2013

Characterization of coconut oil and CLA induced lipolysis

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by

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Dissertation submitted to the Davis College of Agriculture, Natural Resources and Design at West Virginia University in partial fulfillment of the requirements for the degree of

Ph.D.
in
Animal & Food Sciences

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Morgantown, WV
2013

Key words: Conjugated linoleic acid, coconut oil, lipolysis, inhibitors, 3T3-L1

ABSTRACT

Characterization of coconut oil and CLA induced lipolysis

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Obesity is a main health concern and leads to many other health complications. Conjugated linoleic acid (CLA) has been shown to cause a reduction in obesity in several species. CLA-induced body fat loss is enhanced when mice are fed coconut oil (CO). The objectives were to determine if the CLA-induced lipolysis in different oil source-fed mice was time-dependent and to determine the effect of cell signaling inhibitors on CO+CLA-induced lipolysis. Study 1: Male mice (ICR; n=80; 3wk) were fed 7% soybean oil (SO) or CO diets for 6wk and then supplemented with 0 or 0.5% CLA for 3, 7, 10 or 14d. Body fat index (BFI) was calculated as [(epididymal fat pad + retroperitoneal fat pad)/ body weight] and lipolysis was determined by non-esterified fatty acid (NEFA) and glycerol release in 3hr ex vivo cultures. BFI was reduced by CO on d7 ($P<0.01$) and CLA tended ($P=0.09$) to decrease BFI in CO-fed mice on d10. BFI was reduced in both CO and SO-fed mice ($P<0.05$) in response to CLA on d14. NEFA release was increased by CLA in CO-fed mice ($P<0.01$) but not in SO-fed mice on d7 and 10 but on d14 CLA increased NEFA release in both CO and SO-fed mice ($P<0.0001$). Glycerol release was also increased by CLA in CO-fed mice but not in SO-fed mice on d3 and d7 ($P<0.05$). We then determined expression and activation level of proteins involved in lipolysis and lipogenesis. CLA tended to decrease ($P=0.06$) p-perilipin protein expression in CO-fed mice. There was also a trend for CLA ($P=0.06$) to decrease adipose triglyceride lipase (ATGL) protein. CO-fed mice had greater fatty acid synthase, stearoyl CoA desaturase 1 mRNA expression and less acetyl CoA carboxylase mRNA expression ($P<0.01$). Sterol regulatory binding protein 1c and malic enzyme expression was least in CO+CLA-fed mice. Study 2: 3T3-L1 cells were differentiated, exposed to CO or SO for 10d, serum starved overnight, and pre-loaded with 3[H]-oleic acid for 12 hrs. Cells were treated with 50 μM CLA or linoleic acid (LA) with/without cell signaling inhibitors for 12-24 hrs. Lipolysis was measured as the 3-hr release of 3[H]-oleic acid. Without inhibitors, CO+CLA treatment caused more lipolysis ($P<0.01$) than all other treatments, which did not differ. None of the inhibitors tested reduced lipolysis in CO+CLA treated cells. Cyclooxegenase-2 inhibitor increased lipolysis of SO+CLA treated cells ($P=0.05$) to the level of CO+CLA. Phospholipase C inhibitor increased lipolysis in all treatments ($P<0.0001$) except that of CO+CLA. Peroxisome proliferator-activated receptor α inhibitor also increased lipolysis of CO+LA ($P<0.05$) to the level of CO+CLA treated cells and in SO+CLA treated cells. There was no effect of the p42 mitogen-activated protein kinase or protein kinase A inhibitor, compared to absence of inhibitor. Therefore CLA-induced lipolysis occurs more rapidly in CO vs SO-fed mice and the CLA enhanced lipolysis in CO group could involve the PLC pathway.

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INTRODUCTION

Obesity is currently one of the major medical challenges affecting approximately 1/3rd of the adult population. High body lipid content plays a role in other medical complications including metabolic syndrome, diabetes, hypertension, and atherosclerosis. There are several pathways by which the lipid content in tissues can be regulated by the body. When the energy intake exceeds energy expended for various physiological processes, the body has an excess of energy. The excess energy in the body is stored as body fat. The amount of body fat can be decreased by increasing energy expenditure and decreasing food intake. Adipose tissue is the major energy-storing tissue and plays a role in maintaining energy homeostasis. Adiposity can be regulated by regulating lipid metabolism. An increase in lipid oxidation and/or a decrease in *de novo* lipid synthesis would lead to a decrease in adiposity. Since adipose tissue responds rapidly to alterations in nutrient content, dietary composition and the type of lipids can influence the rate of lipid metabolism in the body. Hence, dietary supplements for regulation of body fat have become popular and several of these include fatty acids like conjugated linoleic acid (**CLA**) and the ω-3 fatty acids.

CLA refers to a group of positional and geometric isomers of linoleic acid. The two most biologically active isomers are c9, t11 and t10, c12. CLA was first discovered by Ha et al. [1] and identified as an anti-carcinogen in grilled beef [2]. Since then, CLA was found to have several health benefits including enhancing the immune response [3], reducing atherosclerosis [4], and reducing body fat gain [5, 6]. The t10,c12 isomer is the active form in altering the body composition while the c9,t11 isomer is more effective in causing an anti-carcinogenic effect [7, 8]. Both isomers were found to have similar effects on immune responses. Since obesity has

been a growing problem in recent years, I am going to further discuss the mechanisms by which body fat can be regulated and the effect of CLA on body composition.

CHAPTER 1: LITERATURE REVIEW

MECHANISMS OF BODY FAT LOSS

Several possible mechanisms for inducing body fat loss have been proposed and investigated. It can be achieved by altering energy homeostasis, lipolysis, lipid metabolism, and/or the expression of various proteins. In this section I shall discuss more about some of the possible mechanisms.

Feed intake and energy expenditure:

One of the mechanisms of body fat loss could be by regulation of energy metabolism. Energy stored can be expressed as a function of energy intake and energy expenditure (**EE**). If EE exceeds energy intake there will be a loss in body weight or body fat mass. The reduction in feed intake is mediated by alteration in neuropeptides involved in regulating food intake. CLA has been demonstrated to cause both a decrease in feed intake and/or an increase in energy expenditure. Park et al. showed that mice fed 0.5% t10,c12 CLA (w/w) for 4 wks had reduced feed intake compared to mice fed control diets [5]. Several other studies have shown that CLA at a dose of 0.5% or more can cause a reduction in feed intake in rodents [6, 8]. CLA has been shown to increase expression of propiomelanocortin [9], an appetite decreasing neuropeptide, and decrease in expression of neuropeptide Y [10], a neuropeptide that increases food intake. This reduction in feed intake however is not sufficient to cause the loss of body fat since, mice pair-fed a control diet to the levels of the CLA-fed mice didn't differ in body composition to ad-libitum fed control mice, while the CLA-fed mice were leaner [11]. Also, some studies have reported a decrease in body fat following CLA administration without a reduction in feed intake

[12, 13]. This suggests that reduction in feed intake is not the cause of CLA-induced body fat loss and that other mechanisms are involved in the decline of lipid deposition in adipocytes.

Adiposity can also be reduced by increasing EE through regulating basal metabolic rate, thermogenesis and oxidation in animals. West et al. observed that mice fed 1% CLA had increased EE compared to corn oil-fed mice in both a high and low-fat fed group [6]. It was later confirmed that this increased EE was sufficient to cause the loss in body fat seen in CLA-fed mice [14]. In CLA-fed mice the percentage of energy intake expended as heat was 3% greater than that in sunflower oil-fed mice, when mice were fed 0.93% CLA for 39 d [12]. This enhanced thermogenesis could be associated with alterations in uncoupling protein (**UCP**) expression.

UCPs play an important role in energy expenditure and thermogenesis. UCPs are found in the inner mitochondrial membrane and facilitate proton transport from the mitochondrial inter-membrane space to the matrix, thereby "uncoupling" electron transport from the production of ATP and dissipating the energy of the electrochemical gradient as heat. The different UCP homologs are expressed differentially in various tissues. UCP1 is expressed primarily in the brown adipose tissue. UCP2 is expressed ubiquitously in multiple tissues and is the highly expressed UCP. UCP3 is expressed in skeletal muscle and brown adipose tissue [15, 16]. Supplementation with CLA in rodents caused an increase in UCP2 mRNA expression in brown [14] and white adipose tissue, liver, and skeletal muscle [17]. This increase in UCP2 mRNA was accompanied by a simultaneous decrease in adipose deposition [18, 19]. UCP2 activity has been found to be increased through a phospholipase C (**PLC**) signaling pathway. Activation of PLC increases the Ca^{2+} influx which activates calmodulin. Activation of calmodulin increases UCP2 activity by phosphorylation [20]. Since UCP2 is the predominant UCP in white adipose tissue, a

CLA-induced up regulation of UCP2 expression [17] may be responsible for increased energy expenditure and reduced adipose deposition in animals fed CLA.

Lipid synthesis & oxidation:

A reduction in body fat content could be achieved by inhibiting lipogenesis and lipid uptake, and/or by increasing oxidation of lipids. In this respect, it has been found that treatment of 3T3-L1 cells with 20-200 μM CLA caused an inhibition of lipoprotein lipase (**LPL**) activity [5]. The uptake of fatty acids into the cell is regulated by the activity of LPL, which hydrolyzes circulating triacylglycerides (**TAGs**) to free fatty acids that can be taken up and re-esterified inside the cell. The effect of CLA on LPL activity appears to be dose-dependent [8]. Studies in several species have shown that CLA also has an anti-lipogenic effect. Correspondingly, supplementation of 1% CLA for 5 months in mice, down regulated the mRNA levels of several enzymes involved in lipogenesis including acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase 1 [19]. SREBP-1 and PPAR γ are the major transcription factors that regulate the expression of these lipogenic enzymes. The anti-lipogenic effects of CLA may be exerted in part by its ability to decrease the expression of SREBP-1, and PPAR γ [18]. Hence, evidence indicates that CLA causes a decrease in adiposity by inhibiting lipogenesis.

Several studies have demonstrated the ability of CLA to increase oxidation of fatty acids. Also, dietary supplementation of CLA to rats lowered the respiratory quotient indicating an increase in lipid oxidation [21]. Sergei et al. demonstrated that radiolabelled CLA isomers were oxidized more rapidly than linoleic acid in rats, over a 2h period [22]. In addition, rats supplemented for 6 wks with 1% t10,c12 CLA had increased activity of liver and adipose tissue

carnitine palmitoyl transferase (**CPT**) activity [21]. CPT is the rate limiting enzyme in the mitochondrial β-oxidation of fatty acids. In addition, hepatic acyl-CoA oxidase (ACO) mRNA level, a rate limiting enzyme in peroxisomal β-oxidation, was increased 3-fold in rats fed 1.5% CLA compared to rats fed control diet [23]. These combined results indicate that CLA increases fatty acid oxidation. Thus, the anti-obesity effects of CLA could be at least partially mediated by its regulation of lipid metabolism. The expressions of CPT and ACO genes are regulated by the transcription factor PPAR-α. CLA has been shown to activate PPAR-α, which in turn causes the activation of the PPAR responsive genes ACO, and CPT [24]. Hence, in part, the loss of body fat can be due to down-regulation of lipogenesis and up-regulation of lipid oxidation.

