrast statements were used to make individual comparisons of treatments versus control. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

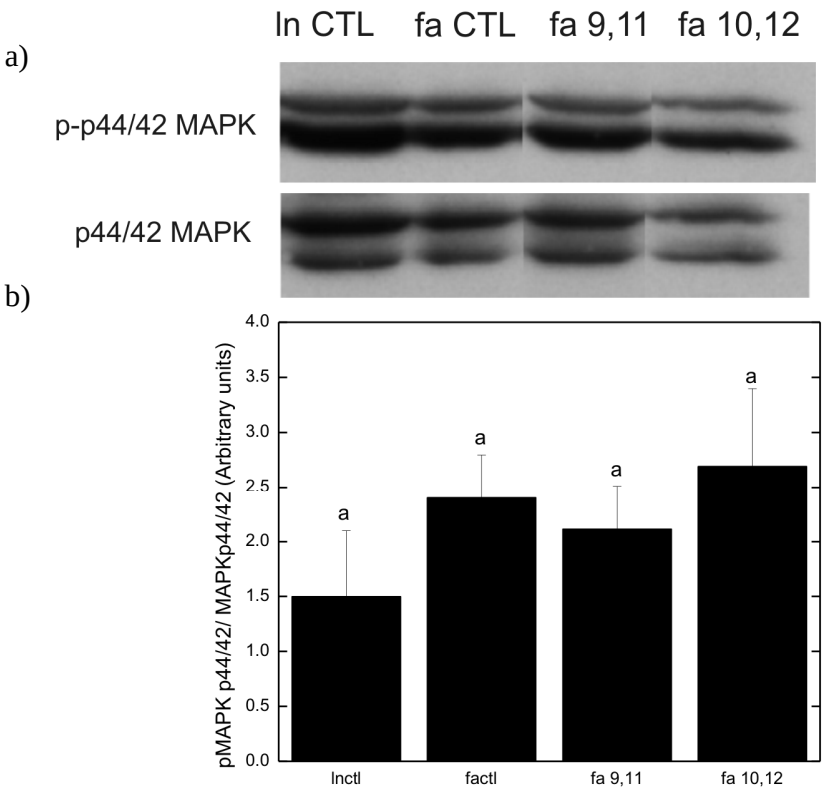
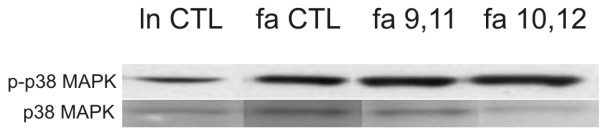


Figure 18: Phospho-p44/42 MAPK (mitogen activated protein kinase) levels in livers of lean and *fa/fa* Zucker rats.

a) Hepatic protein extracts from lean Zucker rats (ln ctl), *fa/fa* Zucker rats (fa ctl), *fa/fa* Zucker rats fed *cis*-9,*trans*-11 CLA (fa 9,11) and *fa/fa* Zucker rats fed *trans*-10,*cis*-12 CLA (fa 10,12) for 8 weeks were analyzed by immunoblotting using antibodies that recognize phospho-p44/42 MAPK and p44/42 MAPK. A representative blot is shown.

b) Corresponding densitometric analysis of the bands (panel a) in arbitrary units. Experimental data were analyzed with SAS software (version 9.1.3), using a mixed model analysis with a random intercept for the repetitions. Data are presented as adjusted means ± SEM (n=6). Contrast statements were used to make individual comparisons of treatments versus control. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

a)



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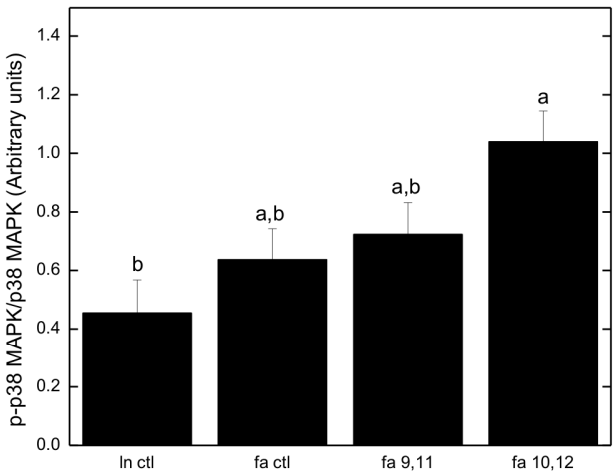
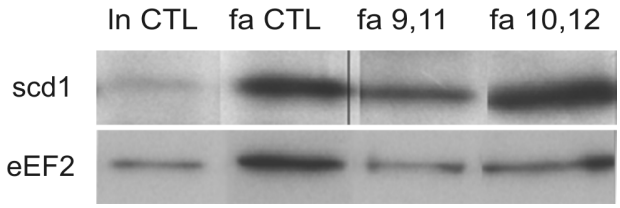


Figure 19: Phospho-p38 MAPK (mitogen activated protein kinase) levels in livers of lean and *fa/fa* Zucker rats.

a) Hepatic protein extracts from lean Zucker rats (ln ctl), *fa/fa* Zucker rats (fa ctl), *fa/fa* Zucker rats fed *cis*-9,*trans*-11 CLA (fa 9,11) and *fa/fa* Zucker rats fed *trans*-10,*cis*-12 CLA (fa 10,12) for 8 weeks were analyzed by immunoblotting using antibodies that recognize phospho-p38 MAPK and p38 MAPK. A representative blot is shown.

b) Corresponding densitometric analysis of the bands (panel a) in arbitrary units. Experimental data were analyzed with SAS software (version 9.1.3), using a mixed model analysis with a random intercept for the repetitions. Data are presented as adjusted means ± SEM (n=6). Contrast statements were used to make individual comparisons of treatments versus control. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

a)



b)

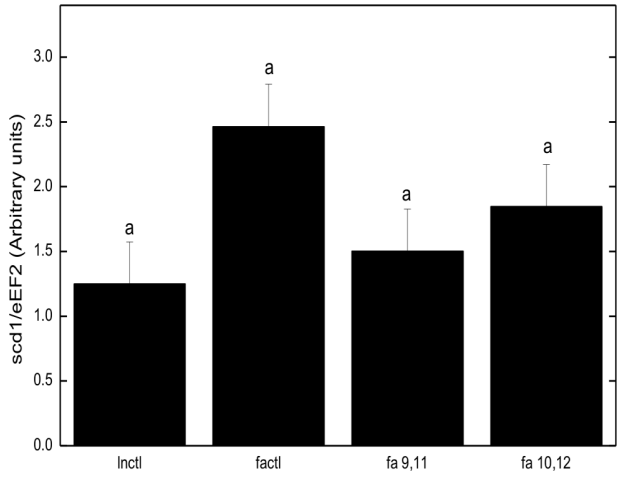


Figure 20: Stearoyl co-A desaturase 1 (scd1) levels in livers of lean and *fa/fa* Zucker rats.

a) Hepatic protein extracts from lean Zucker rats (ln ctl), *fa/fa* Zucker rats (fa ctl), *fa/fa* Zucker rats fed *cis*-9,*trans*-11 CLA (fa 9,11) and *fa/fa* Zucker rats fed *trans*-10,*cis*-12 CLA (fa 10,12) for 8 weeks were analyzed by immunoblotting using an antibody that recognizes scd1. A representative blot is shown.

b) Corresponding densitometric analysis of the bands (panel a) in arbitrary units. Experimental data were analyzed with SAS software (version 9.1.3), using a mixed model analysis with a random intercept for the repetitions. Data are presented as adjusted means ± SEM (n=6). Contrast statements were used to make individual comparisons of treatments versus control. Differences between treatments were considered significant at $p<0.05$ and are indicated by different lowercase letters.

