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Coconut oil enhancement of conjugated linoleic acid induced body fat loss and lipolysis in mice

by

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Thesis submitted to the Davis College of Agriculture, Forestry and Consumer Sciences at West Virginia University in partial fulfillment of the requirements for the degree of

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ABSTRACT

Coconut oil enhancement of conjugated linoleic acid-induced body fat loss and lipolysis in mice

Siri Manasa Ippagunta

Dietary conjugated linoleic acid (CLA) causes a body fat loss that is enhanced when mice are fed coconut oil (CO) compared to soy oil (SO). The objectives were to determine if CLA feeding altered proteins involved in lipolysis. Male mice (n=80; 3 wk old) were fed 7% SO or CO diets for 6 wk then 0 or 0.5% CLA for 12d. A body fat index was calculated: (retroperitoneal (RP) +epididymal (EPI) fat pads)*100/body weight. Lipolysis was determined by non-esterified fatty acid (NEFA) and glycerol release from EPI & RP explants. The relative expression of perilipin, phosphorylated perilipin (P-perilipin), hormone sensitive lipase (HSL), phosphorylated HSL (P-HSL), adipocyte triglyceride lipase (ATGL), and adipocyte differentiation related protein (ADRP) were determined by western blotting. The body fat index was reduced by both CLA ($P < 0.05$) and CO ($P < 0.001$) but there was no interaction. NEFA release was increased by CLA in CO-fed mice (2.94 and 8.63 $\mu\text{mol/g}$; $P < 0.05$) but not in SO-fed mice (1.76 vs 2.26 $\mu\text{mol/g}$ tissue). Glycerol release was not affected by CO or CLA. Total perilipin had a main effect of oil source, where it was decreased by CO feeding ($P < 0.05$) but P-perilipin tended to be increased by CLA in SO-fed mice and decreased by CLA in CO-fed mice ($P = 0.08$). HSL expression had a main effect of oil source where CO feeding decreased the expression ($P < 0.05$), but P-HSL expression wasn't affected by diet. ATGL expression had a main effect of oil source, it was decreased by CO feeding ($P < 0.01$) but wasn't altered by CLA. This may indicate that the CLA-stimulated lipolysis in CO-fed mice is on the decline since P-perilipin, and P-HSL are associated with increased lipase activity. ADRP expression wasn't affected by any diet, suggesting that there is no significant de-differentiation of adipocytes *in vivo*. In conclusion coconut oil enhances the anti-obesity effect of CLA and this effect is, at least in part, due to enhanced lipolysis.

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INTRODUCTION:

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid. The c9, t11 and t10, c12 isomers were found to be the two biologically active isomers known to date. CLA was first discovered as an anti-carcinogen in grilled beef (1). Since their initial discovery, CLA was found to have several other health benefits including enhancing the immune response (2), reducing atherosclerosis (3-5), and reducing body fat gain (6, 7). The main natural sources of CLAs are lipids in the meat and milk of ruminant animals. Dietary linoleic acid (18:2 c9, c12) and α -linolenic acid (18:3 c9, c12, c15) are biohydrogenated to stearic acid (18:0), by the fermentative bacteria in the rumen. The first isomerisation results in the formation of the c9, t11 isomer, also known as rumenic acid (8). Further biohydrogenation yields vaccenic acid (18:1 t11), and finally stearic acid. A mix of CLA, vaccenic acid and saturated products flow from the rumen and are absorbed from the small intestine. Ruminants and non-ruminants can also synthesize CLA in most tissues from vaccenic acid (18:1 t11) by the action of the Δ -9 desaturase (9). Chemically synthesized CLA has 40-41% of c9, t11 isomer, 43-45% of t10,c12 isomer and 4-10% of t9,t11/t10,t12 isomers. The isomer that is the most abundant in natural sources is c9,t11 (8).

The different biological effects of CLA are likely due to the multiple isomers acting individually or synergistically. 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 (10, 11). Both isomers were found to have similar effects on immune responses (2). The t10,c12 isomer causes a loss in body fat. Since CLA has been found to have such a multitude of beneficial effects, I am going to further discuss what these effects are and how CLA is able to elicit these effects. Because obesity has been a growing problem in recent years, the emphasis of

this discussion will be on how CLA causes alterations in body composition.

CHAPTER 1: REVIEW OF LITERATURE

EFFECTS OF CLA

Body composition:

The first study which identified the effect of CLA on body fat showed that, feeding mice 0.5% CLA (by weight) for 4 wks lowered the percentage of body fat by nearly 60% and increased lean mass without affecting body weight (6). The water and ash content also increased with feeding of CLA. Further studies indicated that CLA caused a decrease in fat content in mice (6, 7), rats (12, 13), hamsters (14) and pigs (15, 16) at doses of 0.5–2 % CLA. Several studies indicated that there was also a decrease in the body weight of mice, along with the decrease in body fat (7, 10) while other studies found no effect on weight (12, 13). Regardless of the dietary fat content, CLA caused a reduction in fat deposition in mice (7). 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 (10, 17).

Despite consistent results of CLA feeding in rodents, CLA consumption in pigs has resulted in variable changes in body composition. Body fat was reduced in some studies (18, 19) and was not affected in others (20, 21). This difference might be due to the duration of feeding CLA and also the fat content of the diets. Pigs fed high fat diets were less responsive to CLA when compared to those fed low fat diets (18, 22). It was also reported that CLA caused an increase in the saturated fatty acid, and decrease in unsaturated fatty acid, content of the longissimus muscle and belly fat (19, 20). This caused an improvement in meat firmness. There was also an increase in intramuscular fat while there was a decrease in subcutaneous fat (23). Hence CLA caused an improvement in pork quality.

Conversely, CLA causes a decrease in adipose deposition in lean Zucker rats but an increase in obese Zucker rats (12). These studies indicate that there are differential effects in different species and raises a question if CLA supplementation to humans has any adverse effect. Also, most of these studies were done in young growing animals so the effect in mature animals is not yet clear.