Lipolysis:

Lipolysis is the process by which TAGs stored in the adipocytes are broken down and non-esterified fatty acids (**NEFA**) and glycerol are released. Lipolysis is stimulated by various factors including; glucagon and low circulating levels of catecholamines via cAMP-mediated signaling, which is the "classic" pathway of lipolysis stimulation. Mature adipocytes have unilocular lipid droplets (**LD**) surrounded by the lipid coat protein perilipin (**Plin**). Hormone sensitive lipase (**HSL**) and adipocyte triglyceride lipase (**ATGL**) are the two predominant adipocyte lipases (having acyl-hydrolase activity); collectively they account for about 94% of lipolysis (as characterized by the release of free fatty acids) [25]. ATGL acts on TAGs, while HSL has greater affinity towards diacylglycerides [26]. Plin is the main effector of protein kinase A (**PKA**) stimulated lipolysis. When lipolysis is stimulated perilipin is phosphorylated by PKA. Phosphorylation of Plin causes comparative gene identification 58 (**CGI-58**) to dissociate

from Plin and bind to ATGL, thus activating ATGL (Figure 1.1) [27]. Inhibition of ATGL caused a significant decrease in acyl-hydrolases activity [26]. Knockout of ATGL decreased lipolysis more than knockout of HSL [25]. Also, when lipolysis is stimulated, HSL is phosphorylated at any of four possible sites; upon activation it translocates to the LD surface and this translocation is dependent on the presence of phosphorylated perilipin [27]. Phosphorylation of HSL at sites Ser563, or 660 by PKA or Ser600 by mitogen-activated protein kinase (**MAPK**) increases its activity (Figure 1.1), while phosphorylation at Ser565 by AMP-activated protein kinase (**AMPK**) decreases its activity (Figure 1.1). HSL is required for normal adipose tissue function and structure, as HSL-deficient mice have adipocyte hypertrophy and an increased inflammatory infiltration in white adipose tissue [28].

Chung et al. reported that 30 μM t10,c12 CLA increased lipolysis in human adipocyte cultures [29]. In support of this, several cell culture and animal studies have demonstrated that supplementation of a CLA mixture or t10,c12 isomer caused an increase in lipolysis [30, 31]. The increase in lipolysis could contribute to the decrease in adiposity observed with CLA supplementation.

In contrast to the above studies, there are some reports of CLA not altering lipolysis. Studies in rodent models of CLA supplementation have demonstrated that CLA supplementation has no effect on lipolysis [32] or that there was a reduced level of glycerol release in CLA treated rat adipocytes [33]. In agreement with this, a previous study in our laboratory observed that dietary CLA caused an increase in basal lipolysis in CO-fed mice but not in SO-fed mice [31]. Also, Brown et al. found that in cultures of human adipose tissue, lipolysis was unaltered by either of the CLA isomers after a 3-day supplementation [34]. This discrepancy may be because in the cell culture studies in which an effect on lipolysis was observed, the cultures were serum-

starved for 12-24 h prior to supplementation of CLA which causes a deficiency of fatty acids in the culture. Also in the animal studies, the supplementation of CLA were for either too short (4d) or too long of a period (3wks) at which point either the activation of lipolysis hasn't occurred or the TAG content has been depleted respectively to not elicit a change in lipolysis between control and CLA-fed mice.

In a previous study, we observed a decrease in lipolysis-related protein activation in response to CLA even though NEFA release from cultured adipose tissue was increased [31]. We hypothesize that the effect of CLA is time-dependent and feeding CLA initially causes an increase in lipase activation, followed by a decrease in the expression or activation of lipase. This is based largely on a cell culture study; Chung et al. reported that there was an increase in cytosolic perilipin (which indicates phosphorylated perilipin) accumulation after 12 hr of CLA treatment, followed by a decrease in the overall expression of perilipin and HSL by 24 hr [29].

To fully understand the discrepancy of CLA eliciting an increase in lipolysis in some but not in all studies, an insight into the various signaling pathways which can regulate lipolysis would be helpful. In addition to the cAMP dependent pathway, there are several other pathways which can alter lipolysis in an adipocyte in response to various fatty acids. Phospholipase C (**PLC**) has been known to be activated by unsaturated fatty acids [35, 36]. PLC cleaves phoshotidylinositol bisphosphate into diacylglyceride (**DAG**) and inositol 1,4,5-trisphosphate (IP3). IP3 leads to the opening of calcium channels and release of Ca^{2+} into the cytosol. DAG and Ca^{2+} together activate protein kinase C (**PKC**), which further activates other kinases by phosphorylation. PLC induced PKC activation leads to the activation of p42/p44 MAPK. The activation of p42/p44 MAPK has been shown to stimulate lipolysis in adipocytes by increasing HSL phosphorylation at Ser600 [37].

In addition, there is evidence for pathway mediated by prostaglandin E2 (**PGE_2**) to play a minor role in regulation of lipolysis. Arachadonic acid can be converted into PGE_2 by cyclooxygenase (**COX-2**). PGE_2 binds to a G_i protein coupled receptor and activates the G_i pathway which inhibits adenylyl cyclase thus reducing cAMP generation [38]. This leads to decreased PKA stimulated lipolysis. CLA supplementation has been shown to decrease PGE_2 levels [39, 40]. Some cell culture studies have reported that CLA increases lipolysis in a cAMP independent manner by increasing signaling through the MAPK/ERK signaling pathways [30].

EFFECT OF CLA ON BODY COMPOSITION

CLA appears to play a role in affecting most of the mechanisms involved in adiposity reduction, so its effect on body composition is further described. The effect of CLA on body fat was first identified by Park et al, when mice fed 0.5% CLA for 4 wks had 60% lower body fat and increased lean mass with no effect on total body weight [5]. Later, studies have shown that CLA caused a decrease in body fat in several animal species, including mice [5, 6], rats [13, 41], hamsters [42]and pigs [43, 44]. Mice fed either a mixture of CLA isomers or the t10,c12 isomer alone had a reduction in body fat and weight while those fed the c9, t11 isomer did not differ in their body composition compared to the controls [8, 11]. In pigs, CLA caused an increased the saturated fatty acid, and decreased unsaturated fatty acid, content of the longissmus muscle and adipose tissue [45, 46]. This resulted in an improvement in meat firmness and hence improved pork quality. In previous studies in our lab, it was observed that fat lowering effect of CLA is enhanced when mice were fed coconut oil (**CO**) or fat-free diets as compared to mice fed soybean oil diets (**SO**) [31, 47, 48]. In addition to being deficient in essential fatty acids, CO has

a greater percentage of saturated short and medium chain fatty acids, while SO has saturated and unsaturated 18 carbon fatty acids.

On the other hand, results from human studies on the effects of CLA on body composition are inconsistent. The studies in which there was a decrease in fat mass, subjects were supplemented with a higher percentage of CLA relative to those studies in which no effects were detected. In normal weight humans (BMI 20-25 kg/m^2), CLA supplementation caused a 8% loss of fat mass but the subjects were undergoing physical training in most of these studies [49]. Hence, it is unclear if the body fat loss was an effect of CLA or a synergistic effect of CLA and exercise. Several other studies reported no change in body weight or body fat when CLA was provided at dosage of 0.6-6 g/d [50, 51]. In overweight or obese humans, a few studies showed that CLA dosage of 3.4 g/d caused about a 3% decrease in body fat [52, 53], while in some studies even a dosage of 5-6 g/d didn't produce any effect on body composition [51, 54]. Thus, there is minimal evidence that CLA supplementation induces weight or fat loss in humans.

The effect of CLA on body fat appears to be dependent on species and strain. Hence, a full understanding of the mechanisms by which CLA induces the body fat loss in animal models could provide insight into what the signalling pathway activated by CLA in humans will be (if any) and thus provide an explanation and solution to the discrepancy of effect of CLA in animal and human models.

SUMMARY

Results from animal and cell culture studies indicate that t10,c12 CLA has the ability to reduce body fat content in several different species. The anti-obesity effect of CLA appears to

be dose dependent and species dependent. The differences among studies appear to be due to differences in dosage and type of CLA used, age, genetic composition and metabolic status of animals. There was an increase in body energy expenditure due to dietary CLA. This effect is largely due to alterations in the expression of UCP. CLA causes an increase in UCP2 expression, causing an increase in energy expenditure.

In the liver, CLA decreased FAS, ACC, SCD-1 expression and increased CPT1 expression, which cause a decrease in fatty acid synthesis and an increase in β-oxidation. Thus it appears that lipid metabolism may be regulated by CLA-induced inhibition of transcription factors; SREBP-1 and PPAR-γ and upregulation of PPAR-α.

In the adipose tissue, CLA has been found to increase lipolysis, and decrease adipocyte accumulation of TAG. The effect of CLA on lipolysis was more pronounced in CO-fed mice as compared to SO-fed mice. Hence we hypothesized that CLA may cause an increase in lipolysis in CO-fed mice by inducing signaling through the PLC/MAPK/ERK pathway, and also that the effect on lipolysis is time-dependent.

Since the body fat lowering effect of CLA is not as pronounced in humans, there is a need to elucidate the pathways of CLA action. Also, further research needs to be done to understand the mechanism of action of CLA in CO-fed mice in causing the increase in lipolysis, which could help in devising treatments for obesity.

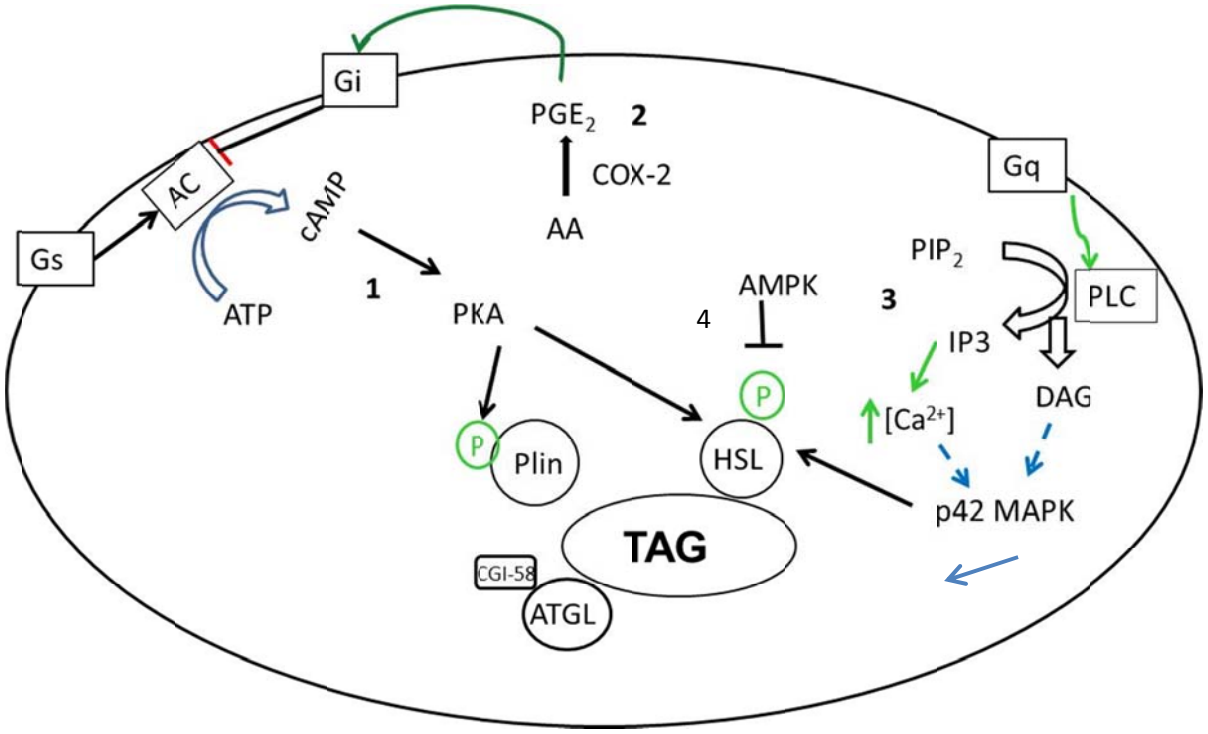


Figure 1.1: Pathways affecting lipolysis in an adipocyte. 1) G_s protein coupled PKA activation pathway. 2) G_i protein coupled inhibition of PKA. 3) PLC mediated activation of p42/p44 MAPK. 4) AMPK mediated inhibition of HSL. ⟶ Represents activation, ⟊ represents inhibition of a protein. AA, Arachadonic Acid; AC, Adenylate Cyclase; AMPK, AMP-activated Protein Kinase; ATGL, Adipose Triglyceride Lipase; CGI-58, Comparitive Gene Identification-58; COX-2, Cyclooxygenase 2; DAG, Diacylglycerol; ERK, Extracellular-signal Regulated Kinase; $G_{s/q/I}$, G protein-coupled receptors; HSL, Hormone Sensitive Lipase; IP3, Inositol triphosphate; MAPK, Mitogen Activated Protein Kinase; PGE_2, Prostaglandin E2; PIP_2, Phosphatidylinositol 4,5-bisphosphate; PKA, Protein Kinase A; PLC, Phospholipase C; Plin, Perilipin; TAG, Triacylglycerol.

