MECHANISMS OF CONJUGATED LINOLEIC ACID ON INSULIN RESISTANCE, HEPATIC STEATOSIS, AND ADIPOSITY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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* * * * *

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ABSTRACT

Conjugated linoleic acids (CLA) are a group of dietary fatty acids that exist as positional and geometric isomers of linoleic acid. CLA decreases body weight primarily through the reduction of adipose mass and is reported to modulate insulin resistance. The mechanisms by which CLA depletes adipose and the roles of adipokines in CLA-mediated insulin resistance are not completely understood. The first objective of this research was to determine the effects of CLA on lipid metabolism and fatty acid composition in the liver of a genetically obese and diabetic model compared to a thiazolidinedione. In this study, the effects of diet supplementation with either 1.5% CLA or 0.2% troglitazone (TZD), an insulin sensitizing thiazolidinedione, on glucose tolerance and lipid accumulation and composition of both lean and Zucker diabetic fatty (fa/fa; ZDF) rats were examined. CLA reduced adipose mass while improving glucose tolerance and hepatic steatosis in ZDF rats. The effects of CLA and TZD on hepatic lipid composition suggest that the effects of these two agents on glucose tolerance may be associated with a reduction in stearoyl-CoA desaturase-1.

Conversely, in both lean and obese mice, CLA rapidly reduces adipose mass and worsens insulin resistance and hepatic steatosis, effects which are preceded by the significant depletion of the adipokines leptin and adiponectin. Therefore, the second objective was
to determine whether effects of CLA on insulin resistance, hepatic steatosis, and inflammation occur depend on the depletion of leptin by CLA. To this end, recombinant leptin was co-administered with dietary CLA in ob/ob mice to control leptin levels and to, in effect, negate the leptin depletion effect of CLA. In a 2x2 factorial design, 6-week old, male ob/ob mice were fed either a control diet or a diet supplemented with CLA and received daily, intraperitoneal injections of either leptin or vehicle for 4 weeks. In both the absence and presence of leptin, CLA significantly reduced adipose mass and depleted adiponectin. In the absence of leptin, CLA worsened insulin resistance without evidence of inflammation in adipose tissue or hepatic steatosis in mice after 4 weeks. In the presence of leptin, CLA failed to worsen insulin resistance, but induced hyperinsulinemia and hepatic steatosis in ob/ob mice. These results suggest that the effects of CLA on hyperinsulinemia, hepatic steatosis, and inflammation may not be dependent on leptin depletion, but perhaps these effects, particularly insulin resistance and inflammation, could be ameliorated with exogenous leptin treatment.

The significant depletion of adipose in mice by CLA may contribute to the lipodystrophic-like effects that accompany. The final objective of this research was to determine mechanisms by which CLA reduces adipose mass. Six-week old, male ob/ob mice were fed either a control diet (CON) or a diet supplemented with 1.5% mixed isomer CLA (CLA) for 4 weeks. A third group of mice (LEPTIN) were fed the control diet and received daily, intraperitoneal injections of 1 mg/kg BW recombinant leptin as a positive control for adipose depletion in ob/ob mice. The depletion of adipose tissue by
CLA was accompanied by the acquirement of brown adipose-like characteristics, such as increased CPT-1b, PGC-1α, and UCP-1, in the white adipose of CLA-fed mice. This alteration may facilitate the reduction of adipose mass by increasing mitochondrial oxidation and energy dissipation. However, it appears that CLA does not increase UCP-1 through β3AR signaling.
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ABSTRACTS