Results from studies in humans are, like the above animal studies, highly variable. Some studies indicate a reduction in body fat (24, 25) while others show no effect (26, 27). The studies in which there was a decrease in fat mass were those studies in which subjects were supplemented with a higher percentage of CLA relative to those studies in which no effects were detected. Whigham et al. (28) performed a meta analysis to analyze the efficacy of CLA as a treatment for altering body composition. They included 20 studies and concluded that CLA does cause a reduction in body fat; however, the reduction at a dose of 3.2 g/day of CLA was small. Hence, further studies should be conducted to determine if CLA can reduce body fat in humans and identify active isomers and doses.

The fat lowering effect of CLA is enhanced by feeding coconut oil (**CO**) or fat-free diets (29, 30). Mice fed a 7% CO diet for 6 wks and then supplemented with CLA had a greater reduction in body fat compared with mice fed soybean oil (**SO**) and then supplemented with CLA (29). In addition to being deficient in essential fatty acids, CO is composed of higher concentration of short and medium chain saturated fatty acids (12 to 16 carbons) compared to SO, which has mostly saturated and unsaturated 18 carbon fatty acids. Similar results have been obtained with fat-free diets (30), indicating that something in SO is counteracting the effect of CLA. The reason for the enhanced effect with CO is not completely known yet, but many involve enhanced lipolysis (31).

Insulin sensitivity:

It is well recognized that obesity impairs insulin sensitivity and promotes the risk of Type 2 diabetes. However, an initial study with 6 wks old male Zucker (fa/fa) rats showed that a 1.5 % CLA diet fed for 14 d normalized glucose tolerance in obese rats thus, preventing or delaying hyperglycemia (32). It was thought that CLA acted similar to thiazolidinediones (**TZD**) by binding to peroxisome proliferator activated receptor- γ (**PPAR- γ**) as it has been reported that CLA can induce activation of PPAR- γ to an extent similar to that of TZD and thus, causing a reduction in circulating lipids. CLA was found to have the same effect when supplemented with high fat (45% Kcal from fat) diets (33). Feeding 1% CLA to high-fat fed Sprague Dawley rats for 8 wks showed that CLA enhanced glucose tolerance. There was no difference due to the type of isomer. This decreased insulin resistance may be due to increased fat oxidation as a result of increased mRNA levels of acyl coenzyme A, which is a key enzyme in the peroxisomal β -oxidation pathway (33).

As opposed to the above studies with rats, mice fed 1% CLA demonstrated no improvement of insulin sensitivity (34). Furthermore, high metabolic rate mice fed CLA had an increase in insulin resistance. Obese mice fed t10, c12 CLA also had an increase in insulin resistance and serum glucose levels, while the c9, t11 isomer reduced serum lipids (TAGs, NEFA) without causing impairment in the insulin action (35). The differential response of mice fed t10,c12 compared to those fed c9,t11 may be due to their differential effects on steroyl element binding protein (**SREBP-1**), a key transcription factor regulating hepatic lipogenesis. The c9,t11 isomer caused a reduction in SREBP-1 mRNA expression while the t10,c12 isomer had no effect on SREBP-1 expression. It was suggested that c9, t11 improved glucose metabolism. However, in a human study, Riserus et al. (36) reported that feeding 3g/day of c9,

t11 CLA to obese men (35-60 y old) caused an increase in insulin resistance and lipid peroxidation. This increased insulin resistance was thought to be the result of impaired cell signaling due to an increase in free radicals. These differences might be due to differences between species or differences in metabolic state.

Blood lipid profile:

Obesity increases the risk of coronary heart disease, hence alterations in the body fat may change the risk of atherosclerosis. Atherosclerosis is a disease characterized by high levels of blood cholesterol, triacylglycerides (**TAG**), and lipoproteins. Dietary CLA was found to alter the serum lipid profile in several models (3-5). Rabbits fed 0.5 g CLA/day showed a marked reduction in total cholesterol (**TC**), low density lipoprotein (**LDL**) cholesterol, and serum TAG concentration (3). Also, there was a decrease in the ratio of LDL to **HDL** (high density lipoprotein) and TC, which would lower the risk of cardiovascular disease. This effect was later confirmed to occur in hamsters (5) and mice (4). A mouse study reported an increase in HDL without effecting TC in CLA-fed animals (4). Studying the individual isomers, it was found that the t10,c12 isomer and not the c9,t11 isomer caused a decrease in LDL and TC content (14). It was also found that CLA had an enhanced positive effect on blood lipids when supplemented to a saturated fat diet (coconut oil) rather than a diet high in unsaturated fat (corn oil) (37).

In contrast to the above results, a pig study reported that feeding a 1% CLA caused an increase in serum very low density lipoprotein (**VLDL**) and LDL with no effect on HDL concentration (38). Another study showed no change in blood lipid measurements when pigs were supplemented with 2% CLA (39). Also, it was found that there was an increase in TAGs and decrease in HDL concentration with 0.66% of either a CLA mix or with the t10,c12 isomer

alone (14). This increase in TAG was thought to be a function of an increase in the liver size found in this study. Hence, the differential effects observed might be due to the different species.

Above we have discussed several biological effects of CLA supplementation. The different effects are likely due to the different isomers acting individually or synergistically. CLA is found to alter the fate of lipids in the body. Thus, reducing the body fat content, the risk of atherosclerosis, and altering glucose tolerance. By understanding the mechanisms of action of CLA we may explain some of the discrepancies noted above regarding the effects of dietary CLA.

MECHANISMS OF CLA ACTION

Although many studies have shown that CLA supplementation reduces fat deposition or body fat content, and in some cases even body weight, the mechanisms of action of CLA are unclear. Several possible mechanisms have been proposed and investigated. It is thought that CLA acts by altering energy expenditure, lipolysis, adipocyte apoptosis, and/or the expression of various proteins. In this section I shall discuss more about the possible mechanisms.