CHAPTER 2: TIME-DEPENDENT EFFECT OF CLA IN COCONUT OIL FED MICE ON LIPOLYSIS AND LIPID METABOLISM

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid. CLA was first discovered in grilled beef and was found to have anti-carcinogenic properties [1]. Since then, dietary CLA has been found to have several other health benefits including enhancing immune response [2], reducing atherosclerosis [3], and reducing body fat [4, 5]. The t10, c12 isomer is solely responsible for the induction of a loss of body fat [6, 7].

The body fat lowering effect of CLA seen in animal models has not been fully reciprocated in humans. One possible reason is that the intake of various other polyunsaturated fatty acids in a typical human diet could possibly have an antagonistic effect on the body fat lowering effect of CLA, as we have found that the body fat lowering effect of CLA is enhanced in mice fed coconut oil (CO) or fat-free diets, as compared to mice fed a soy oil (SO) diet [8, 9]. CO is composed of a higher concentration of short and medium chain saturated fatty acids (12 to 16 carbons) and is deficient in the essential fatty acids linoleic acid and α-linolenic acid, compared to SO which has mostly saturated and unsaturated 18 carbon fatty acids. As similar results were obtained with fat-free diets, it indicates that something in SO, possibly the PUFA, is counteracting the full anti-obesity effect of CLA.

Previously we reported that CLA supplementation caused an increase in basal lipolysis in CO, but not in SO-fed mice [9]. This is supported by cell culture studies that have shown that CLA treatment caused an increase in lipolysis, but only when the cells were serum-starved overnight prior to CLA treatment as this would devoid the medium of any fatty acids which could potentially interfere with CLA [10, 11]. This increase in lipolysis is likely contributing to the enhanced body fat loss observed in CO+CLA-fed mice.

Previously, we have observed a disconnect between NEFA release and protein expression/activation, a decrease in perilipin and HSL phosphorylation in response to CLA in spite of an increase in ex-vivo lipolysis, measured as NEFA and glycerol release [9]. We hypothesize that the effect of CLA is time-dependent and feeding CLA first causes an increase in lipase activation, followed by decrease in the expression or activation of perilipin and lipases, as this has been reported in a cell culture study. Chung et al. reported that there was an increase in cytosolic perilipin (which indicates p-perilipin) accumulation after 12 hr of CLA treatment, followed by a decrease in the expression of perilipin and HSL by 24 hr [11].

Additionally, the increase in lipolysis in CO+CLA-fed mice might not fully explain the enhanced body fat loss. Changes in the rates of lipogenesis could also contribute. CLA has been reported to reduce lipogenesis [10, 12], but the effect in CO-fed mice is unknown. Hence, in this study we determined the effect of CLA and base oil on the mRNA expression of proteins involved in lipogenesis, including fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), malic enzyme, sterol regulatory element binding protein 1c (SREBP1c), and stearyl CoA desaturase 1 (SCD1). Therefore, our overall objective was to determine the time-dependent effect of CLA supplementation on mice-fed different base oil diets on lipolysis and lipogenesis.

METHODS

Animal Protocol:

All animal procedures were approved by the West Virginia University Animal Care and Use Committee. Weanling male mice [n = 80; 3 weeks old; ICR (Imprinting Control Region)] were obtained from Harlan Inc. (Madison, WI) and housed in a temperature-controlled animal

room (25°C) with a 12:12 h light:dark cycle. Mice were housed four per cage for weeks 1–5 and then were individually caged from week 6 on. Animals were randomly assigned to base diets: SO or CO. Mice were fed a modified AIN-93G diet with the composition as shown in Table 1. After 6 weeks, half of each group were fed 0.83% CLA oil (50:50 t10,c12 to c9,t11; 60% purity; BASF, Offenbach/Quiech, Germany) to provide 0.5% CLA isomers, replacing base oil wt/wt, for 3, 7, 10, or 14 days. At the end of the study period the mice were killed by carbon dioxide asphyxiation. One fat pad pool (consisting of one epididymal and one retroperitoneal fat pad) was utilized for lipolysis analysis, liver was homogenized for cytosol extraction, and the remaining fat pad pool and a liver sample were flash frozen in liquid nitrogen and stored at -80°C until further analysis was performed. Blood was collected by cardiac puncture and stored on ice. A body fat index (BFI) was calculated as: [(epididymal + retroperitoneal fat pad weights)/ total body weight] * 100.

Lipolysis:

The lipolysis assay was performed as we described previously [9]. NEFA and glycerol concentrations were corrected for tissue weight and reported as μmol released/g tissue. Blood was centrifuged at 2,000 g for 20 min (model 5415R, Eppendorf, Hauppauge, NY) at 4° C. The serum was collected and the concentration of circulating NEFA was analyzed similar to the method above. Total ketone bodies were also measured spectrophotometrically using the Autokit Total Ketone Bodies (Wako Diagnostics, Richmond,VA).

Western Blotting:

Western blotting was performed as previously described [9], with the exception that adipose tissue samples (n=5/diet/time point) were sonicated (3× for 30 sec, 20W) rather than homogenized. The western blots were analyzed for presence of total perilipin (1:500), ATGL (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated perilipin (1:500), phosphorylated HSL (1:1000), and total HSL (1:1000) (Cell Signaling Technology, Danvers, MA). Blot to blot variability was corrected for by running a pooled sample on each blot and adjusting the densitometry based on the relative expression of the pooled sample. The results are presented as the ratio of protein of interest/β-actin (1:10,000) (Santa Cruz Biotechnology) or in case of the phosphorylated perilipin the results are presented as the ratio of phospho-perilipin/total perilipin.

Co-immunoprecipitation:

To analyze the interactions of CGI-58 with perilipin and ATGL, co-immunoprecipitations were performed according to manufacturer's instructions (Santa Cruz Biotechnology). Briefly, sonicated protein samples (10 μg; n= 5/diet/time point) were pre-cleared by incubation for 30 min at 4 °C with 1 μg of control IgG and 20 μl of protein A/G beads. Samples were centrifuged at 550 g (model 5415R, Eppendorf) for 5 min at 4°C. The supernatant was incubated with the CGI-58 primary antibody (1:50) (Santa Cruz Biotechnology) for 1 hr at 4°C. Then to isolate this protein-antibody complex, 20 μl of A/G beads were added and incubated at 4 °C overnight. The samples were then centrifuged at 550 g for 5 min at 4 °C. Pellets were washed 3 times with 1x PBS. Pellets were resuspended in 20 μl sample buffer (50mM Tris HCl at pH 6.8, 4% SDS, 40% glycerol, 4% β-mercaptoethanol, 0.1% bromophenol

blue) and heated at 95 °C for 3 min. Samples were run on SDS-PAGE gels and protein content of ATGL and perilipin were analyzed by western blotting, as described above. Blots were stripped and re-probed with CGI-58. The results are presented as a ratio of ATGL or perlipin/CGI-58.

FAS activity:

Cytosolic fractions were isolated from fresh liver (n=5/diet/timepoint) as follows [13]. Briefly, liver was homogenized in 5 volumes of homogenization buffer (0.15 M sucrose, 10 mM EDTA, 20 mM Tris at pH 7.4). The homogenates were centrifuged at 500g for 10 min to remove cell debris (model Evolution RC, rotor F13S-14X30C4, Sorvall Instruments, Waltham, MA). The supernatant was centrifuged at 10,000 g (model Evolution RC) for 10 min to remove the mitochondria. The supernatant was further centrifuged at 100,000 g (model Optima LE-80K, 70 Ti rotor, Beckman Coulter, Brea, CA) for 60 min to remove the microsomes and obtain the cytosolic fraction. FAS activity was measured according to method by Bays et al. [14] with minor modifications. Briefly, 10 µl of cytosolic sample was added to 90 µl activity buffer (1 mM DTT, 25 µM acetyl-CoA, 150 µM NADPH, and 100 mM potassium phosphate pH 6.5) with or without 500 µM malonyl CoA. The net absorbance was calculated as the difference between the with malonyl CoA and without malonyl CoA samples. The FAS activity was measured (Spectra Max Plus, Molecular Devices, Sunnyvale, CA) as µmoles of NADPH consumed/min/mg protein. The total protein was measured by the Bradford method.

Real time RT-PCR:

The frozen liver was used for determining the mRNA abundance. The relative abundance of fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), malic enzyme, sterol regulatory element binding protein 1c (SREBP1c), and stearyl CoA desaturase 1 (SCD1) were determined as described previously [15]. The gene-specific mRNA abundance was normalized to acidic ribosomal phosphoprotein (ARP) abundance, and the relative abundance was calculated with respect to that of the 3d SO-fed mice. Primer sequences are presented in Table 1.

Statistical Analysis:

All data were analyzed by two-way analysis of variance (ANOVA) using a fixed model, testing the main effects of oil source (SO, CO), CLA, and day, and all possible interactions. Body weight and feed intake measurements were analyzed by day. F tests, least-squares means, and standard error of means (SEMs) were calculated using the mixed procedure of SAS (SAS Institute Inc., Cary, NC). For all tests $P<0.05$ was considered significant, $P<0.1$ was considered a trend. Pre-determined comparisons of day within treatment and treatment within day were performed.

RESULTS

Body weight, feed intake, and body composition

Body weight was not affected by base oil (Table 2). CLA tended to decrease body weight on d7 ($P<0.1$) and d14 ($P<0.10$). Feed intake was not affected by base oil for the first 5wks when the mice were group caged. After going to individual cages (week 6), there was a

main effect of oil source ($P<0.05$), where CO-fed mice had 9% greater feed intake than SO-fed mice. This did not continue, however, as only a trend for increased consumption of the CO diet was observed in the 0-10d group ($P<0.1$). CLA caused a reduction in feed intake in mice fed for 3 ($P<0.05$) and 7 ($P<0.01$) days but not in mice fed longer (10 or 14 days) (Table 2).

Body fat did not differ across days, but we did detect an overall interaction of oil source × CLA ($P < 0.05$, Fig 1). Body fat did not differ on day 3, but by day 7 the body fat index was reduced in CO-fed mice (Fig 1). This oil effect however did not continue. CLA tended ($P<0.1$) to decrease the body fat index in CO-fed mice only on d10, and on day 14 the body fat index was reduced ($P<0.05$) in both CO+CLA and SO+CLA-fed mice.

Lipolysis

The effect of dietary treatments on lipolysis was determined by the release of NEFA and glycerol during a 3-h period of incubation of the fat pads. There was an interaction of oil source × CLA × day ($P<0.01$) on NEFA release (Fig 2a). On d3 CLA caused an increase in lipolysis in CO-fed mice, but this level of lipolysis was not different than SO-fed mice. CLA caused an increase in NEFA release in CO-fed mice, but not in SO-fed mice on d7 and d10. On d14, CLA increased the NEFA release in both CO (2.12 vs 5.8 μmol/g tissue) and SO-fed mice (1.44 vs 5.3 μmol/g tissue). Within CO+CLA-fed mice lipolysis was increased on d7 and 14 vs d3, with d10 being intermediate.

There was also an oil source × CLA × day interaction ($P<0.01$) on glycerol release, where CLA increased glycerol release in CO-fed mice but not in SO-fed mice on d3 and d7. There was no significant effect of treatments on d10 and d14 on glycerol release (Fig 2b).

The NEFA concentration in serum was not affected by any dietary treatment, although CLA-fed mice tended ($P = 0.05$) to have lower serum NEFA levels, especially on d3 (data not shown). Similarly total ketone body concentration was not affected significantly by dietary treatment but there was a main effect of day ($P<0.05$) where the ketone body concentration was greater on d3 compared to d7,10 and 14 (data not shown).

Protein expression

Since the lipolysis data indicated an effect of diet on NEFA release we also determined the effect of diets on the expression of proteins involved in lipolysis. We observed little differences in protein expression. There was a trend for an oil source × CLA interaction ($P<0.1$) on p-perilipin, where CLA tended to decrease p-perilipin expression in CO-fed mice (Table 3). There was also a trend for a CLA main effect ($P = 0.06$) on ATGL expression where CLA supplementation tended to increase ATGL expression (Table 3). p-HSL expression was below the level of detection in most samples. There was also no significant effect of diet on the association of perilipin or ATGL with CGI-58 (data not shown).