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FIELD OF STUDY

MAJOR FIELD: The Ohio State University Nutrition Graduate Program
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<td>ACOX1</td>
<td>acyl-coenzyme A oxidase 1</td>
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<tr>
<td>AMPK</td>
<td>AMP protein kinase</td>
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<tr>
<td>ATGL</td>
<td>adipose triglyceride lipase</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
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<td>β3AR</td>
<td>beta 3-adrenoceptor</td>
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<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
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<tr>
<td>c9t11</td>
<td>cis-9, trans-11</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CCL2</td>
<td>CC chemokine ligand 2</td>
</tr>
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<td>Cidea</td>
<td>cell-death-inducing DFF45-like effector A</td>
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<td>CLA</td>
<td>conjugated linoleic acid</td>
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<td>CPT</td>
<td>carnitine palmitoyltrasferase</td>
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<td>CRE</td>
<td>cAMP response element</td>
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<td>FABP</td>
<td>fatty acid binding protein</td>
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<td>fatty acid synthetase</td>
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<td>fatty acid transporter</td>
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<td>free fatty acid</td>
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<td>HOMA</td>
<td>homeostasis assessment model</td>
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<td>HSL</td>
<td>hormone sensitive lipase</td>
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<td>interleukin-6</td>
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<td>insulin tolerance test</td>
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<td>LPL</td>
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<td>least square mean</td>
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<td>mitogen activated protein kinase</td>
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<td>MI-CLA</td>
<td>mixed isomer CLA (equal % of c9t11 &amp; t10c12)</td>
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<td>mitochondrial transcription factor A</td>
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<td>nuclear respiratory factor</td>
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<td>ob/ob</td>
<td>B6.V-Lepob/OlaHsd</td>
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<td>PPAR gamma coactivator-1 alpha</td>
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<td>protein kinase A</td>
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<tr>
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<td>protein kinase B (also known as Akt)</td>
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<td>PKC</td>
<td>protein kinase C</td>
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<td>peroxisome proliferator-activated receptor</td>
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<td>standard error</td>
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<td>stromal vascular</td>
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<td>t10c12</td>
<td>trans-10, cis-12</td>
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<td>tumor necrosis factor α</td>
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<td>uncoupling protein</td>
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CHAPTER 1

1.1 INTRODUCTION

The incidence of obesity in the past 20 years has become increasingly prevalent not only in Western cultures, such as the United States, but worldwide. The World Health Organization projected that in 2005 approximately 1.6 billion adults (≥ 15 years old) were overweight (BMI ≥ 25) and an estimated 400 million were obese (BMI ≥ 30) (1). According to the 1999-2004 National Health and Nutrition Examination Survey (NHANES), over 66% of the adults (≥ 20 years old) in the United States were overweight or obese (2). In the United States, $75 billion was estimated to be spent on medical expenditures attributable to obesity in 2003 (3). However, the monetary costs pale in comparison to the health risks. Obesity contributes to the etiologies of a number of co-morbid conditions such as: hypertension, stroke, cardiovascular disease, some cancers, and type 2 diabetes. While the words ‘overweight’ and ‘obesity’ are often described in terms of body weight, the health consequences associated with them are directly derived from an excess of adipose tissue mass. In addition to storing lipid for energy, adipose secretes a variety of adipokines, many of which affect metabolism and inflammation in adipose and non-adipose tissues. Modulation of the endocrine functions of adipose tissue
can contribute to a chronic state of inflammation, which can lead to the pathogenesis of a multitude of disorders, specifically insulin resistance and type 2 diabetes (4).

Type 2 diabetes is clinically defined by elevated fasting glucose, postprandial hyperglycemia, or abnormally increased glucose excursion in response to a defined glucose load (5). An estimated 20.8 million people have diabetes, 90-95% of which is type 2, in the United States, and the incidence is rising at an alarming rate. Approximately 1.5 million people will be diagnosed with diabetes this year alone; meanwhile, diabetes will remain a leading cause of death in the U.S. The indirect and direct costs of diabetes and associated complications were in excess of $130 billion in 2002 (6). Type 2 diabetes is strongly linked to obesity: nearly 80% of diabetics are obese (7). Excess adipose contributes to insulin resistance through dysregulated metabolism resulting in increased circulating nonesterified fatty acids (NEFA). The increased NEFA interfere with insulin signaling decreasing glucose clearance by skeletal muscle and adipose and reducing inhibition of hepatic gluconeogenesis. Adipose tissue also releases a variety of hormones, adipokines, and pro-inflammatory cytokines that are factors in the development of insulin resistance (8).