Feed efficiency and energy expenditure:

Several studies have reported that CLA can cause a reduction in weight gain, fat deposition, and overall body fat content, but the mechanisms are unclear. Some studies have shown that CLA causes a reduction in feed intake (7, 10). This reduction of feed intake however, is not likely to be the cause of the reduction in body fat since, mice pair-fed a control

diet to the intake level of the CLA-fed mice did not differ in body fat compared to ad-libitum fed controls, while the CLA fed mice were leaner (17). Also, a decrease in body fat mass has been observed even when there was no reduction in feed intake (13, 40), suggesting other mechanisms involved in the decline of lipid deposition in adipocytes.

West et al. (7) supplemented 1% CLA to high- and low-fat fed mice and reported that there was increased energy expenditure (**EE**) in mice supplemented with CLA with a simultaneous reduction in food intake. This increased EE may account for the decrease in body fat. Later, West et al. compared the increase in EE and the loss in adipose deposition and found that the increased EE is sufficient to account for the decreased adipose content in CLA fed mice (41). Another study showed that the percentage of energy utilized or stored in the body (calculated as: energy intake-[energy expended as heat + energy lost in excreta]) decreased from 1.9% to - 2.3% in mice fed CLA (40). The negative value indicates that in the CLA group there is no storage but loss of body energy occurring. All these studies showed that increased EE contributes to the body fat loss.

Uncoupling proteins (**UCPs**) play a role in energy expenditure and thermogenesis. UCPs are found in the inner mitochondrial membrane and allow protons to flow from the mitochondrial inter-membrane space to the matrix, thereby "uncoupling" electron transport from the production of ATP. The energy of the electrochemical gradient is dissipated as heat. The different homologs are: UCP1, expressed primarily in the brown adipose tissue; UCP2, expressed ubiquitously in multiple tissues; UCP3, expressed in skeletal muscle and brown adipose tissue; and UCP4 and UCP5, about which not much is known (42, 43). It is possible that these UCPs are involved in the enhanced energy expenditure in CLA-supplemented animals. West et al. didn't detect any effect of CLA on UCP1 expression (41). However, studies have indicated that

increases in UCP1 and UCP3 gene expression don't relate to changes in energy expenditure (7, 44). Additionally a study has shown that feeding male mice 1.5% CLA caused a decrease in UCP1 and UCP3 in brown adipose tissue (45). Hence, UCP1 and UCP3 don't seem to be related to the increased energy expenditure in CLA-fed animals. It was found that CLA supplementation caused an increase in UCP2 expression in brown and white adipose tissue and in skeletal muscle. This increase in UCP2 mRNA was accompanied by a simultaneous decrease in adipose deposition (45, 46). As UCP2 is the predominant UCP in adipose tissue, an increase in UCP2 expression may be a mechanism of increased energy expenditure in animals fed CLA and thus, a mechanism of reduction in adipose deposition.

Lipolysis:

Lipolysis is the process by which TAGs in the fat cells are broken down and non-esterified fatty acids and glycerol are released. This process is a potential target for several therapeutics for obesity and inborn errors (i.e. conditions with errors in lipid energy metabolism). Lipolysis is stimulated by low circulating levels of glucagon and catecholamines and increased expression of tumor necrosis factor α (**TNF- α**). Mature adipocytes have unilocular lipid droplets (**LDs**) surrounded by the protein perilipin (**Plin**). Plin is the main effector of protein kinase A (**PKA**) stimulated lipolysis. When stimulated, Plin is phosphorylated by PKA and allows lipases to act on the TAGs. Hormone sensitive lipase (**HSL**) and adipocyte triglyceride lipase (**ATGL**) are the important adipocyte lipases; collectively they account for about 94% of lipolysis (characterized by the release of free fatty acids) (47). HSL has greater affinity towards diacylglycerides than towards TGs. It is activated by phosphorylation in any of four possible sites (48); upon activation it translocates to the LD surface (49). HSL is also required for normal adipose tissue function and structure, as HSL-deficient mice have adipocyte hypertrophy and an

increase in inflammatory infiltrate in white adipose tissue (50). ATGL was recently discovered to catalyze the first step of TAG hydrolysis; inhibition of ATGL caused a significant decrease in acyl-hydrolases activity (51). ATGL is associated with both basal and stimulated lipolysis (47). Comparative gene identification (**CGI-58**) is a protein associated with the LD and Plin in the basal state and acts as a co-activator of ATGL (49). In the basal state of lipolysis, CGI-58 is bound to Plin, when lipolysis is stimulated CGI-58 dissociates from Plin and binds to ATGL thus activating it.

Chung et al. reported that t10,c12 CLA supplementation increased lipolysis in human adipocyte cultures (52). They also reported that there was an increase in cytosolic perilipin accumulation, followed by a decrease in the expression of perilipin and HSL. It was also reported that along with the decreased expression of Plin there was an increase in adipocyte differentiation related protein (**ADRP**) expression when cells were treated with CLA (53). Similar to Plin, ADRP surrounds the LDs in immature adipocytes which are multilocular, rather than unilocular as in case of mature adipocytes. Thus, an increase in ADRP expression indicates an increase in the number of immature adipocytes. Increased immature adipocytes in a culture system are likely the result of de-differentiation of mature adipocytes. The activation of lipolysis was found to be induced by the activation of the ERK/MAPK pathway (53), indicating that CLA may stimulate lipolysis by a cAMP-independent pathway. This could contribute to the decrease in lipid accumulation in adipose tissue.

In contrast to the above studies, there are also some studies which showed that CLA didn't alter lipolysis. Brown et al. found that lipolysis in cultures of human adipose tissue was unaltered by either of the CLA isomers after a 3-day supplementation (54). This finding was later supported by others where they observed an inhibition in lipogenesis, but no effect on

lipolysis (55-57). This discrepancy may be because the 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.