5. DISCUSSION

The triggers leading to fatty liver and their relation to the molecular mechanisms and respective signaling molecules that modulate hepatic FA metabolism are incompletely understood. Hepatic lipid overloading mainly in the form of TGs is considered a prerequisite for the development of NAFLD (113, 169). The cytotoxicity of CLA isomers, particulary the t10,c12 isomer, has been previously reported for rat hepatomas, however, no direct link to LD formation was shown (247). The nuclear transcription factor, PPARα, and ACS are key players in hepatic FA oxidation and activation, respectively. Thus, they play important roles in the regulation of hepatic lipid metabolism.

Our hypothesis states that an increase in LD formation induced by CLA isomers (c9,t11 or t10,c12) reduces rat hepatoma H4IIE viability. As LD formation in hepatocytes is poorly understood, our first aim was to understand the underlying mechanism. We, therefore, investigated the role of FA and PPARα activation in the regulation of these cellular processes. We compared the effects of different concentrations of the c9,t11 and t10,c12 CLA isomers to their parent FA, LA, in the process of LD induction in quiescent H4IIE cells. We found that CLA isomers as well as LA increased LD formation in a dose-dependent manner up to 120 µM. However, we observed no significant difference in lipid accumulation at higher concentrations (120 and 180 µM) of the FAs, indicating that LD formation reaches a plateau phase. Furthermore, this response to LD formation

was associated with changes in H4IIE viability. For the first time, we report that triacsin C significantly reduces CLA- and LA-induced LD formation in quiescent H4IIE cells implicating an important role of ACS in conditions with increased lipid accumulation such as fatty liver diseases. We also demonstrate that activation of PPARα by Wy14643 produces similar effects and supports previous findings that PPARα plays a primary role in the regulation of hepatic lipid metabolism. It is important to mention that the effect of triacsin C on cell viability was not studied. Triacsin C was able to reduce LD accumulation in the FA treated cells but it is unknown if this effect of triacsin C will affect cell viability. It could be speculated that the inhibition of LD formation with triascin C occurs at an earlier stage, thereby inhibiting cell growth as was observed with the Wy14643 treatment. In addition to our *in vitro* studies, we have also investigated the effects of dietary CLA isomers on PPARα and MAPK phosphorylation in *fa/fa* rat liver and speculate on the importance of this signaling pathway in the anti-steatotic effect of the t10,c12 CLA isomer observed in the *fa/fa* Zucker rats.

CLA isomers and LD formation

Recent findings from our lab have indicated that CLA isomers neither reduce nor prevent but rather induce LD formation in H4IIE cells (205). Therefore, we further investigated the dose response of c9,t11 and t10,c12 CLA isomers on lipid accumulation in quiescent H4IIE cells and subsequent cell viability. Since CLA consists of positional and geometric isomers of LA, we conducted a similar set of experiments using LA to determine CLA-specific effects. Compared to the untreated controls, treatment of H4IIE

cells with individual CLA isomers or LA for 24 hours resulted in significant LD formation in a dose-dependent manner, but after a concentration of 120 µM there was no increase or decrease in LD formation (Figures 1-6). In fact there was no significant difference in lipid accumulation at higher concentrations, i.e. between 120 µM and 180 µM of c9,t11 CLA (Figure 2), t10,c12 CLA (Figure 4) or LA (Figure 6) lipid accumulation remained at a plateau phase. Excess energy is stored as TGs sequestered in LDs during lipogenesis in mammalian cells, but little is understood about how animals package fat within cells. LDs participate actively in a variety of metabolic processes and are, therefore, considered functional and dynamic organelles in cells. When energy is required, the stored TGs are hydrolyzed via activation of lipolytic pathways and the released FAs are then transported by serum albumin to various tissues to provide energy. Zehmer et al. (255) observed that in non-adipocytes, LDs are small, mobile and interact with other cellular organelles, such as the mitochondria and peroxisomes. Therefore, LD formation may be part of a hepatoprotective mechanism at lower lipid concentrations that operates by reducing the cellular levels of non-esterified toxic FAs. Also, since CLA isomers affect hepatic LD formation in a similar dose-dependent pattern as the essential FA, LA, we speculate that both CLA and LA may be metabolized in similar pathways in the liver.

CLA isomers and cell viability

Accumulation of intracellular lipid in non-adipocytes is associated with lipotoxicity (5, 85, 233) We, therefore, evaluated the viability of H4IIE rat hepatomas following exposure to different concentrations of CLA isomers (c9,t11 or t10,c12) or LA

for 24 hours. H4IIE viability was significantly increased at the 60 µM concentration of c9,t11 CLA isomer as detected by the WST-8 dye (Figure 11). However, at a much higher concentration of c9,t11 CLA isomer (180 µM), cell viability was significantly decreased. Although LDs are essential for normal cell function, excess accumulation of intracellular lipid is associated with several metabolic diseases, including hepatic steatosis, insulin resistance and inflammation (7).

A similar statistically significant increase in cell viability was observed for the t10,c12 CLA isomer at 60 µM. Likewise, there was a significant decrease in H4IIE viability at a much higher concentration of 180 µM t10,c12 CLA (Figure 12). The cytotoxicity of CLA isomers, the t10,c12 isomer in particular, has been reported for rat hepatomas (246), however, to date, no link to LD formation has been studied. CLA isomers have been shown to inhibit the proliferation of 3T3-L1 preadipocytes (193), as well as numerous cancer cell lines (30, 99, 116). Recently, the isomer-specific effect of the c9,t11 and t10,c12 CLA isomers was studied and it was found that while both CLA isomers inhibited the proliferation of human breast cancer cells, the c9,t11 isomer had the strongest effect (30). However, we demonstrate for the first time that the effect of CLA isomers on H4IIE viability is mainly dependent on the concentration. From our results, it can be speculated that the CLA isomers have a positive effect on cell growth at low concentrations, however, as the concentration of LD increases, it begins to exhibit a negative effect on cell viability mainly due to the toxic nature of unesterified FAs in the cells.

There was no effect of the 60 µM LA treatment on the viability of quiescent H4IIEs, however, treatment with LA at concentrations higher than 120 µM resulted in a significant decrease in cell viability (Figure 13). The accumulation of lipids in liver and other metabolically relevant organs such as pancreatic beta-cells, heart and skeletal muscle contributes substantially to the pathophysiology of insulin resistance, steatotic liver disease and heart failure (201). Accumulation of lipid in non-adipose tissues under obese conditions, is also associated with ER stress, mitochondrial dysfunction, and ultimately apoptosis (244). In one study, when pancreatic islets from the Zucker diabetic fatty rats were cultured with different FAs (oleate or palmitate), an increase in ceramide level and apoptosis were observed. These effects were blocked by fumonisin B, an inhibitor of ceramide synthesis, suggesting that FAs cause apoptosis *via* a ceramide-mediated pathway (199). Taken as a whole, these studies imply that lipid accumulation can either act as a marker or as a source of toxic lipid products such as ceramide that reduce cell viability or promote apoptosis.

In another study, the cytotoxicity of oleate and palmitate in isolated normal rat islet beta-cells was associated with less TG accumulation, and the authors speculated that the FAs themselves are non-toxic. Rather it is the formation of cytosolic TG from exogenous FA that protects against FA-induced apoptosis (35). Therefore, it is uncertain as to whether TG exerts only positive or negative effects (145). We studied the dose response of CLA isomers (c9,t11 or t10,c12) and LA on hepatoma viability and have determined that the CLA isomers promote cell growth at lower concentrations but reduce cell viability at higher concentrations. There was no effect of LA on H4IIE viability at a low concentration (Figure 13). However, it reduced cell viability at higher concentrations

in a manner similar to that observed with the CLA isomers. Our results are consistent with previous reports that, although a certain amount of lipid storage may be hepatoprotective, prolonged excess lipid storage can result in a loss of metabolic competency (7).