While there are pharmacological therapies for the treatment of diabetes and obesity, improvement in diet and exercise is the initial recommendation in most treatment regimens. Additionally, over the past several decades, there has been an increased awareness of the roles of bioactive components of food in modulating disorders, diseases,
and cancer. A specific group of polyunsaturated fatty acids collectively known as conjugated linoleic acid (CLA) are found primarily in foods derived from ruminants, such as beef, lamb, and dairy, and have received considerable attention due to potential health benefits, in particular decreasing adiposity and improving insulin sensitivity.

CLA exists as positional and stereo-isomers of octadecadienoic acid [18:2 (n-6)]. CLA, specifically the trans-10, cis-12 (t10,c12) isomer (9), significantly decreases body weight primarily through a reduction of adipose tissue in a variety of species (10-12). CLA also ameliorates increased fasting glucose and insulin levels and improves glucose tolerance associated with a rat model of type 2 diabetes (10). However, CLA induces hyperinsulinemia and insulin resistance mice (13-17). In mice, insulin resistance induced by CLA develops in parallel with lipodystrophy, i.e. decreased adipose mass, significant and rapid depletion of the adipokines leptin and adiponectin, and increased hepatic steatosis (15; 18). Despite considerable amount of research, the roles of adipokines in CLA-mediated effects and the mechanisms by which CLA depletes adipose tissue are not completely understood. Therefore, my research has been directed toward elucidating mechanisms relating to these two general deficiencies through the following aims:
1.2 AIMS

Aim 1: To determine the effects of CLA on lipid metabolism and fatty acid composition in the liver of a genetically obese and diabetic model compared to a thiazolidinedione.

Glucose metabolism and insulin sensitivity are associated with reduced hepatic steatosis and decreased SCD-1 activity. While we have shown that CLA normalized fasting glucose levels in ZDF rats similar to the insulin-sensitizing agent thiazolidinedione, the associated effects in hepatic metabolism are unclear. *We hypothesize that many of the effects of CLA on glucose tolerance and hepatic metabolism are similar to a thiazolidinedione.*

Aim 2: To determine whether effects of CLA on insulin resistance, hepatic steatosis, and inflammation occur in a leptin-dependent manner.

Insulin resistance, hepatic steatosis, and inflammation induced by CLA are preceded by a significant and rapid depletion of the adipokines leptin and adiponectin. *Therefore, we hypothesize that chronic administration of exogenous leptin to ob/ob mice prevents CLA-induced insulin resistance, hepatic steatosis, and inflammation.*
Aim 3: To determine mechanisms by which CLA induces delipidation and reduces adipose mass.

CLA is well-known to reduce adipose mass, some of which is thought to accumulate ectopically in tissues such as the liver. We have shown that CLA significantly reduces adipose mass without ectopic distribution of lipid and with little change in lipid metabolism in the liver and muscle. *We hypothesize that reduction of lipid and adipose mass by CLA is associated with markers of increased oxidation and energy dissipation in white adipose tissue.*
CHAPTER 2

LITERATURE REVIEW

2.1 Insulin Resistance

There is a striking relationship between the incidence of obesity and type 2 diabetes and insulin resistance. Insulin resistance is referred to as the inability of insulin to exert its usual biological effects. Insulin resistance is characterized by decreased glucose uptake by the muscle and adipose, increased hepatic gluconeogenesis, and increased lipolysis in adipose. Appropriately, the muscle, liver, and adipose are the primary sites of insulin resistance with consequential effects occurring in the pancreas. Defects in regulation of the above processes contribute to the hallmarks of insulin resistance: impaired glucose tolerance, hyperinsulinemia, and eventually hyperglycemia and β-cell dysfunction (19). While a definitive progression of these events cannot be established, there are several models by which the development of hyperglycemia and β-cell dysfunction are thought to ultimately occur (reviewed in (20)). Classically, however, insulin resistance develops, for example, as a result of obesity, insulin resistant tissues have impaired glucose uptake, resulting in glucose intolerance. The pancreas secretes more insulin to compensate for the attenuated glucose clearance resulting in
hyperinsulinemia. Finally, when insulin can no longer fully compensate for glucose clearance, hyperglycemia occurs and pancreatic β-cells eventually dysfunction (19).