Apoptosis and cell signaling:

In a study by Evans et al., 3T3-L1 preadipocytes cultured with t10, c12 CLA underwent apoptosis, reduced preadipocyte differentiation, and thus, decreased adipocyte cellularity (58). It was found that CLA caused apoptosis and decreased cell viability of 3T3-L1 cells in a dose dependent manner (59). These effects on preadipocytes were thought to contribute to the anti-obesity effect of CLA. Confirming this, a study feeding mice with either a CLA mix, or the isomers alone, found that the CLA mix and the t10, c12 isomer caused an increase in DNA fragmentation indicating increased apoptosis (17). CLA-induced apoptosis was also confirmed by increased amounts of cleaved caspase-3 (active form) (60). However, the degree of caspase-3 activation did not correlate with the degree of fat loss. Also, the increased DNA fragmentation in 3T3-L1 cells was observed only in proliferating cells indicating that preadipocytes are more sensitive to CLA-induced apoptosis than adipocytes (60).

TNF- α is a cytokine that is known to induce apoptosis. The possible mechanism for CLA induced apoptosis was investigated and it was found that feeding 1% CLA to mice led to a 12-fold increase in TNF- α expression in adipocytes, which corresponded with increased apoptosis of adipocyte cells (46). TNF- α has also been found to inhibit synthesis of lipoprotein lipase (**LPL**), acetyl CoA carboxylase (**ACC**), and fatty acid synthase (**FAS**), enzymes involved in lipid uptake and de novo fatty acid synthesis (61, 62).

Prolonged endoplasmic reticulum (**ER**) stress can also lead to apoptosis. The ER stress pathway is regulated by 3 transmembrane proteins – protein kinase-like endoplasmic reticulum-resident kinase (**PERK**), activating transcription factor 6 (**ATF6**) and inositol-requiring enzyme 1 (**IRE1**). On sensing stress these proteins are activated. The mechanism of stress related apoptosis is not clear but caspase-12 and CHOP (a proapoptotic protein) are found to play an important role. CLA was found to induce CHOP and thus activate the IRE1 arm of the stress pathway (63). This could lead to a decrease in cell number and may be one mechanism of reduction in fat content due to CLA.

While the above studies show an increase in apoptosis with CLA, a study feeding rats either 0.25% or 0.5% CLA found that there was a decrease in adipocyte cell size but there was no difference in cell number between the control and CLA supplemented diets (64). The difference observed in this study might be due to the fact that they counted the total number of cells per fat pad and not per unit area, and also, they didn't assess cell viability.

Peroxisome proliferator activated receptors:

The ligand-activated peroxisome proliferator activated receptors (**PPARs**) are a family of transcription factors that modulate lipid metabolism, belonging to the steroid receptor superfamily. Three types of PPAR have been found, in amphibians, rodents, fish, and humans, which are PPAR- α , PPAR- β/δ and PPAR- γ . PPAR- α responsive elements have been identified in regulating regions of various genes like acyl-CoA oxidase (**ACO**), enoyl-CoA hydratase, cytochrome P450 4A family (**CYP4A1**), liver fatty acid binding protein (**L-FABP**), acyl CoA synthase, and LPL and are mainly expressed in liver, kidney, and heart. PPAR- β/δ is expressed in almost every tissue. PPAR- γ is expressed only in adipose tissue and is required for adipocyte

differentiation (65). PPAR- γ is also essential for glucose uptake and TAG accumulation in adipose tissue and regulates the expression of genes like sterol regulatory element binding protein (**SREBP**), stearoyl-CoA desaturase (**SCD**), ACC, LPL, and glucose transporter 4 (**GLUT4**) (66). PPAR- α is activated by hypolipidemic agents of the fibrate family and by leukotrienes. PPAR- β/δ is activated by fatty acids. PPAR- γ is activated by prostaglandins and thioazolidinediones.

CLA is structurally similar to the ligands of PPARs and has been shown to activate the PPARs (32, 65, 67). CLA activation of PPAR- α led to the activation of the PPAR responsive genes ACO, FABP, and CYP4A1 *in vivo* (65). This indicates that at least part of the effects of CLA on cancer, obesity, and atherosclerosis may be mediated by PPAR- α activation. CLA effects on PPAR- γ are variable. Some recent studies have shown that CLA decreases PPAR- γ activity (68) while others have reported that PPAR- γ is activated by CLA (32). t10,c12 CLA was found to decrease ligand-induced activation of PPAR- γ and also decrease its protein levels (68). Further, it caused a reduction in mRNA and protein levels of GLUT4 and LPL. Many studies have also demonstrated that CLA has the capacity to inhibit the expression and activity of SCD, a PPAR- γ responsive gene (69, 70). As SCD is required for monounsaturated fatty acid synthesis in mature adipocytes, a reduction in SCD activity may indicate reduced fatty acid synthesis and hence indicate a reduction in adipocyte differentiation. CLA was found to repress the differentiation of pre-adipocytes into adipocytes *in vitro* in mouse 3T3-L1 cultures (71). As differentiation is required to get mature adipocytes which have the capability to synthesize and store lipids, a decrease in differentiation might attribute to the lowering in TAG content and body fat caused by CLA. In contrast to the above studies, some studies have shown that CLA causes activation of PPAR- γ and improves hyperinsulinemia and increases glucose sensitivity (32, 72).

Most of these studies were done with obese animals. The reason for this discrepancy is not known but it may be that prolonged treatment in genetically obese animals causes an activation of PPAR- γ while in normal mice its activity is decreased.