Triacsin C effectively reduces hepatoma LD formation

To directly address the effect of inhibiting the first essential step in the hepatic synthesis of long-chain FAs, in the presence of an exogenous supply of CLA isomers or LA, we treated quiescent H4IIE cells with triacsin C, a potent inhibitor of ACS. A pre-treatment with 4 μM of triacsin C for 10 minutes was sufficient to diminish CLA isomer or LA stimulated hepatic LD formation (Figure 7). Our results demonstrate that inhibiting ACS and thereby preventing TG synthesis significantly reduces hepatic LD formation in quiescent H4IIE cells (Figure 8).

Our data are consistent with previous reports that exogenously supplied FAs are converted by ACS to their FA acyl-CoAs, which can then be metabolized to form TGs, phospholipids and cholesteryl esters. The predominant pathways for FAs are their storage as TGs in LDs, membrane biosynthesis, and conversion to energy (48). The length of the carbon chain of the FA species defines the substrate specificity for the five different ACS enzymes characterized so far. Mammalian ACS activate FAs with usual chain lengths of 12 to 20 carbon atoms. CLA, which consists of positional and geometric isomers of LA, is an 18 carbon FA and, therefore, CLA isomers may serve as good substrates for this enzyme. The ACS isoforms differ in their subcellular location. Using non-cross-reacting

ACS1, ACS4, and ACS5 peptide antibodies, Lewin et al. (111) showed that ACS4 was the only ACS isoform present in peroxisomes and in mitochondria-associated membrane fractions isolated from livers of gemfibrozil-treated rats and proposed that ACS4 is linked to TG synthesis (110).

Our *in vitro* findings are also in agreement with other *in vivo* reports that have shown that triacsin C can inhibit TG synthesis in hepatocytes from both fed (about 70%) and starved rats (about 40%) (136). Triacsin C also inhibited the synthesis of cholesteryl ester and TG in mouse peritoneal macrophages, leading to a reduction of LDs (141). Recently, Matsuda et al (130) have reported that inhibition of ACS activity in $LDLR^{-/-}$ mice fed a high cholesterol diet has anti-atherogenic activity. Our results have demonstrated for the first time that LD formation in H4IIE cells in response to CLA isomers or LA treatment is directly reduced by triacsin C.

Therefore, we conclude that ACS is an important enzyme in mediating CLA or LA induced hepatic LD formation. Future studies on determining the effects of CLA isomers or LA on ACS activity may thus be useful.

Wy14643 reduces lipid accumulation in CLA isomer as well as LA treated cells

H4IIE cells specifically express the PPARα isoform (253), which is an important transcription factor regulating genes of FA oxidation. In the liver, PPARα plays a pivotal role in numerous processes including gluconeogenesis and lipid metabolism (124). PPARα mediates the effects of hypolipidemic fibrate drugs, which decrease plasma TGs

and increase plasma HDL concentrations. Currently, the mechanisms by which CLA isomers affect fatty liver or hepatic steatosis are unclear. CLA may be working either independently or dependently of PPARα. H4IIE cells are thus a valuable tool for elucidating the direct effects of PPARα activation on CLA isomer- or LA-induced hepatic lipid accumulation by using a potent PPARα activator, Wy14643.

Similar to our results with triacsin C, we observed that treatment with Wy14643 prior to supplementation with individual CLA isomers or LA significantly reduced LD formation (Figures 9-10). These studies were achieved using quiescent H4IIE cells treated with 250 μM of Wy14643 for 10 minutes prior to the addition of 60 μM c9,t11 CLA or 60 μM t10,c12 CLA or 60 μM LA, followed by a further 24 hour incubation period. However, it was interesting to note that Wy1463 reduced LD formation in LA treated cells to the level of the untreated controls while only partially inhibiting LD formation in response to the CLA isomers (Figures 9-10).

Contrary to our *in vitro* results, several studies in rats showed that CLA decreases hepatic TG levels by increasing the gene expression of lipid oxidative genes (180-181, 228), which are target genes of PPARα. Furthermore, recent evidence from our lab demonstrated that the t10,c12 CLA isomer reduces hepatic steatosis in obese insulin-resistant *fa/fa* Zucker rats, possibly by decreasing hepatic adipophilin levels and reducing LD size in the liver (205). It is interesting to note that adipophilin is transcriptionally regulated by PPARα in mouse liver and rat and human hepatoma cells through a highly conserved direct repeat-1(DR-1) element (37). Taken together, these results indicate that PPARα plays a significant role in the regulation of hepatic TG level in both *in vivo* and *in vitro* models.

Wy14643 reduces H4IIE cell viability

As shown previously, compared to untreated control cells, treatment with 60 µM of the CLA isomers significantly increased cell viability but there was no effect of 60 µM LA treatment. Interestingly, combined treatment of Wy14643 (PPARα activator) with CLA isomers or LA decreased H4IIE viability. However, Wy14643 in the absence of the FAs had no effect on cell viability (Figure 14). Our findings indicate that PPARα activation by Wy14643 in the presence of CLA isomers or LA was associated with decreased cell viability thereby implicating the importance of this nuclear receptor in the survival of the H4IIE cells. From our results, it can be speculated that the inhibition of LD formation by Wy14643 occurs at an early stage, thereby also inhibiting cell growth at an early stage. Consequently, this may decrease cell viability at low concentrations (60 µM) of the CLA isomers that otherwise promote cell viability.

Dietary CLA isomers and PPARα expression and phosphorylation in liver of *fa/fa* Zucker rats

Our previous observation that PPARα activation is associated with reduced LD formation in H4IIE cells suggests that PPARα effectively modulates FA metabolism under conditions associated with elevated FAs such as obesity and steatosis. PPARα can exist in a phosphorylated form, and its phosphorylation is increased by ciprofibrate in Fao cells (106). In addition to its ligand-induced activation, PPARα is regulated by ubiquitination (176) and phosphorylation *via* ERK-MAPK, PKA and PKC (49). CLA, primarily the c9,t11 CLA isomer, has been shown to be a potent ligand for human

PPARα (134). However, the effect of CLA isomers on PPARα phosphorylation is currently unknown. Therefore, we investigated the effect of dietary CLA isomers on PPARα level and its phosphorylation in the livers of 7 week old male *fa/fa* and lean Zucker rats fed either a control or a CLA isomer (0.4% wt/wt c9,t11 or 0.4% wt/wt t10,c12) diet for 8 weeks (kindly provided by Danielle Stringer).

Immunoblotting results showed that feeding the *fa/fa* rats the c9,t11 CLA isomer (fa 9,11) had no significant effect on PPARα phosphorylation compared to the lean control (ln Ctl) (Figures 15). Also, compared to the ln Ctl, levels of phospho-PPARα S21 were significantly lower in the t10,c12 CLA-fed *fa/fa* rats (fa 10,12) while there was no significant effect of t10,c12 on phospho-PPARα S12 (Figure 16). However, PPARα level was not significantly affected by any of the dietary CLA treatments (Figure 17). These results suggest that only the t10,c12 CLA isomer affects PPARα phosphorylation at the serine 21 position. Interestingly, treatment of quiescent H4IIE rat hepatoma cells with the potent PPARα agonist, Wy14643 reduced phospho-PPARα S12 and S21 levels (Figures A.1-A.2 in the Appendix section). Other investigators (208) have shown that PPARα mRNA and protein levels as well as PPARα transcriptional activity are increased through decreasing receptor phosphorylation at serines in the AF1 region of human hepatoma cells (HepG2) (discussed in detail in Review of Literature 2.1.3-iii PPARs- PPARα section). These results show that both Wy14643 and the t10,c12 CLA isomer similarly reduce hepatic PPARα phosphorylation at Ser 21whereas the c9,t11 CLA isomer has no significant effect on PPARα phosphorylation.