2.2 The effects of NEFAs on glucose homeostasis

As aforementioned, both type 2 diabetes and obesity are strongly associated with insulin resistance. While virtually all people with type 2 diabetes are insulin resistant, most are also obese (21), suggesting a causal role for excess adiposity in the development of insulin resistance. Elevated NEFAs associated with obesity and type 2 diabetes and are perhaps the most critical effectors in the modulation of insulin sensitivity (8) (Fig. 2.1). NEFAs are released from the adipose during fasting to provide a source of energy for other tissues. Normal fluctuations in circulating NEFA concentrations result in impaired insulin sensitivity with in three hours of increased NEFA (22). NEFA-induced insulin resistance, acute or chronic, reduces glucose uptake in both muscle and adipose and promotes hepatic gluconeogenesis, all of which contribute to increased levels of circulating glucose (22). NEFAs, thus, facilitate the metabolic shift from the utilization of glucose as energy to fatty acids in most tissues, sparing glucose for those tissues which are dependent on it, such as the central nervous system. This effect is usually transient and compensated for by an acute increase in pancreatic insulin production stimulated by NEFAs. However, increased adiposity generates a chronic state of elevated NEFAs leading to sustained insulin resistance resulting in decreased glucose uptake,
increased glucose output, and eventual destruction of pancreatic β-cells (23). Additionally, insulin resistance in the adipose tissue results in increased lipolysis and the release of more NEFA, exacerbating insulin resistance.

Figure 2.1. Effects of NEFAs on systemic insulin resistance (24).
2.2.1 Glucose uptake in skeletal muscle

Skeletal muscle accounts for over 80% of glucose uptake in the body (25). However, in individuals with type 2 diabetes, glucose uptake can be significantly ameliorated by 30-40% (26). Skeletal muscle, therefore, is a major contributor to impaired glucose tolerance. Additionally, skeletal muscle is a major site for β-oxidation of fatty acids. In 1963, Randle and colleagues (27) first demonstrated that the competition between NEFAs and glucose as energy substrates for the muscle could contribute to insulin resistance. They showed that increased NEFA concentrations promoted fatty acid oxidation rather than glycolysis, which was associated with decreased insulin-stimulated glucose. The current paradigm expands on Randle’s glucose-fatty acid cycle: McGarry (28) first postulated that increased malonyl-CoA concentrations suppressed carnitine palmitoyltransferase-1 (CPT-1) activity, thereby decreasing fatty acid oxidation and increasing lipid accumulation which contributed to insulin resistance. Specifically, the increased availability of NEFAs causes acetyl-CoA to accumulate from increased β-oxidation. Acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-coA allosterically inhibits carnitine palmitoyltransferase-1, thus reducing the transfer of long-chain fatty acyl-CoA (LCFA-CoA) into the mitochondria for β-oxidation. The resulting accumulation of LCFA-CoA can be esterified into diacylglycerol (DAG). DAG allosterically activates protein kinase C theta (PKCθ). PKCθ phosphorylates insulin receptor substrate-1 (IRS-1) on serine/threonine residues. This alternative phosphorylation (as opposed to phosphorylation of a tyrosine residue) interferes with the association of IRS-1 with the
insulin receptor, thus decreasing activation of phosphatidylinositol 3-kinase (PI3-K) and the subsequent insulin signaling cascade. Diminished insulin signaling decreases translocation of the glucose transporter GLUT4 to the plasma membrane, ultimately reducing glucose uptake.