Lipogenesis

FAS activity was affected by diet only on d14 (oil × day interaction, $P<0.05$). CO increased FAS activity, but this was driven by an increased activity in CO+CLA-fed mice (Fig 3).

CO-fed mice had greater ($P<0.001$) mRNA expression of FAS compared to SO-fed mice, with no effect of CLA (Table 4). Similar to FAS, the expression of ACC was also affected only by oil source, where CO-fed mice had a less expression of ACC ($P<0.01$) compared to SO-fed mice (Table 4). The expression of malic enzyme was increased by both coconut oil ($P<0.01$) and

CLA ($P<0.001$), so that CO+CLA fed mice had the greatest expression of malic enzyme (Table 4). There was an oil source × CLA interaction ($P<0.05$) on SREBP1c expression, where dietary CLA caused a decrease in SREBP1c expression but only in the CO-fed mice (Table 4). CO-fed mice had increased expression ($P<0.0001$) of SCD1 compared to SO-fed mice on each day, but on d14 CO+CLA fed mice had an increased expression compared to all other diets, where there was also a trend ($P=0.09$) for CLA to cause an increase in SCD1 expression in CO-fed mice alone on d14. There was also a main effect of day ($P<0.05$) where on d14 the SCD1 expression was greater than expression on other days (Table 4).

DISCUSSION

We have previously reported that feeding coconut oil enhanced the CLA-induced body fat loss in mice [9, 16]. We have confirmed this finding in the current work, as mice fed CO and supplemented with CLA had less body fat compared to SO-fed mice supplemented with CLA starting on d7 of CLA supplementation, although the CLA response on d10 was not significant (Fig 1). Following 14 days of CLA supplementation the body fat index was also reduced in the SO-fed mice in response to CLA. This might indicate that SO-fed mice have a delayed response to CLA compared to CO-fed mice. In this study we also observed that CO diets caused a decrease in body fat even without CLA supplementation on d7 which could be the reason we did not detect a significant interaction of oil source × CLA. This effect of CO diets itself causing a decrease in body fat was also observed in our previous study [9] and also in a study where CO-fed group had lower body fat compared to mice fed olive or safflower oil [17]. This could be due to the presence of small and medium chain fatty acids in CO which would be oxidized at a faster rate than longer chain fatty acids [18]. Also, it has been observed that UCP1 mRNA expression

was increased in CO-fed mice which would indicate an increase in energy expenditure and thus a mechanism for lower body fat in CO-fed mice [19].

One of the mechanisms for body fat loss is altering the rate of lipolysis, which is the process by which stored TAGs are hydrolyzed into free fatty acids and glycerol by the action of lipases. We have previously observed that CLA induces lipolysis in CO but not in SO-fed mice [9]. We hypothesized that CLA-induced lipolysis in CO fed mice was time-dependent based on a cell culture study, where CLA supplemented to human adipocytes caused an increase in cytosolic perilipin, indicating activation of lipolysis, by 12 hrs [11]. This was then followed by a decrease in the expression of perilipin and HSL by 24 hrs, indicating a decline in lipolysis. In support of this we observed that supplementation of CLA for 7 days enhanced NEFA release in CO-fed mice, but this level of lipolysis decreased with 10d of supplementation of CLA although the CO+CLA group continued to have the highest NEFA release. Glycerol release was also significantly increased in CO+CLA fed mice on d3 and d7. We didn't observe any significant differences between treatments on d10 and d14. In the current study, the enhanced body fat loss appears to involve CLA inducing lipolysis earlier in CO-fed mice compared to SO-fed mice. As it was observed that 14d of supplementation of CLA also induced lipolysis (increased NEFA release) in SO-fed mice, which corresponds with the reduction in the body fat index. SO contains large amounts of linoleic acid, well in excess of the dietary requirement, that could interfere with the actions of CLA and could be the reason for a delayed response of CLA-induced lipolysis seen in SO-fed mice. CLA can be metabolized by the same desaturase and elongase enzymes that convert LA to AA [20] and as it has been shown that activity of Δ6 desaturase is required for CLA-induced body fat loss [21]. It has also been reported that conjugated metabolites of CLA including conjugated eicosatrienoic acid (20:2), caused a

decrease in body fat when supplemented to mice similar to that of CLA supplementation [22]. The higher ratio of LA: CLA in the SO-fed mice could lead to competition for Δ6 desaturase and therefore less metabolization of CLA and a reduced responsiveness to CLA in the SO-fed mice.

Classically it has been found that when lipolysis is activated, perilipin and HSL are phosphorylated by PKA, and ATGL is translocated to the lipid droplet surface. CGI-58 dissociates from phosphorylated perilipin and then associates with ATGL and activates it [23]. The activated lipases hydrolyze the TAGs in the lipid droplet. Based on our lipolysis measurement which showed maximal NEFA release in CO+CLA-fed mice on d7 and a reduction in this release on d10 and 14, we expected a time-dependent increase, followed by a decrease in the activation of the lipases and perilipin. However, we observed little differences in protein expression. There was a trend for an increase in ATGL expression in CLA-fed mice which could contribute to the increase in lipolysis. The lack of changes in the activation of the lipases and perilipin is consistent with our previous work [9] and Moon et al [24]. Others have indicated an increase in HSL expression in CLA treated adipocytes [11]. But this was *in vitro* and a measure of total HSL alone doesn't indicate on increase in its activation levels. We could not detect p-HSL in our samples, which might indicate that changes in phosphorylation of HSL are very time-sensitive and had dissipated by the time we measured them or that HSL may be activated via a non PKA-mediated manner. HSL is also phosphorylated by ERK (Ser 600) and AMPK (Ser 565) at other sites than PKA-phosphorylated sites (Ser 563,659) [25]. Phosphorylation by ERK activates HSL and phosphorylation by AMPK inhibits its activity. We measured the phosphorylation levels of PKA-mediated phosphorylation sites on perilipin and HSL (Ser 659), and didn't observe any changes; this could indicate that the enhancement of lipolysis in CO-fed mice might occur by an alternate pathway than the PKA-dependent signaling.

This PKA-independent activation of lipolysis by CLA has also been proposed by Moon et al in 3T3-L1 adipocytes in culture [24].

Given the enhancement in lipolysis in the CO+CLA-fed mice, we speculated that these mice may also have an elevated rate of lipid oxidation. Therefore we measured serum ketone bodies as an indication of fatty acid oxidation, but did not find any differences between dietary groups. We previously have showed that CPT activity was decreased by CLA in the CO-fed mice (Kanosky KM et al, unpublished data). Hence it appears that lipid oxidation is not being enhanced in the CO+CLA fed mice.

Another potential mechanism for body fat loss is by regulating lipogenesis. Lipogenesis is the process by which fatty acids are synthesized, elongated, and desaturated *de novo*. The rate limiting enzyme of fatty acid synthesis is ACC. ACC catalyzes the irreversible reaction of malonyl CoA synthesis. ACC mRNA expression was decreased in CO-fed mice compared to SO-fed mice. A previous study has shown that when mice where fed high-fat diets containing saturated fatty acids there was a decrease in ACC expression compared to the mice fed diets containing olive oil (which would be primarily monounsaturated fatty acids). Hence it is possible that the greater degree of saturation in CO could lead to the decrease in ACC expression seen in the current study.

We also simultaneously observed an increase in FAS and malic enzyme expression due to CO-diets with the CO+CLA-fed mice having the highest expression of both these genes. This is in contrast to previous studies where CLA induced a decrease in FAS expression [12, 26]. However, mice fed high fat diets containing beef tallow (majorly saturated fatty acids) had increased FAS expression compared to mice fed diets where the fat source was fish oil (primarily unsaturated fatty acids) [27]. In other studies we have seen that 56d of CO feeding cause a

change in fatty acid profile with the mice in CO-fed diet group having a greater saturated: unsaturated FA ratio (34.2:12.29 vs 25.71:35.48, CO vs SO respectively) [8, 16], so the increase in FAS mRNA in CO-fed mice detected in our study could be due to this shift in FA profile. Corresponding to the FAS expression we observed that the FAS activity was also greater in CO+CLA-fed mice. This could indicate that there is a greater turnover of fatty acids in these CO-fed mice as it has been observed that the rate of oxidation of short and medium chain fatty acids (found in CO diets) is greater compared to long chain fatty acids (found in SO diets) [18, 28]. This potential increase in turnover could also contribute to the increase in lipolysis in these CO+CLA-fed mice.

Mice fed CO diets had greater expression of SCD1, which is the enzyme that catalyzes the conversion of saturated fatty acids into unsaturated fatty acids. CO diets consist mainly of saturated fatty acids, so these fatty acids can be further desaturated in the cell and thus in CO-fed mice we observe an increase in SCD1 expression as it has been observed that PUFAs lower SCD1 expression while saturated and MUFAs increase SCD1 expression [29].

SREBP1c is a transcription factor which regulates the expression of lipogenesis-related genes including FAS, and ACC. Previous studies have indicated that CLA exerts an anti-lipogenic effect by decreasing SREBP1c and FAS expression [10, 12]. In the current study we observed an increase in SREBP1c expression due to CLA in SO-fed mice, while there was a decrease in its expression in response to CLA in CO-fed mice. The decrease in expression due to CLA in CO-fed mice is similar to previous studies in which CLA has been reported to decrease SREBP1c [10, 12]. The increase in SREBP1c mRNA in SO+CLA vs SO-fed mice could relate to PUFA level; in the SO+CLA-fed mice we observed a numeric decrease in SCD1 mRNA expression compared to SO-fed mice, and CLA has been reported to inhibit SCD1 expression

and activity, which could indicate that there are less unsaturated fatty acids. SREBP1c expression is known to be attenuated by PUFAs [30], hence a decrease in PUFAs as indicated by decreased SCD1 expression might be the reason why SO+CLA mice had greater SREBP1c expression compared to SO-fed mice. Also the presence of high amount of PUFAs in SO diets could explain why the SO-fed mice had lower expression of SREBP1c compared to CO-fed mice.

In conclusion, we have confirmed that coconut oil enhances the anti-obesity effect of CLA and this effect is, at least in part, due to enhanced lipolysis. The effect of CLA in response to different lipid sources appears to be time-dependent with lipolysis in SO+CLA-fed mice delayed compared to CO+CLA-fed mice. The mechanism of this enhancement of lipolysis in CO+CLA mice appears to be independent of PKA signaling pathway. There also appears to be a simultaneous decrease in lipogenesis and increase in fatty acid turnover in the CO-fed mice especially when supplemented with CLA. Further studies looking into the different signaling pathways which could potentially regulate lipolysis in an adipocyte might be helpful to determine the mechanism of the CLA-induced lipolysis in CO-fed mice.

Table 1: Composition of experimental diets

Ingredients[1] (g/kg)	SO	SO+CLA	CO	CO+CLA
Isolated soy protein	200	200	200	200
L-cystine	2.54	2.54	2.54	2.54
L-methionine	2.54	2.54	2.54	2.54
Cornstarch	395.406	395.406	395.406	395.406
Maltodextrin	132	132	132	132
Cellulose	100	100	100	100
AIN-93G mineral mix	35	35	35	35
AIN-93G vitamin mix	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
Soybean oil	70	61.7	-	-
Coconut oil	-	-	70	61.7
CLA[2]	-	8.3	-	8.3

[1]All diet ingredients except CLA were obtained from Dyets, Inc., Bethlehem, PA.