There was no significant difference in the levels of stearoyl co-A desaturase1 (scd1) in the fa Ctl and the fa 10,12 groups compared to the ln Ctl (Figure 18).

Furthermore, we observed no significant difference in the phosphorylation of p44/42 MAPK (Figure 19) in the fa Ctl and the fa 10,12 groups compared to the ln Ctl group. The t10,c12 CLA isomer significantly increased the phosphorylation of p38 MAPK in the *fa/fa* rats (fa 10,12 group) compared to all the other groups (ln Ctl, fa Ctl and the fa 9,11 groups) (Figure 20). However, neither of the CLA isomers affected p44/42 MAPK phosphorylation in quiescent H4IIE rat hepatoma cells (Figures A.5-A.6 in the Appendix section). These data imply that the effects of the t10,c12 CLA isomer on p44/42 MAPK phosphorylation observed in the *fa/fa* Zucker rats may be an indirect effect of this CLA isomer.

A recent study demonstrated that aldose reductase (which catalyzes the reduction of a variety of aldehydes and carbonyls, including monosaccharides) regulates hepatic PPARα phosphorylation and activity mainly by p44/42 MAPK signaling in mouse hepatocytes (179). The MAPK signal transduction pathway has an important role in cell growth. Thus, discovering if CLA isomers mediate PPARα functions either in a MAPK-dependent or -independent manner could provide useful information regarding the actions of this important bioactive compound. We have demonstrated that the activation of MAPK signalling molecule (increased p38 phosphorylation) in fa 10,12 rats was associated with reduced PPARα phosphorylation at S-21 indicating that the t10,c12 CLA isomer affects these molecules through independent pathways. It is also interesting to note that a similar response in PPARα (reduced phosphorylation) and MAPK (increased phosphorylation) was observed with the PPARα activator, Wy14643 (Figure A.3 in the Appendix section). Therefore, we speculate that Wy14643 and the t10,c12 CLA isomer

increase PPARα transcriptional activity via reduced receptor phosphorylation in a MAPK independent manner.

The importance of PPARα as a dietary FA sensor and in regulating hepatic FA content and composition was highlighted in another study under low fat intake conditions (4.8% w/w) which showed that PUFAs reduced hepatic fat stores in wild-type PPARα mice but not PPARα null mice (126). NAFLD is closely linked with obesity and an anti-obesity role for PPARα is well supported by several studies in rodents administered synthetic PPARα agonists (71, 123, 226). Murakami et al. (137) indicated a protective effect of PPARα agonism against abnormal lipid metabolism in the liver of obese Zucker *fa/fa* rats.

Previous studies from our laboratory have demonstrated that the PPARα ligand, t10,c12 CLA, reduces hepatic steatosis in obese insulin-resistant *fa/fa* Zucker rats. These changes were associated with a significant decrease in hepatic adipophilin levels and a reduction in LD size (205). This effect was not observed with the c9,t11 CLA isomer. Our results, therefore, contribute to an understanding of the roles of FA and PPARα in CLA-induced LD formation in H4IIE cells and in understanding the mechanism of the anti-steatotic effect of the t10,c12 CLA isomer in *fa/fa* Zucker rats. Priore et al. (177) have also suggested that increased FA oxidation with consequent decreased FA availability for TG synthesis is a potential mechanism by which CLA isomers reduce TG levels in rat liver. PPARα activation up-regulates genes involved in FA oxidation pathways, as well as increases hepatic uptake and esterification of FFAs (59). Therefore,

future studies need to focus on expression of PPARα target genes involved in FA oxidation in response to CLA isomer treatment.

These results indicate that PPARα activation has important implications in treating NAFLD mainly by the reduction of hepatic LD formation via decreased receptor phosphorylation which possibly increases its transcriptional activity and affects hepatic gene transcription. The H4IIE cells served as a useful model to examine the direct effects of CLA isomers, particularly on signal transduction while the animal model looked at the long-term exposure of dietary CLA. Thus these two systems play different roles.

6. CONCLUSIONS

The findings from this study support our first hypothesis that CLA isomers (c9,t11 or t10,c12) induce LD formation via ACS which is prevented by triacsin C or Wy14643. Since a similar effect on lipid accumulation was observed with LA, it can be concluded that LD formation in H4IIE cells is not unique to CLA isomers but more likely a FA effect.

ACS regulates the first essential step of TG formation by converting FAs into their acyl CoA forms in the endoplasmic reticulum, which is also the site associated with the biogenesis of LDs in cells. We show for the first time that ACS inhibition with triacsin C is linked to significant reductions in hepatic LD formation by LA or CLA isomers. In addition to ACS, we show that in the presence of Wy14643, LD formation was significantly reduced. These results indicate that FA and PPARα activation have a modulating role in hepatic lipid accumulation.

The increase in LD formation induced by CLA isomers, particularly at high concentration, was found to be associated with reduced rat hepatoma viability and thereby supports our second hypothesis.

From our results, we conclude that the physiological effects of CLA isomers on LD formation and cell viability are related and depend largely on the concentration of the FA. At low dosage, LD formation is hepatoprotective by activating the storage pathway via ACS and subsequent sequestering of the excess FAs as TGs into LDs. CLA isomers

(c9,t11 or t10,c12) promote quiescent H4IIE viability at low concentrations while LA has no such effect. However, the ability of the hepatocytes to store excess FAs declines at higher doses of CLA and LA and this effect was associated with reduced cell viability. Consequently, concentrations higher than 120 µM of the CLA isomers or LA decreased the viability of the hepatoma cells.

In addition, we demonstrate for the first time that dietary t10,c12 CLA isomer significantly reduced PPARα phosphorylation which is contrary to our last hypothesis. The t10,c12 CLA isomer specifically increased p38 MAPK phosphorylation *in vivo* but this effect was not observed with the c9,t11 isomer. These results indicate that the t10,c12 CLA isomer mediates hepatic PPARα phosphorylation and thereby its activity in a MAPK-independent manner. Since these changes were only observed between the ln Ctl and the obese insulin resistant *fa/fa* rats fed the t10,c12 CLA diet, the group with decreased hepatic steatosis, we speculate that activation of PPARα through decreased receptor phosphorylation at serine 21 in the AF1 region may be an independent effect of the t10,c12 CLA isomer in obesity and insulin resistance.

Therefore, results of this thesis provide novel insights into the mechanism of the isomer-specific effects of CLA in LD formation, cell viability and help to shed light into the underlying mechanism, particularly with respect to the role of ACS and PPARα activation in these two important cellular processes.

7. STRENGTHS AND LIMITATIONS OF THE RESEARCH

STRENGTHS

One of the greatest strengths of this study was the use of the rat H4IIE hepatoma cell line as the experimental model. Firstly, H4IIE cells specifically express the PPARα isoform. Secondly, *in vitro* models enable the investigation of the effects of various compounds under similar conditions and timelines, which is not feasible with animal models.