2.2.2 Endogenous glucose production in liver

While muscle utilizes glucose for its expansive energy requirement, the liver accounts for nearly 85% of endogenous glucose production (29). Endogenous glucose production is reported to be 25% higher in individuals with type 2 diabetes (30; 31). Importantly, increased endogenous glucose production, rather than decreased glucose uptake, accounts for most of the elevated fasting glucose levels in people with type 2 diabetes (19). While the mechanisms of insulin resistance in the liver are still being elucidated, Bays and colleagues (32) proposed the following model by which NEFAs contribute to hepatic insulin resistance and dysregulated hepatic glucose production. 1.) NEFA increase gluconeogenesis and glucose release: The increased availability of NEFAs drives β-oxidation resulting in increased acetyl-CoA. Acetyl-CoA stimulates pyruvate carboxylase (PC) and phosphoenolpyruvate carboxylase (PEPCK), rate-limiting enzymes for gluconeogenesis (33-35). Acetyl-CoA also stimulates glucose-6-phosphate activity which controls the release of glucose from the hepatocyte (36). 2.) NEFAs provide energy for gluconeogenesis: The increase in β-oxidation increases ATP production and reduces NADH, which provide energy to drive gluconeogenesis. 3.) NEFAs induce hepatic insulin resistance: Similar to muscle, increased NEFAs in the
liver ultimately result in the inhibition of insulin signaling. In the liver, increased DAGs activate PKC to alternatively phosphorylate IRS-1 and ultimately reduce insulin signaling (37-39).

2.2.3 Lipolysis in adipose

Insulin resistance in adipose results in the relief of inhibition of lipolysis. Lipolysis is the sequential hydrolysis of the ester bonds in triglycerides. Several different lipases participate in lipolysis including adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and others. The degradation of triglycerides by lipolysis yields NEFAs (and glycerol) for release into circulation to be used as energy for other tissues (40; 41). Lipolysis is primarily regulated by catecholamines and insulin, which stimulate or inhibit lipolysis, respectively. Activation of β-adrenergic receptors by catecholamines, such as epinephrine or norepinephrine, results in the increase of cyclic AMP (cAMP) and sequential activation of PKA and HSL. Insulin, however, activates phosphodiesterase 3B (PDE3B) which degrades cAMP to AMP, preventing the activation of PKA and HSL, thus inhibiting lipolysis (42). Without suppression of lipolysis by insulin, as in an insulin resistant state, lipolysis increases, releasing more NEFAs, further exacerbating insulin resistance.

2.2.4 Insulin secretion by the pancreas

The secretion of insulin by pancreatic β-cells is regulated by numerous nutrients, neurotransmitters, and hormones (43). Glucose is the main nutrient regulating the
secretion of insulin; however, NEFAs stimulate insulin release in response to glucose and non-glucose secretagogues. The stimulated release of insulin by NEFAs may be mediated by activation of intracellular signaling subsequently inducing a release of intracellular calcium and insulin granule exocytosis (44). Alternatively, fatty acyl-CoA generated from NEFAs can directly simulate secretory granule exocytosis or indirectly through the release of intracellular calcium by activation of PKC (45). While NEFAs are essential for normal insulin release, chronic exposure to NEFAs coupled with elevated glucose are detrimental to β-cell function and can cause β-cell apoptosis (45). The progressive decline in β-cell health and increased apoptosis culminates in the reduction of insulin production.

2.3 Adipose tissue

Over 80% of people with type 2 diabetes are obese and nearly all have insulin resistance (21). A causal link between increased adiposity and the development of insulin resistance has been established as weight loss or gain positively correlates with the attenuation or development/worsening of insulin resistance (46; 47). Adipose was traditionally considered a storage depot for excess energy in the form of lipid. However, now adipose is recognized as a metabolically active organ with substantial endocrine functions (48). Numerous adipose-derived factors are now known to provide an important link between adiposity and insulin resistance. Of them, NEFAs, which were described previously, the adipokines leptin and adiponectin, and inflammation are perhaps the best studied and have the strongest associations with insulin resistance.
2.3.1 Non-esterified fatty acids

NEFAs have been proposed to induce insulin resistance by altering lipid metabolism to increase levels of intracellular diacylglycerol (DAG). Increased DAG activates protein kinase C (PKC) which phosphorylates IRS-1 on a serine residue instead of the tyrosine residue. This alternative phosphorylation reduces the ability of IRS-1 to mediate downstream events resulting in decreased insulin signaling (49).