METABOLISM OF CLA

Even though the effects of CLA and a few of its mechanisms are known, the fate of CLA in a biological system is not clear. It is not known if CLA is eliciting its effects directly or through its metabolites. CLA has a backbone structure similar to oleic acid (18:1) owing to its conjugated diene double bond system. Hence, it is incorporated into tissues similar to oleic acid, primarily into neutral lipids in adipose and mammary tissues (73). Despite an incorporation pattern similar to oleic acid, CLA is metabolized like linoleic acid (**LA**) (18:2). It has been shown that CLA can undergo $\Delta 6$ desaturation, elongation, and $\Delta 5$ desaturation and produces metabolites with a conjugated diene structure in animal and human tissues (74, 75). It has been reported to form conjugated 18:3, 19:2, 20:2, 21:2, 20:3 and 20:4 fatty acids (75, 76). Rats fed either a CLA mixture or pure isomers had 18:3 and 20:3 conjugated isomers in liver tissues (75). The further conversion into 20:4 has been found to occur to a greater extent in animals that were fed diets low in LA and hence might be dependent on LA/CLA ratio in the diets (11, 73, 75). CLA has also been found to undergo β -oxidation to yield 12:2, 16:2, 16:3 isomers (76, 77). CLA isomers may compete with LA for the metabolizing enzymes. This has been supported in a study where supplementing t10,c12 isomer was found to inhibit the $\Delta 9$ desaturation of stearic acid into oleic acid and also the $\Delta 6$ desaturation of linoleic acid but only at a high non-physiological concentration (74). The c9,t11 isomer also inhibited the $\Delta 6$ desaturation of linoleic

acid. Also, another study showed that feeding rats with increasing levels of CLA caused a significant dose-dependent decrease in the content of LA metabolites in adipose and mammary tissues (78). However, this decrease in LA metabolites was not observed in liver and plasma and, above 1% CLA there were no further decreases. This indicates that CLA may compete with LA, especially in tissues where it is maximally incorporated.

The metabolism the c9,t11 and the t10,c12 isomers of CLA appears to be different. Feeding t10,c12 CLA produces large amounts of 18:3 fatty acid and little 20:3, while c9,t11 CLA is converted to both 18:3 and 20:3 isomers in similar amounts (79). This difference in metabolism might account for the differential biological effects exerted by the two isomers.

Recently, it has been found that CLA metabolites and other conjugated fatty acids can also exert potent biological effects. Conjugated eicosadienoic acid (CEA) a 20:2 fatty acid formed possibly by the elongation of CLA, was found to cause changes in body composition similar to CLA. CEA caused a significant decrease in body fat content and tended to increase water and protein content (76). In cultures of 3T3-L1 adipocytes CEA caused an inhibition in LPL activity, increased glycerol release (indicating increased lipolysis), and reduced total TAG content. Of the two conjugated isomers of CEA, the t12,c14 isomer was found to be the active form. However, CEA requires longer incubation periods and/or higher concentrations to give similar effects to CLA. Conjugated 20:3 Δ c8,t12,c14 (CETA), the Δ 6 desaturation and elongation product of t10,c12 CLA also inhibited LPL activity and enhanced glycerol release but to a lesser extent than CLA. Conjugated nonadecadienoic acid (CNA), a 19:2 fatty acid was also found to cause a reduction in body fat content and LPL activity similar to CLA (80).

The observed effects of CLA may be direct or due to one or more of its metabolites. It is also possible that the CEA and CETA may be converted back to CLA to cause the effect, since in cultures supplemented with CEA or CETA there was CLA detected. However, we cannot be sure if this would occur in animals as conversion of CEA to CLA requires peroxisomal β -oxidation and therefore, has to escape from the mitochondrion to elicit its effects.

SUMMARY

CLA has been found to cause multiple biological effects including anti-inflammatory, anti-obesity and anti-carcinogenic effects. The complete mechanisms are not yet clear but, several possible mechanisms explaining the effect of CLA on body composition have been reported (Figure 1). CLA has been found to cause alterations in both adipose tissue and liver. In the adipose tissue CLA has been found to increase lipolysis, and decrease adipocyte cell number, accumulation of TAG, and glucose uptake. There was also an increase in body energy expenditure. These effects are largely due to alterations in the expression of different proteins. Dietary CLA may cause an increase in lipolysis by inducing signaling through the MAPK/ERK pathway, and in a few studies have also been found to lower perilipin expression. CLA causes an increase in UCP2 expression, causing an increase in energy expenditure. It has also been found that consumption of CLA lowers the expression of PPAR- γ , which in turn causes a decrease in differentiation of adipocytes and also effects the expression of several proteins that lower TAG accumulation and hence adipocyte size.

In the liver, CLA has decreased FAS and increased CPT1 expression, which cause a decrease in fatty acid synthesis and an increase in β -oxidation. It has also been found to alter the

expression of PPAR- α , increasing its expression which in turn causes an increase in fatty acid oxidation by causing an increase in ACO and CYP4A1 expression.

The isomers of CLA are metabolized differentially and hence may be the basis for the different effects of the isomers. Some of the conjugated metabolites of CLA have been found to lower body fat and to effect lipolysis. Consequently, more research is needed on the mechanism of action of CLA to fully understand its beneficial and any potential detrimental effects.

Fig 1. Possible mechanisms by which CLA causes body fat loss. → Represents mechanism reported by several studies, — → represents mechanism reported by just one study. FAS, fatty acid synthase; SREBP, sterol regulatory element binding protein; CPT, carnitine palmitoyl transferase; LPL, lipoprotein lipase; PPAR, peroxisome proliferator-activated receptor; ACO, acyl-CoA oxidase; CYP4A1, cytochrome P450IV A1; FABP, fatty acid binding protein; UCP, uncoupling protein; Plin, perilipin; TNF, tumor necrosis factor; SCD, stearoyl CoA desaturase; ACC, acetyl CoA carboxylase; GLUT, glucose transporter; C/EBP, CCAT enhancer binding protein; FA, fatty acid; TG, triacylglycerides.