By using H4IIE cells, we have demonstrated for the first time that LD formation by CLA isomers and LA is dose-dependent. We further showed that this effect is markedly reduced by triacsin C, a potent inhibitor of acyl CoA synthetases, as well as the PPARα agonist, Wy14643. This *in vitro* model has enabled us to examine the direct effects of CLA isomers on hepaocyte viability with respect to LD formation and lipid accumulation.

Furthermore, cell viability was assessed with WST-8, which is much more sensitive than the conventional MTT, XTT, MTS or WST-1 and the kit does not require harvesting, washing or solubilization steps. Thus, experimental error is greatly reduced. The cck assay can also be used to indicate cell growth, however, when applied to quiescent and non-growing cells, it can be used to measure survival or cell viability.

LIMITATIONS

One of the main limitations of this study was regarding the cell viability assay. Cell viability was measured indirectly with the cck-8 kit which is based on the reduction of tetrazolium salts (WST-8) to formazan by dehydrogenases. It therefore implicates a role of the mitochondria in this process, but we did not study the effects of CLA isomers on mitochondria function/permeability.

Another limitation of this study was that the effects of the inhibitors or activators were not examined on the expression of their target genes or directly on the enzyme activity.

8. FUTURE DIRECTIONS

1. To determine the isomer-specific effects of the c9,t11 and t10,c12 CLA on the expression of PPARα target genes in hepatic LD formation.
2. To measure the activity of the hepatic ACSLs in response to PPARα agonists and/or CLA isomers in order to gain a better understanding of the mechanism that triggers FA channeling into specific catabolic or synthetic pathways.
3. To perform hepatic FA profiling in order to determine the effects of c9,t11 and t10,c12 CLA isomers and Wy14643 on FA composition, and determine the total amount of lipid.
4. Since the mitochondria play an important role in FA oxidation and apoptosis, it would be interesting to study the effect of these compounds on mitochondrial activity or permeability.

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10. APPENDIX

NAFLD is closely linked with obesity and an anti-obesity role for PPARα is well supported by several studies in rodents administered synthetic PPARα agonists (71, 123, 226). PPARα can exist in a phosphorylated form, and its phosphorylation is increased by ciprofibrate in Fao cells (106). In addition to its ligand-induced activation, PPARα is regulated by ubiquitination (176) and phosphorylation *via* MAPK, PKA and PKC (49). The MAPK signal transduction pathway has an important role in cell growth. Thus, discovering if MAPK signal transduction pathway mediates PPARα activation *via* affecting the phosphorylation of this nuclear receptor could provide useful insights into the role of PPARα in NAFLD and its associated hepatocellular damage.

Results from the studies presented in this thesis have demonstrated that the CLA isomers (c9,t11 and t10,c12) differentially affect the phosphorylation of PPARα with the t10,c12 isomer decreasing phosphorylation at Ser 21 position and the c9,t11 isomer reducing PPARα phosphorylation at both the serine sites (S12 as well as S21). However, this effect was associated with the activation of the p44/42 MAPK, particularly with the t10,c12 CLA isomer. These results suggest that the CLA isomers affect PPARα phosphorylation and thereby its transcriptional activity in a MAPK-independent manner. Other investigators (208) have shown that PPARα mRNA and protein levels as well as PPARα transcriptional activity are increased through decreasing receptor phosphorylation at serines in the AF1 region of human hepatoma cells (HepG2) (discussed in detail in

Review of Literature 2.1.3-iii PPARs- PPARα section). However, the effect of the PPARα agonist, Wy14643, on PPARα and MAPK phosphorylation was not studied.

Therefore, preliminary studies were undertaken to investigate the effects of Wy14643 on PPARα and MAPK phosphorylation using quiescent H4IIE rat hepatoma cells. Cellular protein extracts were used for immunoblotting. Our results showed that treatment with 250 µM Wy14643 significantly reduced the level of phospho-PPARα S12 compared to the untreated control or to the 50 µM Wy14643 group (Figure A.1). Similarly, levels of phospho-PPARα S21 were significantly lower in the quiescent H4IIE cells treated with higher concentrations of Wy14643 (100 µM and 250 µM groups) compared to the untreated control or to the 50 µM Wy14643 treatment group (Figure A.2). Interestingly, treatment with 250 µM Wy14643 significantly increased the phosphorylation of p44/42 MAPK compared to all the other groups (Figure A.3).

Therefore, in order to gain more insight into the underlying molecular mechanism of Wy14643-induced p44/42 MAPK phosphorylation, we studied the effects of the potent p44/42 inhibitor, PD98059 on p44/42 MAPK phosphorylation. Interestingly, treatment with 10^{-5} M PD98059 significantly reduced Wy14643-induced phosphorylation of p44/42 MAPK compared to the Wy14643 treatment group (Figure A.4).

We next compared the effects of the individual CLA isomers (c9,t11 or t10,c12) on Wy14643-induced p44/42 MAPK phosphorylation. It was found that neither of the CLA isomers affected p44/42 MAPK phosphorylation compared to the untreated controls (Figures A.4 and A.5). However, concomitant addition of 250 µM Wy14643 in the t10,c12 CLA isomer treated cells significantly increased p44/42 MAPK phosphorylation (Figure A.5). These results indicate that Wy14643-induced phosphorylation of p44/42

MAPK is not affected by the t10,c12 CLA isomer. Interestingly, combined treatment of the c9,t11 CLA isomer and Wy14643 significantly reduced p44/42 MAPK phosphorylation compared to the Wy14643 treatment group (Figure A.6). Thus, the c9,t11 CLA isomer potentially suppresses Wy14643-induced phosphorylation of p44/42 MAPK similar to the p44/42 inhibitor, PD98059.

To conclude, our preliminary results have demonstrated that the PPARα agonist, Wy14643 reduced the phosphorylation of PPARα at serines 12 and 21 in the AF1 region of the H4IIE rat hepatoma cells. We also observed that this effect of Wy14643 was associated with increased phosphorylation of p44/42 MAPK. Thus, we report that Wy14643 reduces hepatic PPARα phosphorylation at both serine 12 and 21 positions whereas the t10,c12 CLA isomer reduces PPARα phosphorylation only at Ser 21. Therefore, we speculate that these compounds increase PPARα transcriptional activity *via* reduced receptor phosphorylation in a MAPK independent manner, since these changes were associated with increased MAPK phosphorylation. It is also interesting to note that only the c9,t11 CLA isomer suppressed the Wy14643-induced p44/42 MAPK phosphorylation. This effect was not observed with the t10,c12 CLA isomer. In addition, Wy14643 affects p44/42 MAPK in a MEK-dependent manner since PD 98059 inhibited Wy14643-induced p44/42 MAPK phosphorylation. It would be interesting to see if the c9,t11 CLA isomer affects p44/42 MAPK phosphorylation in a similar manner. Future research may help to further clarify the underlying molecular mechanism of CLA isomers and Wy14643 on the MAPK signalling pathway and PPARα transcriptional activation.