2.3.2 Adipokines

Since the discovery of leptin in 1994 (50) and the many adipokines subsequently identified, the adipose tissue is now considered an endocrine organ rather than a lipid storage depot. Of the many adipokines that contribute to the regulation of glucose homeostasis (Fig. 2.2), leptin and adiponectin are perhaps the best described.

Figure 2.2. Effects of adipose-derived proteins on glucose homeostasis (24).
i. Leptin

In 1994, Friedman’s group (50) first identified leptin as the adipose-secreted factor deficient in ob mice. Mutations in this gene and the gene for the leptin receptor result in obesity, hyperphagia, hyperlipidemia, hyperinsulinemia, and insulin resistance. Subsequent studies by Friedman’s group (51) and others (52) solidified the role of leptin regulating adiposity and insulin sensitivity by normalizing these levels with exogenous leptin treatment in leptin deficient ob/ob mice. Leptin was first thought to regulate adiposity through the suppression of appetite, and while this is a major role of leptin, studies in pair-fed mice suggest other factors contribute to the mechanisms by which leptin reduces or prevents adipose accretion (51). Similarly, the improvement in hyperglycemia by leptin may not be due to the reduction of adipose tissue, as leptin improves insulin resistance within hours of administration and much before significant weight reduction (52) and in lipodystrophic mice (53). Rather, the improvement in hyperglycemia by leptin may be mediated by a reduction in both intramuscular and hepatic lipid levels, improving insulin sensitivity and thus enhancing glucose uptake into the muscle and inhibiting gluconeogenesis in the liver. The reduction of intra-tissue lipid is most likely through direct activation of AMP-activated kinase (AMPK) by leptin (54). Activation of AMPK increases fatty acid oxidation and glucose uptake and inhibits lipogenesis and hepatic glucose production, overall contributing to the reduction of lipid and circulating glucose.
**ii. Adiponectin**

Shortly after the discovery of leptin, another adipose-secreted hormone that contributed to energy expenditure was identified by Scherer and colleagues in 1995 (55) and subsequently by other groups (56). Like leptin, adiponectin enhances lipid oxidation and insulin sensitivity in the liver and muscle by activating AMPK. The induction of adiponectin by thiazolidinediones (TZDs), a class of anti-diabetic drugs, is required for the improvement of insulin sensitivity by TZDs. Furthermore, Scherer’s group (48) showed that the overexpression of adiponectin in leptin-deficient ob/ob mice resulted in complete normalization of glucose clearance, fasting insulin levels, and β-cell function. The increased adiponectin also contributed to enhanced clearance of triglycerides and reduced ectopic lipid accumulation. Interestingly, these mice were considerably larger than their littermates (48). The increased adiposity induced by the overexpression of adiponectin may, paradoxically, contribute to the increased insulin sensitivity by promoting lipid accumulation in the adipose rather than ectopically.

**2.3.3 Inflammation**

Both insulin resistance and obesity are associated with inflammation marked by increased levels of TNFα, IL-6, and other cytokines produced by the adipose tissue. A causal relationship between inflammation and insulin resistance was established by reports showing administration of TNFα impaired insulin action in mice (57), and conversely, mice lacking TNFα or its receptor have improved insulin sensitivity (58). However, treatment of insulin resistant individuals with TNFα-neutralizing antibodies did
not result in improvement of insulin sensitivity (59). This, combined with the low expression of TNFα, suggests that TNFα may act primarily in a paracrine rather than endocrine manner. Appropriately, TNFα inhibits lipogenesis and increases lipolysis in the adipose contributing to the mobilization of lipid and an increase in NEFA, which exacerbates insulin resistance. TNFα directly contributes to insulin resistance by impairing insulin signaling. TNFα, similarly to NEFA, stimulates phosphorylation of insulin receptor substrate-1 (IRS-1) on serine residues instead of tyrosine. This alteration reduces the ability of IRS-1 to associate with the insulin receptor resulting in decreased insulin signaling (60).