**CHAPTER 2: COCONUT OIL ENHANCEMENT OF CONJUGATED LINOLEIC ACID
INDUCED BODY FAT LOSS AND LIPOLYSIS IN MICE**

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 anti-inflammatory (2), anti-atherogenic (3-5), anti-carcinogenic (1, 10, 11), and anti-obesity effects (6, 7). The 18:2 c9, t11 and t10, c12 isomers are the two biologically active isomers known to date. The t10, c12 isomer is solely responsible for the induction of body fat loss (10, 17).

The loss of body fat induced by dietary CLA can be enhanced when mice are fed a coconut oil (CO) containing diet compared to mice fed a soybean oil (SO) containing diet (29). 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 and an abundant supply of linoleic acid. We have also reported increased sensitivity to CLA-induced body fat loss in mice consuming fat free diets (30), indicating that something in SO, and not in CO, interferes with the full effect of CLA. The mechanism behind the enhanced response with CO feeding is not yet completely known.

Preliminary data from our laboratory indicated that dietary CLA caused an increase in basal lipolysis, in CO- and fat free-fed mice, but not in SO-fed mice (31). This is in agreement with several cell culture studies that have shown that CLA supplementation causes an increase in lipolysis (52, 53). Lipolysis can be stimulated by low circulating levels of glucagon and catecholamines and increased expression of tumor necrosis factor α (TNF- α) (81). Mature adipocytes have unilocular lipid droplets (LDs) surrounded by the protein perilipin (Plin). In the

basal state of lipolysis, perilipin and comparative gene identification-58 (CGI-58) form a complex on the LD, adipose triglyceride lipase (ATGL) is localized partially to the LD, and hormone sensitive lipase (HSL) is localized to the cytoplasm (49). When lipolysis is stimulated, cAMP-dependent protein kinase (PKA) activation results in phosphorylation of HSL and perilipin. Phosphorylation of perilipin causes the release of CGI-58, which binds ATGL, activating ATGL, and initiating lipolysis. Phosphorylated HSL translocates to the LD, associates with phosphorylated perilipin, and degrades diacylglycerol (**DAG**). CLA has been found to cause an increase in cytosolic perilipin (activated form of perilipin), while causing a decrease in HSL and total perilipin expression (52).

Adipose tissue is composed of multiple cell types including preadipocytes, immature adipocytes, and mature adipocytes. Immature adipocytes are multilocular with adipocyte differentiation related protein (ADRP) surrounding the LDs instead of perilipin. It has also been reported that there is an increase in ADRP expression with a simultaneous decrease in perilipin expression in CLA-treated adipocytes, indicating that cells are undergoing de-differentiation (52). It is unclear if this also occurs *in vivo*. In the current study, we determined the effect of base oil and CLA on lipolysis, the expression and activation of perilipin and associated lipases, and if CLA caused any de-differentiation of adipocytes *in vivo*.

METHODS AND PROCEDURES:

Animal protocol

All animal procedures were approved by the West Virginia University Animal Care and Use Committee. Weanling male mice (n = 80; 3 wks old; ICR) were obtained from Harlan Inc.

Madison, WI and housed in a temperature-controlled animal room with a 12 h light:dark cycle. Mice were fed a purified base diet containing (g/kg): isolated soy protein (200), L-cystine (2.54), L-methionine (2.54), cornstarch (395.406), maltodextrin (132), cellulose (100), soybean oil (70), AIN-93G mineral mix (35), AIN-93G vitamin mix (10), choline bitartrate (2.5) (Harlan Teklad, Madison, WI). A modification of the purified AIN-93G diet with soy protein instead of casein was used so as to avoid possible intake of CLA from the casein. For the coconut oil (CO) diet, soybean oil was replaced by fully hydrogenated coconut oil (wt/wt). CLA replaced the base oil (wt/wt) and was added in the diets to provide 0.5% CLA isomers in the diet. The CLA mixture contained an equal amount of c9,t11 and t10,c12 (Nu-Check-Prep, Elysian, MN).

Mice were housed four per cage for weeks 1 - 5 and then were individually caged during weeks 6 - 8. Animals were randomly assigned to base diets: SO (soy oil) or CO (coconut oil). After 6 wks, half of each of these two groups was given a 0.5% CLA diet for a period of 12 days. Body weight and feed intake were measured weekly. At the end of 54 days, the mice were killed by carbon dioxide asphyxiation. Epididymal and retroperitoneal fat pads, livers, and blood were collected and tissues were weighed. One fat pad pool, consisting of one epididymal and one retroperitoneal fat pad, for eight mice from each diet was utilized for lipolysis analysis and the remaining tissues were flash frozen in liquid nitrogen. A body fat index was calculated as:

$$[(\text{epididymal} + \text{retroperitoneal fat pad weights}) / \text{total body weight}] * 100.$$

Lipolysis

One each of retroperitoneal and epididymal fat pads per mouse (n = 8/diet) were pooled, minced, and incubated in 0.8 ml of DMEM (containing 1 Kunits/ml adenosine deaminase) for 3 h at 37° C in a shaking water bath. This was done in triplicate for both baseline and stimulated lipolysis (10 µM isoproterenol). Media samples were collected and analyzed for non-esterified

fatty acids (NEFA- HR kit, Wako Chemicals, Richmond, VA) and glycerol (free glycerol reagent, Sigma-Aldrich Inc., St Louis, MO) by colorimetry. NEFA and glycerol concentrations were corrected for tissue weight and reported as μmol released per gram tissue.

Blood was centrifuged at $2,000 \times g$ for 20 min at 4°C . The serum was collected and the concentration of NEFA was analyzed similar to the method above.

Western blotting

One fat pad pool per mouse ($n = 12/\text{diet}$) was homogenized in 1 ml of lysis buffer (25 mM HEPES, 5 mM EDTA, 5 mM MgCl_2 , 10 $\mu\text{l}/\text{ml}$ of protease and phosphatase inhibitor mix (Thermo Scientific, Rockford, IL)) and centrifuged at $14,000 \times g$ for 20 min at 4°C . Protein content was analyzed by the BCA method (BCA kit, Thermo Scientific, Rockford, IL). Equal amounts of protein (5 μg) were resolved on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were probed with antibodies for perilipin, ATGL, ADRP (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-PKA substrate (for phospho-perilipin), HSL, and phospho-HSL (Cell Signaling Technology, Danvers, MA). Then the membranes were probed with a HRP-conjugated secondary antibody. Membranes probed with β -actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used for normalization.