[2]0.83% CLA oil (50:50 t10,c12 to c9,t11; 60% purity), provided by BASF, Offenbach/Quiech, Germany

Table 2.2: Primer[1] sequences for real-time RT-PCR

Gene[2]	GenBank accession no.	Primer Sequence
ARP	NM_017404	Forward: 5'- GCGACCTGGAAGTCCAACTA-3' Reverse: 5'-GCTCCCACAATGAAGCATTT-3'
FAS	NM_007988	Forward: 5'-GACTGAAACCTGACGGCATC-3' Reverse: 5'-CAGTAAGCTGCAAGCACAGC-3'
ACC	NM_133904	Forward: 5'-GTGGTCTTCGAGTGGATTGG-3' Reverse: 5'-AGCTGCCTTCAGACCATCAT-3'
SCD1	NM_009127	Forward: 5'-GCGTTCCAGAATGACGTGTA-3' Reverse: 5'-GTCGGCGTGTGTTTCTGAG-3'
SREBP1c	NM_011480	Forward: 5'-CTCCCAGAGTAGCCCCTTGT-3' Reverse: 5'-GGCTTTGACCTGGCTATCCT-3'
Malic enzyme	NM_001198933	Forward: 5'-GAGGCAGCGTCTTCCAAATA-3' Reverse: 5'-CTTGTTCAGGAGACGAAATGC-3'

[1]Primer sequences were designed using Primer3 software
[2] FAS(Fatty acid synthase); ACC (Acetyl CoA carboxylase); SREBP (sterol regulatory element binding protein); SCD (sterol CoA desaturase); ARP (Acidic ribosomal protein)

Table 2.3: Effect of oil source and/ or CLA on feed intake, and body weight

	Diets[1]					**P-values**		
	SO	SO+CLA	CO	CO+CLA	SEM	Oil	CLA	Oil × CLA
Body weight (g)								
Initial[2]	18.15		18.75		0.27	0.13		
Before CLA[2]	33.78		32.88		0.55	0.25		
Final[2]								
d3	34.53	33.74	33.3	32.51	0.79	0.12	0.32	0.99
d7	36.84	34.97	35.16	33.67	0.95	0.12	0.08	0.84
d10	36.83	34.35	35.52	34.33	1.28	0.61	0.16	0.62
d14	38.86	33.79	36.34	33.33	2.06	0.48	0.07	0.62
Feed intake (g/day)								
Wk1-5 avg	2.51		2.54		0.04	0.6		
Wk 6	5.17[b]		5.66[a]		0.15	**0.03**		
Final avg								
d0-3	5.79[ab]	4.73[b]	5.85[a]	5.03[ab]	0.39	0.64	**0.02**	0.75
d0-7	5.38[a]	4.4[b]	5.51[a]	4.94[ab]	0.29	0.26	**0.01**	0.48
d0-10	5.69	4.89	6.45	5.85	0.44	0.06	0.13	0.82
d0-14	5.35	4.8	5.11	5.39	0.29	0.58	0.65	0.17

[1]Diets: SO (7% Soy oil); SO+CLA (6.5% soy oil+0.5% CLA); CO (7% coconut oil); CO+CLA (6.5% coconut oil+0.5% CLA)
[2] Initial = Day -42; Before CLA = Day 0; Final = Day 3, 7, 10 or 14.
[ab]Different letters within a row indicate significant differences, $P < 0.05$

Table 2.4: Effect of oil source and/ or CLA on lipolysis related proteins

Protein[1]	Diets[2]					P-Values		
	SO	SO+CLA	CO	CO+CLA	SEM	Oil	CLA	Oil x CLA
Perilipin/β-actin						0.37	0.78	0.49
d3	1.59	0.83	0.89	0.88	0.07			
d7	0.62	0.35	1.21	0.38	0.23			
d10	0.83	1.12	0.96	0.51	0.32			
d14	0.57	0.77	1.78	0.42	0.2			
P-perilipin/perilipin						0.69	0.68	0.06
d3	1.54	1.45	3.02	1.23	0.98			
d7	1.56	1.69	2.05	2.38	0.75			
d10	2.07	4.15	3.65	1.78	0.8			
d14	1.5	1.31	1.68	1.28	0.6			
HSL/β-actin						0.42	0.38	0.53
d3	1.79	1.21	0.7	2.26	0.8			
d7	2.34	1.60	0.13	0.75	0.39			
d10	1.6	3.02	0.16	0.26	0.18			
d14	1.58	0.53	1.79	1.69	0.59			
ATGL/β-actin						0.64	0.06	0.53
d3	2.66	5.98	1.63	2.05	0.9			
d7	5.28	3.82	3.27	4.21	1.3			
d10	1.99	2.53	2.94	2.72	0.86			
d14	1.18	2.34	4.02	3.23	0.32			

[1]HSL (Hormone Sensitive Lipase); ATGL (Adipose Triglyceride Lipase)
[2]Diets: SO (7% Soy oil); SO+CLA (6.5% soy oil+0.5% CLA); CO (7% coconut oil); CO+CLA (6.5% coconut oil+0.5% CLA)
[ab]Different letters within a row indicate significant differences, $P < 0.05$

Table 2.5: Effect of oil source and/or CLA on lipogenesis-related gene expression

Genes[1]	Diets[2]					P-Values		
	SO	SO+CLA	CO	CO+CLA	SEM	Oil	CLA	Oil x CLA
FAS						**<0.0001**	0.42	0.85
d3	1.52	0.83	3.58	3.52	1.04			
d7	1.33	2.03	3.44	3.68	1.03			
d10	1.64	1.98	3.81	3.11	1.02			
d14	1.88^{b}	3.03^{b}	3.77^{ab}	6.58^{a}	1.04			
ACC						**0.0024**	0.75	0.13
d3	1.04^{ab}	1.68^{a}	0.78^{ab}	0.69^{b}	0.32			
d7	1.41^{ab}	1.50^{a}	0.49^{bc}	0.31^{c}	0.33			
d10	0.83	0.73	0.82	0.34	0.35			
d14	0.81	0.95	0.97	0.49	0.34			
Malic enzyme						**0.0031**	**0.0006**	0.13
d3	0.825^{b}	1.32^{b}	1.88^{b}	3.86^{a}	0.55			
d7	1.47	2.12	1.41	2.98	0.57			
d10	0.77	1.94	1.35	2.07	0.58			
d14	1.37^{b}	2.01^{ab}	1.51^{ab}	3.54^{a}	0.58			
SREBP1c						0.72	0.12	**0.01**
d3	1.06^{b}	2.06^{a}	2.28^{a}	1.14^{b}	0.39			
d7	1.28	1.39	1.91	1.06	0.39			
d10	1.88	1.89	1.68	1.16	0.4			
d14	2.003	1.5	2.09	1.19	0.42			
SCD[3]						**<0.0001**	0.26	0.089
d3	1.13^{bc}	0.83^{c}	3.85^{a}	2.71^{ab}	0.63			
d7	1.21^{b}	1.28^{b}	3.39^{a}	4.48^{a}	0.62			
d10	1.62^{ab}	1.44^{b}	2.84^{ab}	3.51^{a}	0.68			
d14	2.37^{b}	2.27^{b}	3.09^{b}	6.26^{a}	0.62			

[1]FAS (Fatty acid synthase); ACC (Acetyl CoA carboxylase); SREBP (sterol regulatory element binding protein); SCD (sterol CoA desaturase)

[2]Diets: SO- (7% Soy oil); SO+CLA (6.5% soy oil+0.5% CLA); CO (7% coconut oil); CO+CLA (6.5% coconut oil+0.5% CLA)

[3]Main effect of day $P < 0.05$; d14 greater than d3,7,10.

[ab]Different letters within a row indicate significant differences, $P < 0.05$. Although there were significant main effects there were no significant differences observed between treatments on some days.

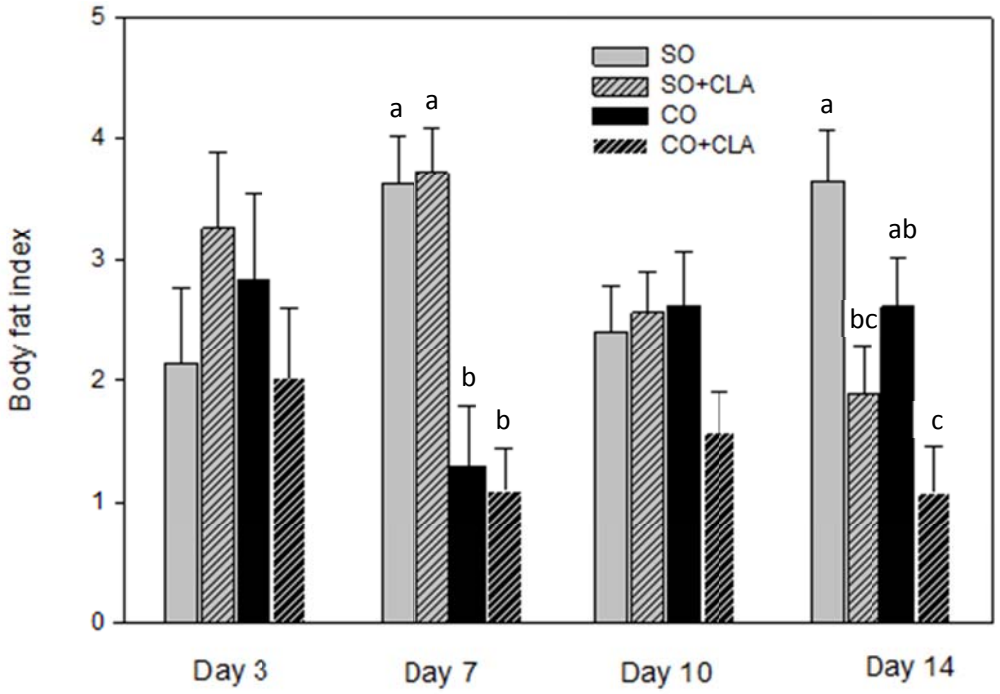


Figure 2.1: Effect of dietary CLA and oil source on body composition. Body fat index = ((Retrperitoneal +Epididymal fat pad weights)/body weight) x 100; n = 5/diet/day. Diets: SO (7% Soy oil); SO+CLA (6.5% soy oil+0.5% CLA); CO (7% coconut oil); CO+CLA (6.5% coconut oil+0.5% CLA). Oil*CLA, $P<0.05$; CLA×day, $P=0.06$. Data is shown as mean ± SEM. Different letters indicate significant differences between treatments within a day.