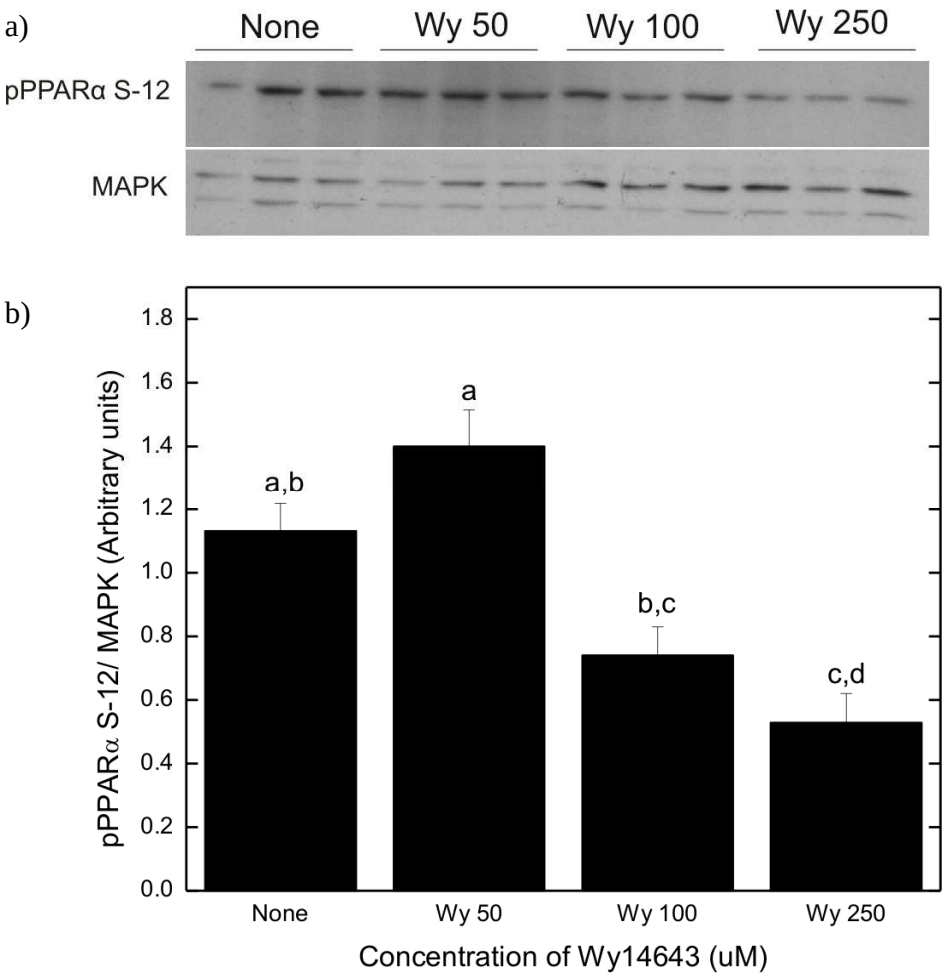

Figure A.1: Phospho-peroxisome proliferator activated receptor (PPAR) α S-12 levels in quiescent H4IIE rat hepatoma cells.

a) Quiescent H4IIE rat hepatoma cells were treated with 50 µM, 100 µM and 250 µM Wy14643 (Wy) in triplicate for 30 minutes to study the dose response of this PPARα agonist on the phosphorylation of PPARα. Cellular protein extracts were analyzed by immunoblotting using an antibody that recognizes phospho-PPARα S-12. A representative blot is shown.

b) Corresponding densitometric analysis of the bands (panel a) in arbitrary units. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SEM (n=3). Statistical significance ($p < 0.05$) was determined by one-way ANOVA and Tukey's test. Bars with different letters are significantly different.

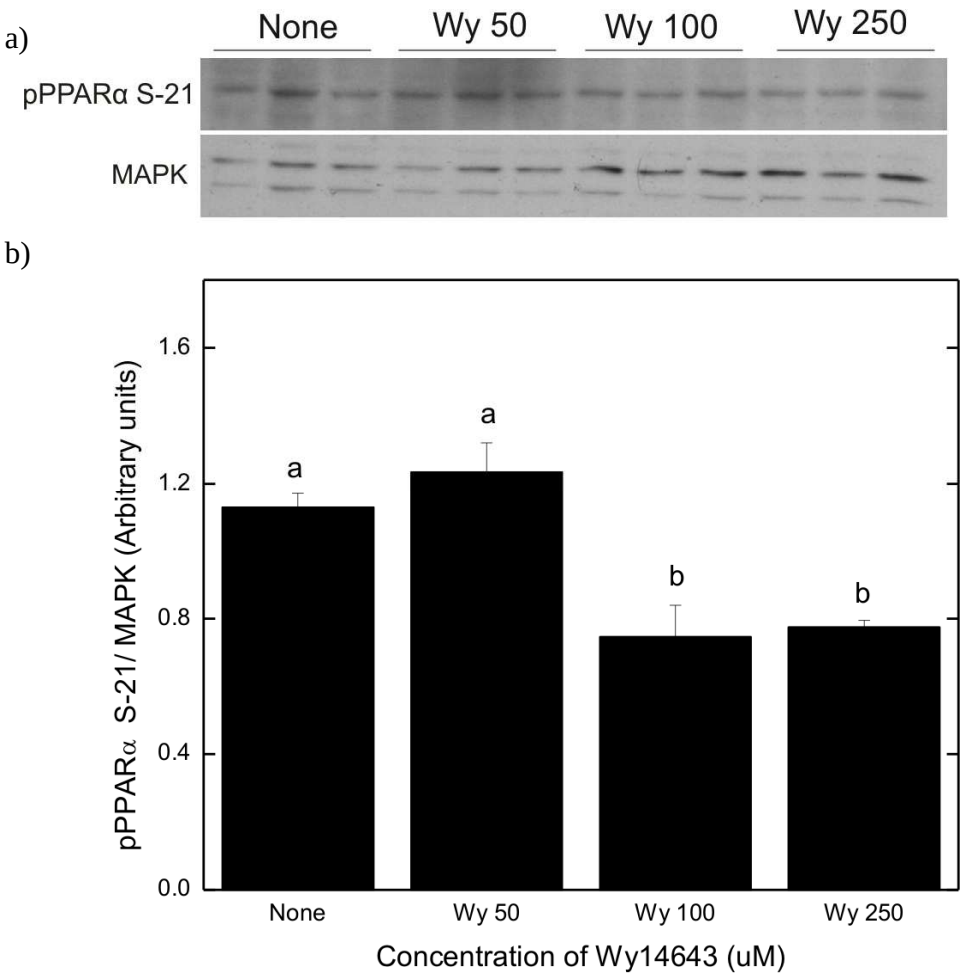

Figure A.2: Phospho-peroxisome proliferator activated receptor (PPAR) α S-21 levels in quiescent H4IIE rat hepatoma cells.

a) Quiescent H4IIE rat hepatoma cells were treated with 50 µM, 100 µM and 250 µM Wy14643 (Wy) in triplicate for 30 minutes to study the dose response of this PPARα agonist on the phosphorylation of PPARα. Cellular protein extracts were analyzed by immunoblotting using an antibody that recognizes phospho-PPARα S-21. A representative blot is shown.

b) Corresponding densitometric analysis of the bands (panel a) in arbitrary units. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SEM (n=3). Statistical significance ($p < 0.05$) was determined by one-way ANOVA and Tukey's test. Bars with different letters are significantly different.