Chemiluminescence was produced using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL). The density of the bands obtained was analyzed by densitometry.

Statistical analysis

All data were analyzed by a two-way analysis of variance (ANOVA) using a fixed model testing the main effects of oil source (SO vs. CO), CLA, and the interaction of oil source $\times$ CLA.

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.

RESULTS

Body weight, feed intake, and body composition

Body weight was not affected by diet except on the last week where there was an oil source $\times$ CLA interaction ($P < 0.05$), where CLA caused a 6% increase in weight in SO fed mice while causing a 5% decrease in CO fed mice (Table 1). Feed intake was not affected by oil source until week 6, but during the last 2 wks there was a main effect of oil source ($P < 0.05$) where CO fed mice had a 5% higher feed intake compared to SO fed mice. Also, on the final measurement there was oil source $\times$ CLA interaction ($P < 0.05$) where CLA caused a decrease in feed intake, but only in CO-fed mice.

There was no significant interaction on the body fat index, but both CO ($P < 0.001$) and CLA ($P < 0.05$) caused decreases in the body fat index (Fig 2). CO caused a 32% decrease in the body fat index while CLA caused a 20% decrease in body fat index.

Lipolysis

The effect of treatments on lipolysis were determined by the release of NEFA and glycerol during a 3-h period of baseline and isoproterenol incubation of the fat pads. There was an interaction of oil source $\times$ CLA ($P < 0.05$) on the amount of NEFA released under basal conditions. CLA caused a 3-fold increase in NEFA release in CO-fed mice, while the increase in SO-fed mice due to CLA was not significant (Fig 3a). There was no significant effect on the

amount of glycerol released, although it followed the same trend as NEFA release (Fig 3b). There were no differences in either NEFA or glycerol release under stimulated conditions.

The NEFA concentration in serum was not affected significantly by any dietary treatment, though CO fed mice tended ($P = 0.07$) to have higher serum NEFA levels compared to the SO fed mice (Fig 3c).

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. The western blots showed that the total protein concentration of perilipin was affected by oil source ($P < 0.05$) alone where CO-fed mice had a decreased expression of perilipin (Fig 4a). The activation of perilipin (concentration of p-perilipin) had a tendency toward an interaction of CLA $\times$ oil source ($P = 0.08$), as CLA tended to increase the amount of p-perilipin but only in SO-fed mice (Fig 4b). SO+CLA-fed mice had a 255% of the p-perilipin expression found in SO-fed mice, while CO+CLA-fed mice had only 76% of the p-perilipin expression found in CO-fed mice (Fig 4b). HSL expression and activation weren't altered by CLA, but there was a decrease in total HSL expression in CO fed mice ($P < 0.05$) (Fig 4c,d). ATGL expression was decreased in CO-fed mice ($P < 0.01$) but there was no effect of CLA (Fig 4e). ADRP expression was not altered significantly by any of the four diets (Fig 4f).

DISCUSSION

It has previously been reported that feeding coconut oil enhanced the CLA-induced body fat loss in mice (29, 30). In support of this, mice in this study fed coconut oil and supplemented with CLA had the lowest body fat index (Fig 2). The enhanced body fat loss appears to involve CLA-induced lipolysis in CO-fed mice. Some studies have reported that CLA enhances lipolysis in adipocyte cultures (52). In our study we observed an enhanced lipolysis in CO+CLA-fed mice compared to SO+CLA-fed mice.

In this study we saw that CO diets caused a decrease in body fat even without CLA supplementation, which is in contrast to the previous reports (29, 30). Therefore, we did not detect a significant interaction of oil source $\times$ CLA, as was seen in previous studies. The reason for this is not known but possible explanations could be that these were a different strain of mice compared to those used in previous studies (ICR vs a four strain composite of ICR, C57BL/6, NIH Swiss, CF-1). Also, in this study the mice didn't consume as much food or gain as much as weight as previous experiments. In contrast to previous coconut oil-CLA studies, a study in which mice were fed different fat sources after a period of energy restriction, reported that the coconut oil-fed group had a lower body fat compared to groups fed other high fat diets (82). In fact, the level of body fat, and serum cholesterol were similar to mice fed low fat diets. Later another study showed that there was an increase in UCP1 expression in energy restricted and coconut oil fed mice, which may be a possible mechanism for the cause of lower body fat with coconut oil feeding (83). Coconut oil is deficient in essential fatty acids and this has been hypothesized to be the reason for the enhanced response to CLA, but a previous study showed that adding essential fatty acids at the level of their requirement to CO diets didn't affect the enhanced response to CLA (30). Hence the enhanced effect of CO is not dependent on a true

deficiency of essential fatty acids. SO, however, contains large amounts of linoleic acid, well in excess of the dietary requirement, that could still interfere with the actions of CLA.

In support of our preliminary data (31), we observed a CLA-induced increase in basal lipolysis in CO-fed mice, but not SO-fed mice. This differential effect of CLA likely contributes to the overall enhanced body fat loss. The CLA supplemented CO diets caused a tremendous increase in NEFA release but a significant effect was not detected on the amount of glycerol released. This might be because the overall amount of lipolysis occurring was relatively low and differences between diets are more pronounced in NEFA release than glycerol as their concentration would be at a 3:1 ratio. We detected no differences in stimulated lipolysis, indicating no differences in the total amount of lipases present in the cell, because under these conditions all available protein should be activated. Several studies have shown that CLA causes an increase in lipolysis (52, 53), while others have reported no effect of CLA on lipolysis (55-57). The difference between these studies may be that, those studies in which there was an effect of CLA, the cells were serum starved prior to supplementation of CLA. This is similar to our treatment with CO, as serum starving makes the cultures devoid of fatty acids. Explants cultures of CLA-fed pigs didn't have any CLA induced increase in lipolysis (55), similar to our SO+CLA-fed mice.