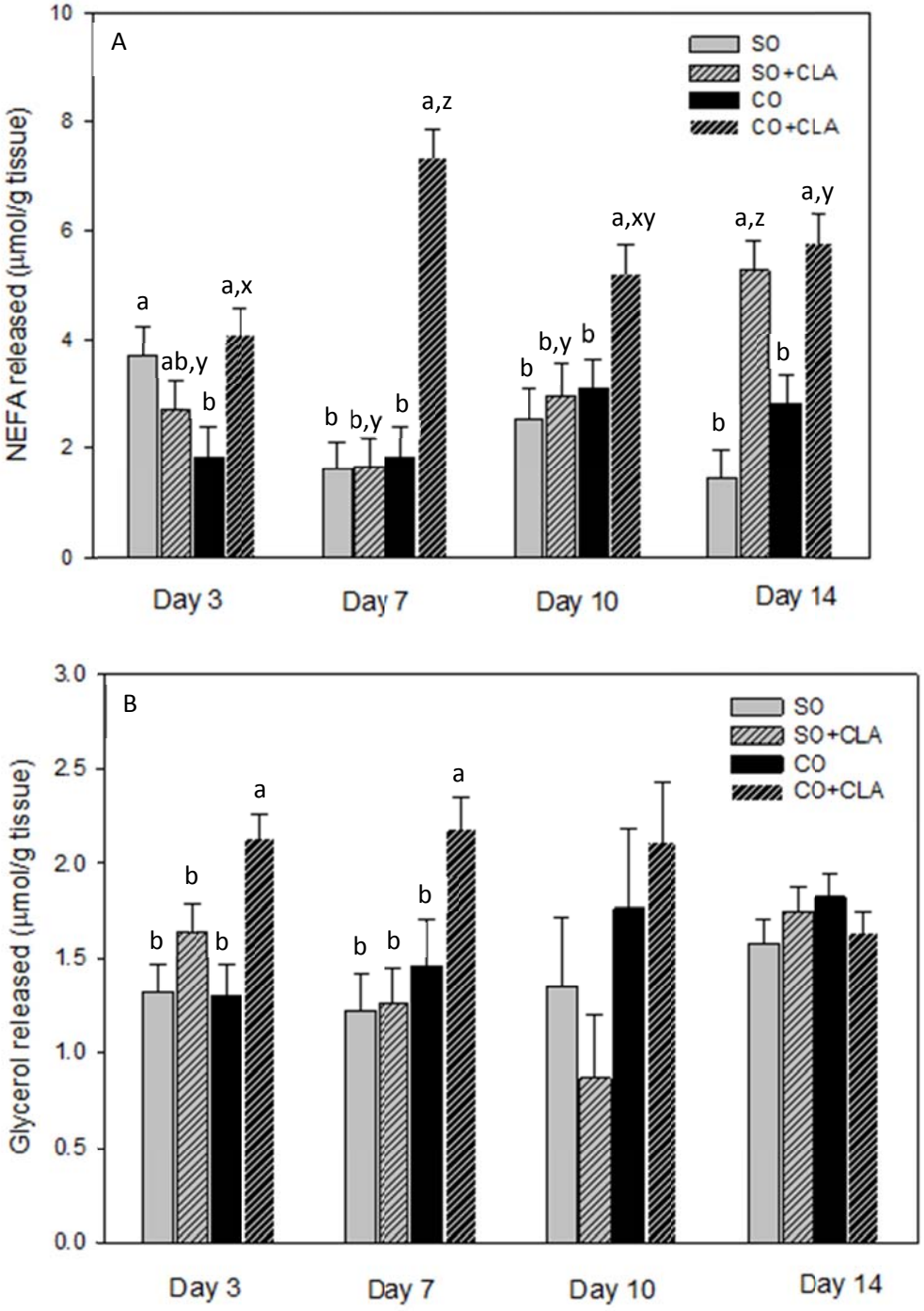


Figure 2.2: Effect of dietary CLA and oil source on lipolysis. Fat pads were cultured for 3 hrs ex vivo. Diets: SO (7% Soy oil); SO+CLA (6.5% soy oil+0.5% CLA); CO (7% coconut oil); CO+CLA (6.5% coconut oil+0.5% CLA). A) NEFA release, Oil*CLA*day P=0.0015 and B) Glycerol release, Oil×CLA×day P=0.008; n =5/diet/day. Data is shown as mean ± SEM. ^{abc} different letters indicate significant differences between treatments within a day. ^{xyz} indicates differences within treatment between days.

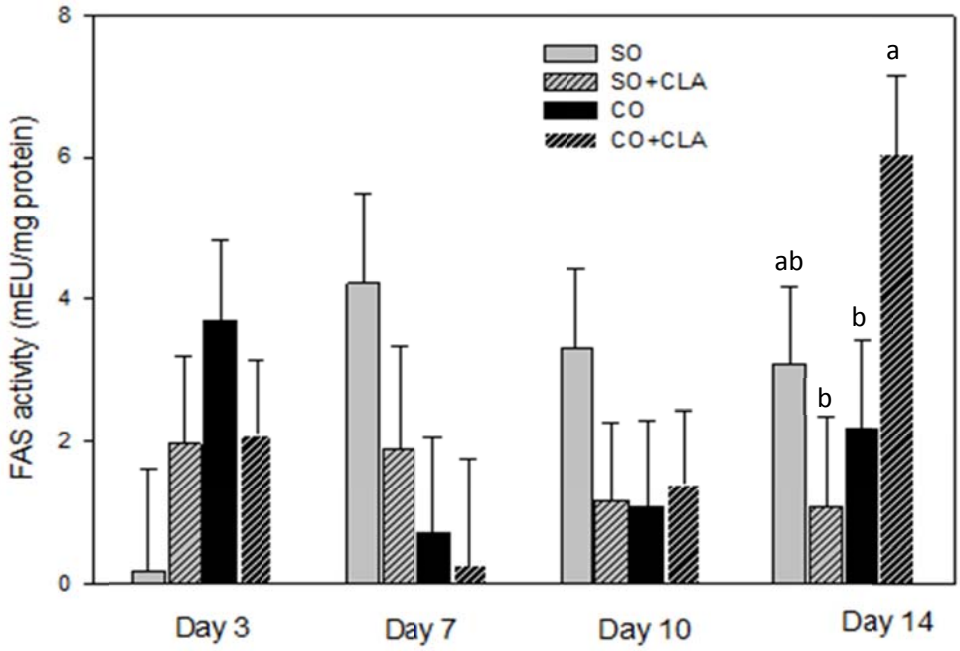


Fig 2.3: Effect of dietary CLA and oil source on FAS activity. n= 5/diet/day. Oil*day, $P<0.05$. Data is shown as mean ± SEM. Different letters indicate significant differences between treatments within a day.

CHAPTER 3: LIPOLYSIS IN 3T3-L1 ADIPOCYTES IN RESPONSE TO DIFFERENT LIPID SOURCES AND CLA

INTRODUCTION:

Adipose tissue is a metabolically active tissue; one of its major functions is the storage and mobilization of fatty acids based on the energy requirement of the body. In the adipocyte the lipid is stored in the form of triacylglycerides (TAG) in lipid droplets (LD). When energy demand is increased, the TAGs are hydrolyzed to free fatty acids via the process of lipolysis. Adipocytes have unilocular LD surrounded by the protein perilipin (Plin) which prevents the action of cytosolic lipases on the TAGs. Under basal conditions (fed state), perilipin is associated with a co-regulator comparative gene identification 58 (CGI-58). In a stimulated state Plin and hormone sensitive lipase (HSL) are phosphorylated thus activating them [60]. CGI-58 dissociates from phosphorylated Plin and binds to adipose triglyceride lipase (ATGL) to activate it. The activated lipases then hydrolyze the ester bonds in TAG to yield free fatty acids. The most well-known signaling cascade activating lipolysis is the cAMP dependent protein kinase A (PKA) pathway, where stimulation of Gs-protein coupled receptors activate adenylate cyclase, thus increasing intracellular cAMP levels, and leading to PKA activation. PKA then phosphorylates Plin and HSL.

In addition to this pathway, lipolysis has been shown to be activated by a phospholipase C (PLC) induced protein kinase C (PKC) activation. Activated PKC leads to activation of p42/p44 mitogen-activated protein kinase (MAPK), which phosphorylates HSL [37]. Recent studies have also shown that prostaglandin E2 (PGE_2) has an inhibitory effect on lipolysis [38, 67]. Archadonic acid is converted to PGE_2 , which stimulates Gi-protein coupled signaling thus inhibiting adenylate cyclase, and decreasing PKA stimulated lipolysis.

Dietary conjugated linoleic acid (CLA) induces a body fat loss in several species which is increased in mice fed coconut oil (CO) diets compared to those fed soybean oil diets [31, 47,

48]. It has been observed that this enhanced body fat loss involves enhanced lipolysis in CO+CLA fed mice [31]. The mechanism by which CLA induces lipolysis in CO-fed mice is unknown but we have not detected increased PKA-phosphorylation of perilipin or HSL in the adipose tissue of CO+CLA-fed mice. In cell culture studies, cells treated with CLA had increased lipolysis and activation of MAPK signaling [29, 30]. We hypothesize that CLA supplementation increases lipolysis in CO treated cells primarily through a MAPK-dependent, PKA-independent pathway.

METHODS:

Cell culture and differentiation:

3T3-L1 cells (ATCC, Manassas, VA) were seeded at a density of 4×10^4cells/cm^2 into 24-well plates and grown to confluence in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% Pen-Strep at 37^{o}C. Two days after confluency, cells were stimulated to differentiate by exposure to differentiation media (DMEM containing 1% Pen-Strep, 10% fetal bovine serum (FBS), 5 μg/ml insulin, 1 μM dexamethasone, 0.5 μM 3-isobutyl-1-methylxanthine (IBMX) and 1 μM rosiglitazone) for 2d. Cells were then exposed to DMEM with 1% Pen-Strep, 10% FBS, 5 μg/ml insulin, and 1 μM rosiglitazone for an additional 2d; thereafter, cells were maintained for 10d in DMEM with 1% Pen-Strep and 10% FBS or delipidated bovine serum with experimental oils to allow them to accumulate lipid.

Preparation of serum with different oils:

Coconut and soybean oils (0.3g) were saponified in 50 ml ethanol and 5 ml 10N KOH for 14 hrs at room temperature [68]. The lipid was extracted with 10 ml hexane in 3.5 ml of 37% HCl. Samples were centrifuged (model Evolution RC, rotor F13S-14X30C4, Sorvall Instruments, Waltham, MA) for 1 hr at 900×g and the organic layer was collected and evaporated to obtain the free fatty acids. The fatty acids were coupled to bovine serum albumin (BSA; 3:1 ratio) and combined with delipidated bovine serum (300mg/100ml serum), to achieve a similar concentration of lipid as in bovine serum (based on a published value) [56].

Lipolysis assay:

Cells were seeded in 24-well plates and allowed to differentiate as described above. The lipolysis assay was performed based on methods reported by Chung et al. with a few modifications. Briefly, 3T3-L1 adipocyte were serum-starved overnight in DMEM before pre-loading with 6.25 nmol of [^{3}H]-oleic acid for 12 hrs. Cells were rinsed 3x with HBSS (Hanks Buffered Saline Solution;) to remove excess oleic acid. Cells were then treated with BSA-coupled linoleic acid, or t10, c12 CLA (3:1 fatty acid methyl esters to BSA; Nu-chek Prep, Elysian, MN) in DMEM. The media was removed and cells were treated with DMEM with 1% penicillin/streptomycin for 3hrs. 200 µl of medium was collected from each well and transferred to a 5ml liquid scintillation counting vial with 1.5 ml of scintillation fluid (Ecolume, MP biomedicals, Santa Ana, CA) to measure [^{3}H]-oleic acid release to the medium using a β-counter (model LS 6500, Beckman Coulter, Brea, CA). Data are presented as either CPM/mg protein or CPM/cell count. Protein content was determined by Bradford method. Cell count was determined by a normalization stain (Sapphire700 (1:1000) which stains both live and dead cells

and DRAQ5 (1:10000) which stains DNA in live cells) and the count of live cells was determined.

Experimental design:

DOSE CURVE: Cells were differentiated and the lipolysis procedure was followed as described above. Cells were treated with bovine serum albumin (BSA)-coupled linoleic acid, or t10, c12 CLA (3:1 fatty acid to BSA) at concentrations of 20, 30, 50, or 75 μM for 12 hrs followed by the measurement of lipolysis.

TIME CURVE: The optimum dosage of CLA determined in the dose curve (50 μM) was used and cells were treated with CLA or LA for 6, 12, 24, or 36 hrs followed by the measurement of lipolysis.

SERUM CONDITIONS: Cells were grown and differentiated as above. Prior to pre-loading with [^{3}H]-oleic acid for 12 hrs, cells were serum-starved, treated with delipidated serum, or 10% FBS overnight. The reason for treating cells under different serum conditions was because some cell culture studies have reported that CLA supplementation increases lipolysis [29, 34] but others have shown that lipolysis in cultures of human adipocytes was not altered by CLA [32, 33]. This discrepancy may be because the studies in which an effect on lipolysis was observed, the cultures were serum-starved for 12-24 hr prior to supplementation of CLA. Cells were then exposed to 50 μM CLA/LA for 12 hrs, followed by the measurement of lipolysis.

EFFECT OF OILS: After differentiating, cells were treated with SO or CO containing delipidated serum, during lipid accumulation for 10d. Cells were then treated with 50 μM CLA/LA for 12 hrs. Lipolysis assay was performed as described above.

EFFECT OF CELL SIGNALLING INHIBITORS: Cells were exposed to different oils during lipid accumulation as above. During treatment with CLA or LA, cells were treated with either no inhibitor or various inhibitors [5μM NS-398 (Cox-2 inhibitor [69]) for 6 hrs; 50μM H-89 (PKA inhibitor [70]) for 12 hrs (Cayman Chemicals, Ann Arbor, MI); 75μM PD-98059 (p42 MAPK inhibitor [71]) for 12 hrs (Enzo life sciences, Farmingdale, NY); 10μM U73122 (PLC inhibitor [72]) for 12 hrs; 10μM GW6471 (PPAR-α inhibitor [73]) for 12 hrs (Santa Cruz Biotechnology, Santa Cruz, CA)]. The time period of inhibitor treatment was determined by a time-curve experiment performed with each inhibitor present (data not shown). Concentrations of inhibitors were determined based on previous literature. Lipolysis was measured as described above.