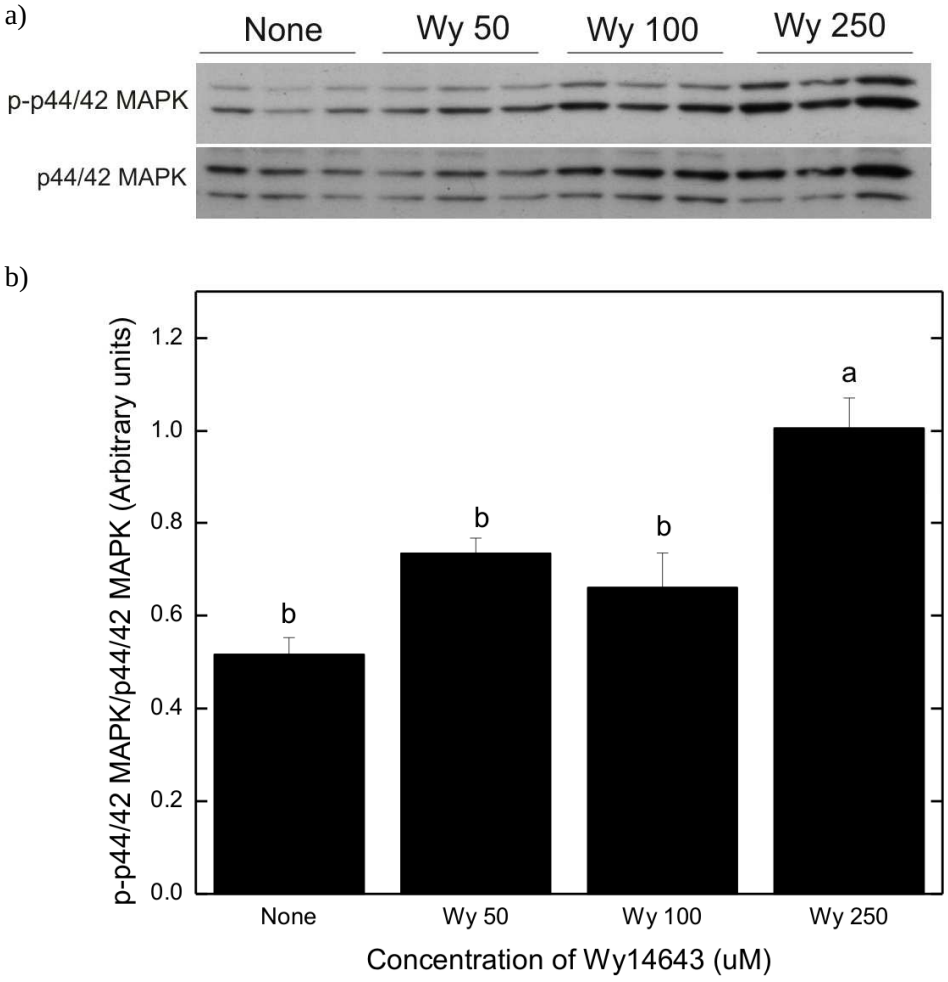

Figure A.3: Effects of Wy14643 on phospho-p44/42 MAPK (mitogen activated protein kinase) levels in quiescent H4IIE rat hepatoma cells.

a) Quiescent H4IIE rat hepatoma cells were treated with 50 µM, 100 µM and 250 µM Wy14643 (Wy) in triplicate for 15 minutes to study the dose response of this PPARα agonist on the phosphorylation of p44/42 MAPK. Cellular protein extracts were analyzed by immunoblotting using antibodies that recognize phospho-p44/42 MAPK and p44/42 MAPK. A representative blot is shown.

b) Corresponding densitometric analysis of the bands (panel a) in arbitrary units. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SEM (n=3). Statistical significance ($p < 0.05$) was determined by one-way ANOVA and Tukey's test. Bars with different letters are significantly different.

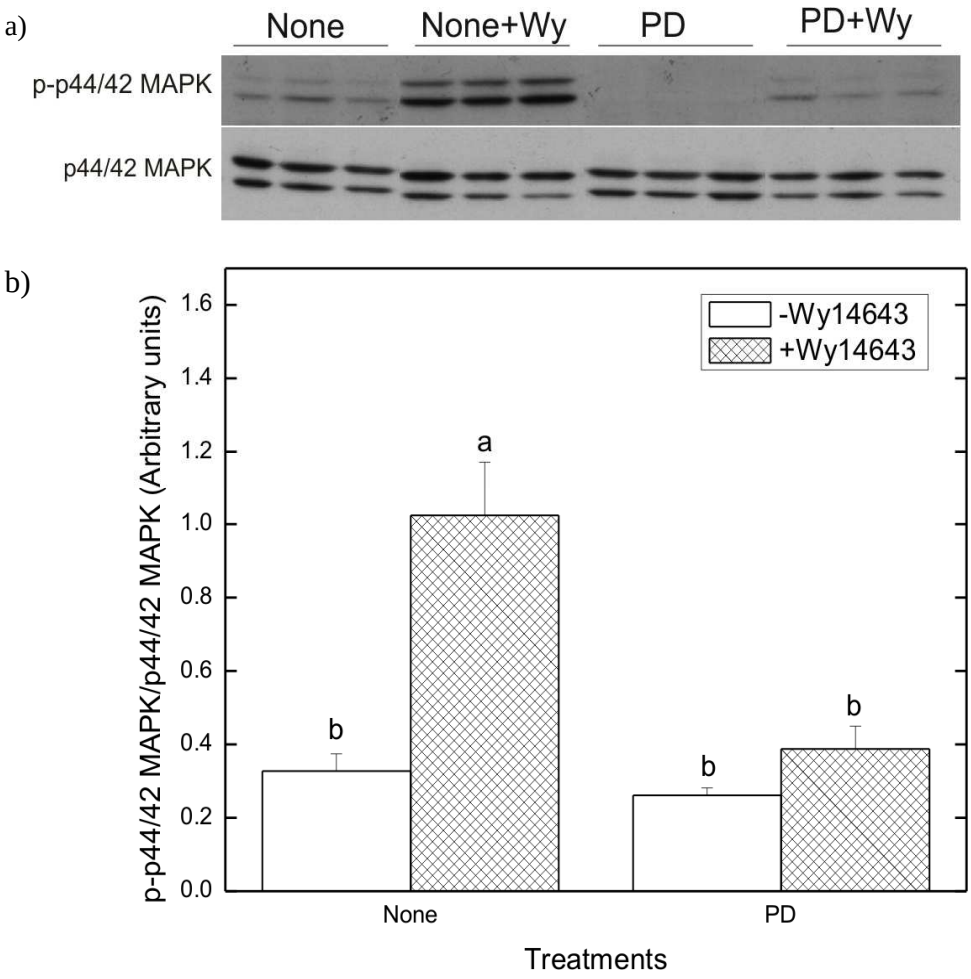

Figure A.4: Effects of PD98059 on phospho-p44/42 MAPK (mitogen activated protein kinase) levels in quiescent H4IIE rat hepatoma cells.

a) Quiescent H4IIE rat hepatoma cells were pre-treated with 10^{-5} M PD98059 (PD) for 30 minutes followed by the addition of 250 µM Wy14643 (Wy) for another 30 minutes (in triplicate) to assess the effects of this potent p44/42 MAPK inhibitor on Wy-induced phosphorylation of p44/42 MAPK. Cellular protein extracts were analyzed by immunoblotting using antibodies that recognize phospho-p44/42 MAPK and p44/42 MAPK. A representative blot is shown.

b) Corresponding densitometric analysis of the bands (panel a) in arbitrary units. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SEM (n=3). Statistical significance ($p < 0.05$) was determined by one-way ANOVA and Tukey's test. Bars with different letters are significantly different.