Based on the lipolysis data we expected an increase in the activation of the lipases and perilipin. However, we found that CLA did not increase lipolysis associated protein expression in CO-fed mice. We found that CLA caused an increase in expression of p-perilipin but only in SO-fed mice. There was no effect of CLA on any of the other proteins. 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, as this has been reported in a cell culture

study (52). CLA was supplemented to human adipocytes, causing an increase in lipolysis and in cytosolic perilipin, indicating activation, following 12 hr. This was followed by a decrease in the expression of perilipin and HSL by 24 hrs. It is possible that after 12 days of CLA consumption our mice were beginning to experience a decline in perilipin and lipase expression. Similar to the body fat index, there was an effect of CO itself on the expression of perilipin, HSL, and ATGL.

Some previous studies have shown that CLA caused a de-differentiation of mature adipocytes, as they observed a simultaneous increase in ADRP expression with a decrease in the expression of perilipin (52, 53). Immature adipocytes are characterized by having multilocular lipid droplets surrounded by ADRP. In this study we didn't observe any effect of CLA on ADRP expression. This might be because unlike cell cultures, there are an abundance of immature adipocytes *in vivo* and any slight alterations in their number due to CLA might not be detectable.

In conclusion, coconut oil enhances the anti-obesity effect of CLA and this effect is, at least in part, due to enhanced lipolysis. The effects of CLA on the expression and activation of lipases and other lipolysis-related proteins may be time-dependent and therefore, were not clear in the current experiment. Further studies evaluating the effect of different feeding durations of CLA might be helpful in determining the mechanism of CLA action on lipolysis.

Table 1. 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	9.62	9.42	9.58	9.53	0.07	0.64		
Before CLA ²	31.73	32.8	32.75	31.65	0.76	0.76		
Final ²	33.60 ^{ab}	35.88 ^a	35.05 ^{ab}	33.31 ^b	0.86	0.51	0.75	0.02
Feed intake (g/day)								
Initial ³	2.85	2.82	2.83	2.87	0.02	0.59		
Before CLA ²	4.05	4.21	4.33	4.41	0.11	0.03		
Final ²	2.88 ^b	3.04 ^{ab}	3.25 ^a	2.99 ^b	0.09	0.09	0.61	0.04

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 0; Before CLA = Day 42; Final = Day 57.

³ Initial is feed intake measured from day 0 to day 7.

^{ab} Different letters within a row indicate significant differences, P < 0.05

Fig 2. Effect of dietary CLA and oil source on body composition. Body fat index = ((Retrperitoneal + Epididymal fat pad weights)/body weight) x 100; n = 20/diet. Data is shown as mean ± SEM. Different letters indicate significant differences between treatments. Oil source, $P < 0.001$; CLA, $P < 0.05$. 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).

Fig 3. Effect of dietary CLA and oil source on lipolysis. Fat pads were cultured for 3 hrs *ex vivo*. Basal = 0 and Stimulated = 10 μM Isoproterenol; n = 8/diet. (A) NEFA release, expressed as μmol/g tissue. Oil*CLA, P < 0.05. (B) Glycerol release, expressed as μmol/g tissue. (C) Serum NEFA, expressed as mmol/l. Data is shown as mean ± SEM. Different letters indicate significant differences between treatments. 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).

Fig 4. Effect of dietary CLA and oil source on adipose tissue protein expression. Densitometry analysis of each protein, relative to β -actin was performed; $n = 12$ /diet. (A) Perilipin expression, Oil source, $P < 0.05$. (B) P-Perilipin expression, Oil source*CLA, $P = 0.08$. (C) HSL expression, Oil source, $P < 0.05$; CLA $P = 0.05$. (D) P-HSL expression. (E) ATGL expression, Oil source, $P < 0.01$. (F) ADRP expression. Data is shown as mean $\pm$ SEM. Different letters indicate significant differences between treatments. 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).

CHAPTER 3: FUTURE DIRECTIONS

FUTURE RESEARCH

We have established, with the current work, that coconut oil enhances the body fat lowering effects of CLA, also that this involves an enhanced lipolysis. In a future study, we would like to determine the effect of CLA on the expression and activation of proteins involved in lipolysis by feeding CLA for different periods of time. This would let us determine the effective time period of feeding CLA to elicit the required response on lipolysis and also if feeding longer than this time period causes any harmful effects. We hypothesize that there would be a time-dependent effect based on a cell culture study, which reported that supplementation with CLA to the adipocyte cultures caused an increase, followed by a decrease, in expression of perilipin (52).

The reason for the effect of CO enhancement is not known clearly; hence another future study would concentrate on validating the effect of coconut oil on the metabolism of CLA. We would like to determine if there are differential effects of the base oil on the amount of CLA being metabolized. This was thought to be possible based on a study where inhibiting $\Delta 6$ -desaturase prevented the CLA-induced reduction of body fat, implying that a metabolite of CLA causes this anti-obesity effect (84). If there is any difference in metabolism of CLA between the two base oils, then further studies feeding mice the metabolites and assessing their effects on body fat and lipolysis would give us a better picture of how CO is able to enhance the effect of CLA. Some previous studies have shown that CLA can be metabolized similar to linoleic acid. These conjugated metabolites when supplemented have also been shown to cause a significant decrease in body fat content. Hence it might be possible that the different base oils are affecting metabolism of CLA.

These studies would give a better understanding of the effective time of supplementation of CLA to elicit a good effect on the loss of body fat and an understanding of how CO enhances the CLA induced fat loss. This would add to the knowledge of the possible mechanisms of action of CLA. It might be helpful in devising a possible treatment of obesity with CLA or another compound in the future.

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