In-cell western procedure:

An in-cell western protocol was performed, as follows, with slight modifications to the manufacturer's instructions for the Odyssey imaging system (Model ODYSSEY CLx, Li-Cor Biosciences, Lincoln, NE). Cells were fixed with 3.7% formaldehyde in 1x PBS for 20 min at room temperature. Cells were then washed 3 times with 1x PBS containing 0.1% Triton X-100 for 5 min to permeabilize the cells. Cells were blocked with 5% dry milk in 1x PBS for 90 min at room temperature. Cells were probed with antibodies for perilipin (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA), and phosphorylated-HSL (Ser 660) (1:200) (Cell Signaling Technology, Danvers, MA) overnight at 4 °C. Protein expression was detected with a fluorescent-labeled secondary antibody [IR 800CW (1:800), Li-CoR Biosciences] and normalization stains [Sapphire700 (1:1000) which stains both live and dead cells and DRAQ5 (1:10000) which stains DNA in live cells] for 1 hr at room temperature. The intensity was measured with the Odyssey Imaging System (Li-COR Biosciences). Protein expression was

normalized to live cell count as determined by the intensity measured at 700nm due to the normalization stains.

Statistical analysis:

All data were analyzed by two-way analysis of variance (ANOVA) using a fixed model, testing the main effects of treatment (LA, CLA) and time, dosage, or oil source (SO, CO), and the interaction of oil source, time, or dose × treatment. For the data using inhibitors, the main effects of treatment, oil source, and inhibitor and all possible interactions were tested. F tests, least-squares means, and standard errors of means (SEMs) were calculated using the mixed procedure of SAS (SAS Institute Inc., Cary, NC). For all tests $P < 0.05$ was considered significant, $P < 0.10$ was considered a trend.

RESULTS:

Optimal conditions for CLA treatment:

Dose curve: There was an interaction of Dose × CLA ($P<0.0001$), where CLA treated cells had greater lipolysis than LA treated cells for doses above 20μM (Fig 3.1a). The optimum dosage selected was 50μM, since at this dosage there was the greatest difference in lipolysis between the 2 treatments.

Time curve: There was a main effect of time ($P<0.0001$), where lipolysis was greatest at 12hrs. There was also a main effect of treatment ($P<0.0001$), where CLA treatment induced an increase

in lipolysis compared to LA at 12 and 24 hrs (Fig 3.1b). The optimal time period of CLA/LA treatment was thus determined to be 12 hrs as it was the time at which maximal lipolysis was observed in both treatments.

Serum conditions: There was a main effect of serum ($P<0.0001$), where serum-starving cells had increased lipolysis as compared to treating cells with fetal bovine or delipidized serum (Fig 3.1c). In serum-starved cells, CLA treatment induced greater lipolysis than LA treatment (397,922 vs 206,176 CPM/mg protein).

Effect of different oils on lipolysis:

An interaction of oil source × treatment ($P<0.05$) was observed on lipolysis when no inhibitor was added (Fig 3.2a). CLA caused an increase in lipolysis compared to LA but only in cells exposed to CO.

Effect of signaling pathway inhibitors on lipolysis:

The PKA inhibitor increased lipolysis in CO+CLA and SO+LA –treated cells (oil × treatment × inhibitor, $P<0.001$) (Fig 3.2a). In PKA inhibitor-treated cells, SO+LA caused a greater rate of lipolysis than SO+CLA; CO+CLA-treated cells still had increased lipolysis compared to CO+LA.

There was no effect of p42 MAPK inhibitor treatment compared to absence of inhibitor. There remained an oil × treatment interaction ($P<0.05$) in p42 MAPK inhibitor (PD-98059)-treated cells on lipolysis, where the CO+CLA treated cells had the greatest lipolysis (Fig 3.2a).

The PLC inhibitor (U73122) caused an increase in lipolysis in all treatments except CO+CLA (oil × treatment × inhibitor, $P<0.0001$; Fig 3.2a). Within the PLC inhibitor-treated

cells, there was an oil × treatment interaction where CLA caused a decrease in lipolysis in cells exposed to CO.

There was a treatment effect ($P<0.05$) observed with the COX-2 inhibitor (NS-398; Fig 3.2b). NS-398 caused the lipolysis of the SO+CLA treated cells to increase to the level of that of CO+CLA treated cells (oil×trt×inhibitor, $P=0.05$). The PPARα inhibitor (GW6471) increased lipolysis in the CO+LA treated cells to the level of CO+CLA treated cells and also increased lipolysis in SO+CLA treated cells (oil×trt×inhibitor, $P<0.05$; Fig 3.2b).

Effect of inhibitors on lipolysis-related protein expression:

When cells were not treated with an inhibitor, no effect of treatment was observed on perilipin expression (Table 3.1). Cells exposed to the COX-2 inhibitor had an oil source × treatment interaction ($P<0.05$), where CLA treatment caused an increase in perilipin expression in SO-treated cells. Addition of the COX-2 inhibitor increased expression of perilipin in SO+CLA treated cells and decreased perilipin in CO+LA treated cells. There was a main effect of the p42 MAPK inhibitor ($P<0.0001$) on perilipin expression, where the addition of inhibitor increased the expression of perilipin. Cells treated with the PLC inhibitor also had a greater expression of total perilipin compared to cells not treated with an inhibitor ($P<0.0001$). There was also a main effect of oil ($P=0.0001$), where SO-treated cells had greater perilipin expression than CO-treated cells. The presence of the PPARα inhibitor caused an increased perilipin expression in SO-treated cells. There was an oil × treatment interaction on perilipin expression in the presence of the PKA inhibitor, where CLA decreased perilipin expression in SO but not CO-treated cells. Addition of the PKA inhibitor increased perilipin expression in all treatments compared to absence of inhibitor (oil×treatment×inhibitor, $P<0.05$)

There was no effect of lipid treatments on the expression of p-HSL (Table 3.1). There was a main effect of inhibitor ($P<0.0001$) where the addition of all inhibitors increased the expression of p-HSL.

DISCUSSION:

Dietary supplementation of CLA has been shown to cause a body fat loss in several species. This effect of CLA has been observed to be enhanced in mice fed coconut oil diets as compared to mice fed soybean oil diets [31, 47]. In the current study, we developed a cell culture model of this phenomenon, 3T3-L1 adipocytes exposed to CO and supplemented with CLA had increased lipolysis compared to LA treated cells, but cells exposed to SO did not respond to CLA.

In the current study we found that there was no alteration of CLA induced lipolysis by the PKA inhibitor but the lipolysis was increased further in the CO+CLA treated cells which might indicate that when the PKA pathway is inhibited, lipolysis is being stimulated by another pathway. It has been found that catecholamine-stimulated lipolysis was inhibited by only 20-30% by addition of a PKA inhibitor [74]. This is supported by a study which reported that addition of a cAMP inhibitor to cells did not cause a reduction in the lipolysis of CLA treated cells [30], indicating that CLA enhanced lipolysis is not PKA-mediated. We also found that addition of the PKA inhibitor increased the total perilipin and p-HSL expression indicating that in the presence of these lipid treatments the lipase is activated by a PKA-independent pathway. It has been reported that catecholamine-stimulated lipolysis is inhibited 50% by a p42 MAPK

inhibitor [74], hence the enhanced lipolysis seen in the current study in CO+CLA treated cells could be mediated by the p42 MAPK pathway.

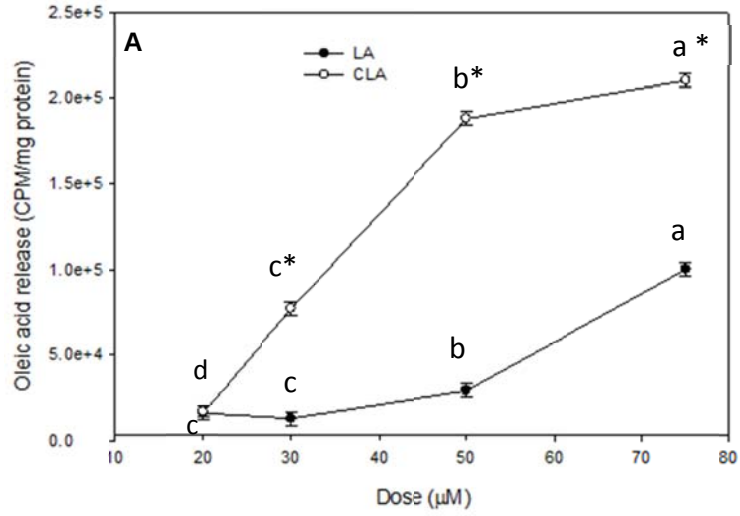
Previous cell culture studies have indicated that CLA enhanced lipolysis is mediated through a cAMP-independent pathway and that it might involve the activation of the MAPK/ERK pathway [29, 30]. In contrast to previous studies, we didn't find an effect of a p42 MAPK inhibitor on lipolysis, but the expression of perilipin and p-HSL were found to be increased in the presence of this inhibitor with no treatment effects that differ from the no inhibitor results. The previous work observed an increase in phosphorylated ERK (p-ERK) in response to CLA, but did not determine whether the increase in p-ERK was associated with an increase in lipolysis [29]. Our data indicate that CLA enhanced lipolysis in cells exposed to CO is not dependent on the MAPK pathway.

In the current study we also found that addition of a COX-2 inhibitor increased the lipolysis and expression of perilipin in SO+CLA treated cells. COX-2 catalyzes the synthesis of PGE_2, which is known to inhibit PKA stimulated lipolysis by activating the G_i protein coupled pathway [75]. Soy oil contains long chain unsaturated fatty acids like linoleic acid which could be converted to arachadonic acid (AA), the precursor for PGE_2 synthesis. Therefore, it is possible that the lack of stimulation of lipolysis in SO-treated cells could be due to the PGE_2-mediated inhibition of lipolysis. When the COX-2 inhibitor was added, the prevention of PGE_2 production may have allowed CLA to induce lipolysis. We didn't observe an effect of the COX-2 inhibitor on lipolysis in CO+CLA treated cells, possibly because of reduced substrate for COX-2 in CO-treated cells. Mice fed CO have reduced LA and AA stores [48]. A previous study has also shown that t10,c12 CLA treatment of 3T3-L1 cells increased the expression of COX-2 [76]. This increase in COX-2 expression has also been observed with other PUFAs like

DHA and EPA [77]. Hence the effect of CLA in CO-treated cells doesn't appear to depend on the COX-2 pathway.

Addition of a PLC inhibitor increased lipolysis in cells exposed to all treatments except the CO+CLA. It has been reported that the PLC-induced release of Ca^{2+}, can activate Akt by phosphorylation through Ca^{2+} calmodulin kinase [78]. Akt activation has been shown to be required for insulin mediated inhibition of lipolysis [79]. Therefore, based on our data, it is possible that SO is activating PLC and inhibiting lipolysis through the insulin-signaling pathway as it has been previously shown that fatty acids like oleic acid and arachadonic acid can stimulate PLC activity [80, 81]. Also, it appears that CO+CLA treatment is inhibiting the PLC mediated signaling in an unknown manner, thus causing the enhanced lipolysis observed in CO+CLA treated cells. This is also supported by the fact that we observed no further increase in lipolysis in the CO+CLA treated cells when they were treated with isoproterenol (data not shown), indicating that lipolysis had been maximally stimulated in these cells. It has been shown that addition of a PLC inhibitor to 3T3-L1 cells exposed to insulin can partially alleviate the inhibition of lipolysis by insulin [72]. Hence the increase in lipolysis in CO+CLA treated cells could be in part due to PLC inhibition. Further studies into the PLC activation and insulin signaling pathway in CO+CLA treated cells are needed to fully understand the mechanism.