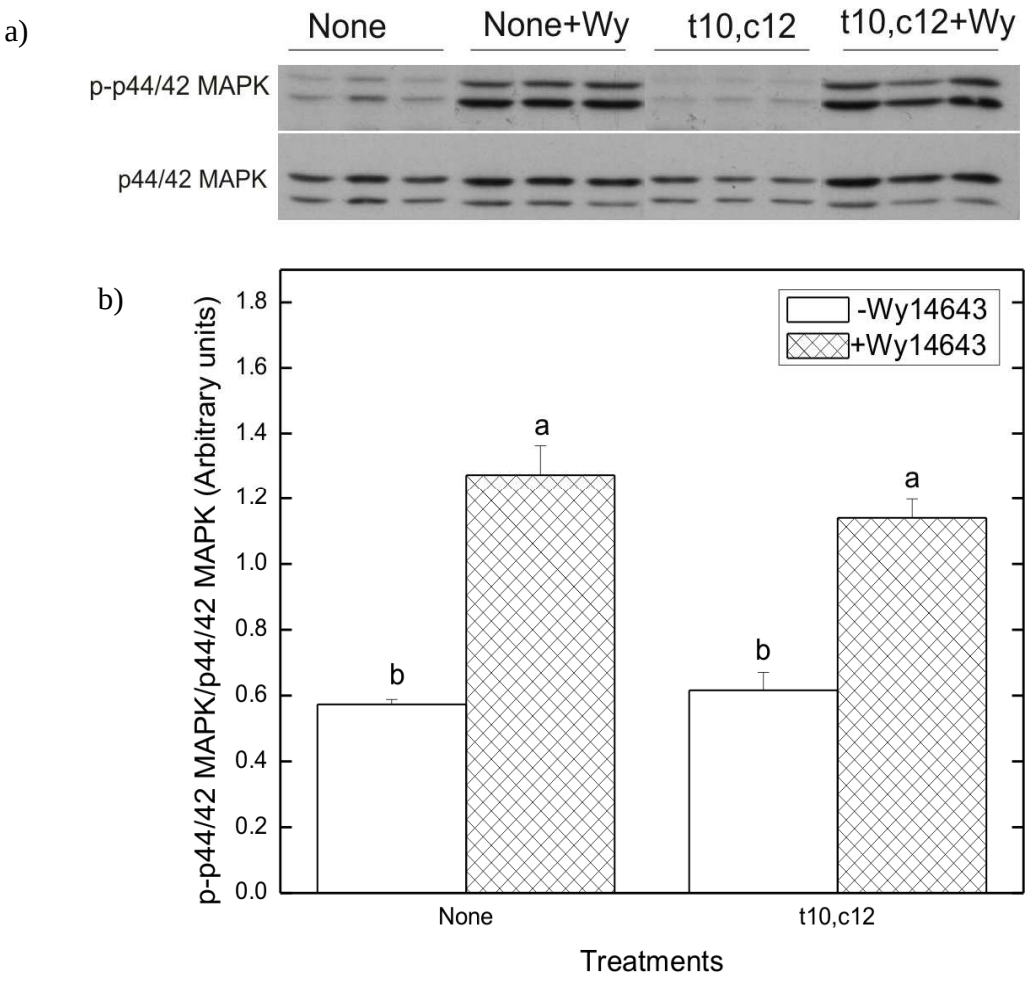

Figure A.5: Effects of *trans*-10,*cis*-12 (t10,c12) CLA isomer on phospho-p44/42 MAPK (mitogen activated protein kinase) levels in quiescent H4IIE rat hepatoma cells.

a) Quiescent H4IIE rat hepatoma cells were pre-treated with 60 µM *trans*-10,*cis*-12 CLA (t10,c12) for 30 minutes followed by the addition of 250 µM Wy14643 (Wy) for another 30 minutes (in triplicate) to assess the effects of this CLA isomer on Wy-induced phosphorylation of p44/42 MAPK. Cellular protein extracts were analyzed by immunoblotting using antibodies that recognize phospho-p44/42 MAPK and p44/42 MAPK. A representative blot is shown.

b) Corresponding densitometric analysis of the bands (panel a) in arbitrary units. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SEM (n=3). Statistical significance ($p < 0.05$) was determined by one-way ANOVA and Tukey's test. Bars with different letters are significantly different.

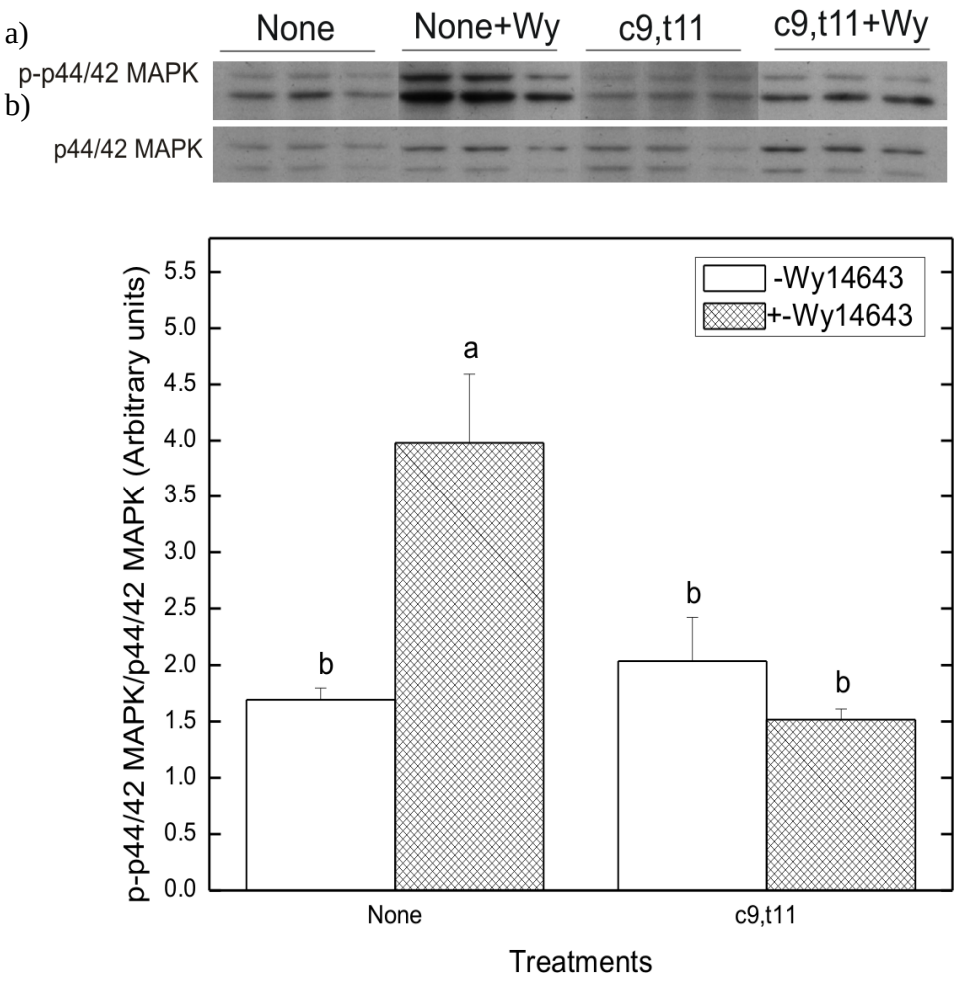

Figure A.6: Effects of *cis*-9,*trans*-11 (c9,t11) CLA isomer on phospho-p44/42 MAPK (mitogen activated protein kinase) levels in quiescent H4IIE rat hepatoma cells.

a) Quiescent H4IIE rat hepatoma cells were pre-treated with 60 µM *cis*-9,*trans*-11 CLA (c9,t11) for 30 minutes followed by the addition of 250 µM Wy14643 (Wy) for another 30 minutes (in triplicate) to assess the effects of this CLA isomer on Wy-induced phosphorylation of p44/42 MAPK. Cellular protein extracts were analyzed by immunoblotting using antibodies that recognize phospho-p44/42 MAPK and p44/42 MAPK. A representative blot is shown.

b) Corresponding densitometric analysis of the bands (panel a) in arbitrary units. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SEM (n=3). Statistical significance ($p < 0.05$) was determined by one-way ANOVA and Tukey's test. Bars with different letters are significantly different.