In conclusion, it appears that the CLA enhanced lipolysis in CO treated cells is not mediated by the activation of MAPK or PKA. Removal of the inhibitory effects of PGE_2 and/or inhibition of PLC-mediated insulin signaling could be mechanisms for the enhanced lipolysis observed in CO+CLA treated cells.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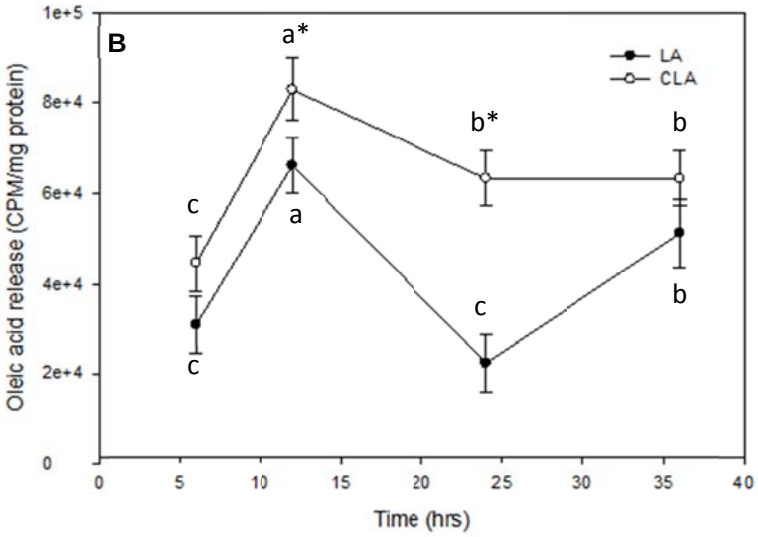
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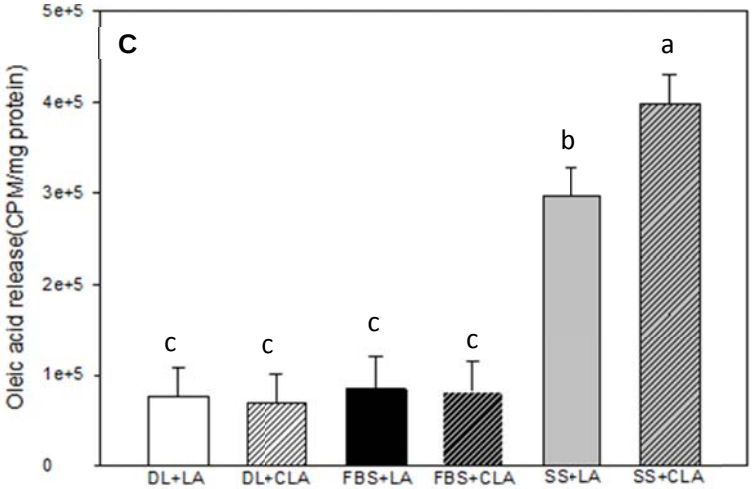
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Fig 3.1: Optimal conditions for CLA treatment in 3T3-L1 cells. (A) Dose-response curve, CLA× dose, $P<0.001$. Differentiated cells were serum starved and treated with different doses of CLA/LA for 12 hrs (B) Time curve, CLA, $P<0.0001$; Time, $P<0.0001$. Differentiated cells were serum starved and treated with 50 μM CLA/LA for various time periods and lipolysis was performed. (C) Serum conditions, serum $P<0.0001$ (DL- delipidated serum, FBS- fetal bovine serum, SS- serum starved). Differentiated cells were treated under different serum conditions prior to treatment with 50 μM CLA/LA for 12 hrs. Data are shown as mean ± SEM. Different letters indicate differences between dose/time points/ serum conditions within treatment. * indicates difference between treatments.

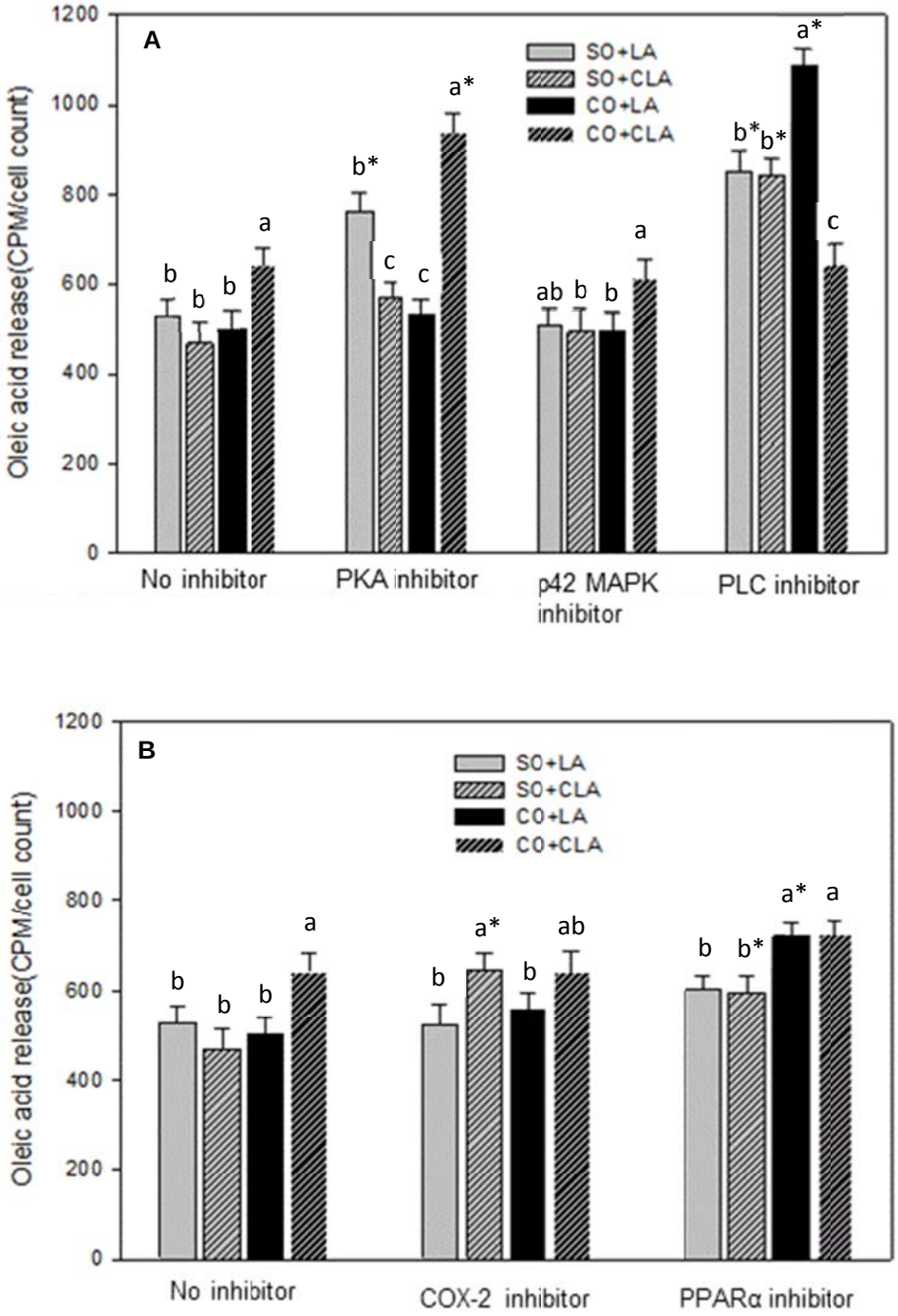

Fig 3.2: Effect of cell signaling inhibitors on CLA and oil source induced lipolysis in 3T3-L1 cells. (A) oil×trt, P<0.05; PKA, oil×trt×inhibitor, P<0.001; p42, oil×trt, P<0.05; PLC, oil×trt×inhibitor, P<0.0001, (B)oil×trt, P<0.05; COX-2, oil×trt×inhibitor, P=0.05; PPARα, oil×trt×inhibitor, P<0.05. Data are shown as mean ± SEM. Differentiated cells were serum-starved and treated with 50 μM CLA/LA for 12 hrs in the presence or absence of various inhibitors and lipolysis assay was performed. Different letters indicate differences between treatments within an inhibitor treated group. * indicates difference within treatment with presence or absence of inhibitor.

Table 3.1: Effect of cell signaling inhibitors on lipolysis related protein expression

	Treatments					P-Values		
	SO+LA	SO+CLA	CO+LA	CO+CLA	SEM	Oil	Trt	Oil x Trt
Perilipin								
No inhibitor	40.6	29.8	42.2	33.6	2.9			
COX-2	42.8[b]	83.9[a*]	23.2[c*]	24.3[c]	4.6	**0.0001**	0.17	**0.03**
p42 MAPK	181.5[*]	216.7[*]	178.7[*]	186.3[*]	15.1	0.59	0.65	0.62
PLC	168.6[a*]	137.9[a*]	79.9[b*]	85.7[b*]	17.2	**0.0007**	0.24	0.31
PPARα	91.2[*]	91.9[*]	73.6	58.4	9.7	0.12	0.25	0.64
PKA	320.3[a*]	228.9[b*]	172.9[c*]	158.3[c*]	14.1	**<0.0001**	**0.003**	**0.0179**
p-HSL								
No inhibitor	205.8	207.0	200.4	206.3	11.9			
COX-2	261.7[*]	231.35	254.6[*]	251.1[*]	14.7	0.86	0.47	0.40
p42 MAPK	420.9[*]	396.6[*]	419.2[*]	419.4[*]	33.6	0.88	0.87	0.77
PLC	452.9[*]	418.9[*]	403.5[*]	416.6[*]	25.3	0.46	0.86	0.51
PPARα	384.6[*]	373.0[*]	391.7[*]	439.0[*]	26.6	0.41	0.59	0.43
PKA	486.8[*]	474.9[*]	447.7[*]	505.1[*]	32.1	0.85	0.52	0.37

[ab]Different letters within a row indicate significant differences, $P < 0.05$

* indicates effect of inhibitor within a treatment. Main effect of inhibitor $P<0.0001$

CHAPTER 4: OVERALL DISCUSSION

In the document we have reported that CLA-induced lipolysis is time-dependent with maximal lipolysis occurring at 7 days of CLA supplementation in CO-fed mice. This is similar to another study where they observed a time-dependent effect on perilipin expression with CLA treatment in adipocyte cell culture [29]. We also observed that CLA induces lipolysis earlier in CO compared to SO-fed mice (d7 vs d14). This could be due to the activation of Gi-coupled protein receptor by PGE_2 in SO+CLA group as we showed that inhibiting COX-2, the enzyme that catalyzes the conversion of arachadonic acid to PGE_2, increases the lipolysis in SO+CLA group to the level of CO+CLA group. We speculate that since SO contains large amounts of LA which can be converted to arachadonic acid, the presence of this precursor for PGE_2 is inhibiting CLA mediated lipolysis in the SO group.

We have also shown that CLA-induced lipolysis in CO fed mice isn't mediated through the PKA or the MAPK-mediated pathways but appears to involve the PLC pathway. Inhibition of PLC had increased lipolysis in the other three treatment groups except CO+CLA. Hence we speculate that the enhanced lipolysis in CO+CLA group is mediated by an inhibition of PLC pathway in an unknown manner. It has been shown that PLC induced Ca^{2+} release can activate calmodulin kinase which phosphorylates and activates Akt. Akt is required for insulin-mediated inhibition of lipolysis [79]. Therefore, it is possible that the activation of PLC in the SO group is inhibiting lipolysis. Hence, future work needs to concentrate on the PLC pathway activation in response to CLA and different oil sources. We plan to measure the release of Ca^{2+} and activation of Akt and calmodulin kinase in response to CLA in different base oils. Also, we would like to test if the activation of PLC would ablate the CLA-induced lipolysis in CO group. It is still unknown how the PLC pathway could be inhibited with the CO+CLA treatment. It has been shown that several fatty acids can activate PLC including oleic acid and arachadonic acid.

Hence, an analysis of fatty acid metabolite composition in the different oil groups in response to CLA would give us a better understanding of how these pathways are being regulated by the different dietary group.

These studies would give a better understanding of the mechanism behind enhanced lipolysis in CO vs SO group and thus the mechanism behind enhanced body fat loss in response to CLA in CO-fed mice. An understanding of these mechanisms might aid in future development of targets for obesity treatments.

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