TNFα also induces IL-6 release from adipocytes (61). As opposed to TNFα, IL-6, produced by adipose and activated macrophages, circulates and induces systematic effects on peripheral tissues, such as muscle and liver (62). Serum plasma IL-6 concentrations correlate positively with BMI, hyperinsulinemia (63), and insulin resistance (64), it may not be the cause of these disturbances. This is evident by research showing treatment with IL-6 did not impair insulin signaling or induce insulin resistance in rats (65). Equally, IL-6 knockout mice became obese and had reduced glucose tolerance (66). This suggested IL-6 may have a protective role in the development of obesity and insulin resistance.

Increased adiposity also begets the recruitment of macrophages to the adipose tissue by inducing macrophage chemoattractant protein-1 [MCP-1; also known as CC chemokine ligand 2 (CCL-2)] (67). Targeted overexpression of MCP-1 in the adipose of mice resulted in increased levels of macrophage infiltration, inflammation (marked by
TNFα and IL-6), and plasma NEFA. These mice had increased hepatic glucose output and decreased glucose uptake by muscle suggesting the development of insulin resistance (68). The co-localization of adipocytes and macrophages likely contributes to a concerted production of cytokines that leads to and worsens insulin resistance.

2.4 Alteration of adipose metabolism

A reduction in adipose mass is generally understood to contribute to the improvement of insulin sensitivity. While adipose releases a number of factors that modulate whole body metabolism and glucose homeostasis, it can also modulate energy balance through alterations in its own metabolism. While increased fatty acid oxidation consumes substrate, to be effective in weight reduction the ATP generated must be utilized or the process halts. Instead of conserving the energy of fatty acids as ATP, uncoupling of oxidative phosphorylation dissipates the energy as heat resulting in complete expenditure of energy. This process has been implicated as a possible mechanism to drive adipose depletion. In adipose, especially brown adipose tissue, as well as other tissues, a family of proteins, appropriately named uncoupling proteins (UCPs) mediate this energy expenditure.

2.4.1 Uncoupling proteins

Uncoupling proteins are a collective group of at least five proteins (UCP-1, UCP-2, UCP-3, UCP-4, UCP-5) that share similar homology (69). While the role of UCP-1 in adaptive thermogenesis is undisputable, the functions and ‘uncoupling’ abilities of the other UCPs are less definitive. UCPs generate a proton leak which can uncouple ATP
production from mitochondrial fatty acid oxidation and respiration (69). The energy, which would usually drive ATP synthesis, is dissipated as heat (Fig. 2.3). While the production of heat is important in thermogenesis, the energy wasting effect of UCP-1 and UCP-2 found in adipose tissues may contribute to alterations of lipid metabolism and adiposity.

Figure 2.3. Role of UCPs in the uncoupling of oxidative phosphorylation.
i. UCP-1

UCP-1 is a 32 kDa protein exclusively expressed in brown adipose tissue and is responsible for adaptive, or non-shivering, thermogenesis (70). Additionally, UCP-1 is induced by feeding a high-fat diet to rodents, suggesting a role for UCP-1 in energy homeostasis (71). In 1993, Lowell et al (72) showed that ablation of brown adipose tissue (and UCP-1) caused mice to become obese. A subset of mice, in which brown adipose tissue regenerated, lost weight, suggesting a protective role of UCP-1 against obesity. However, subsequent studies showed that UCP-1 deficient mice did not become obese as expected (73; 74). Liu and colleagues (74) postulated that the compensatory mechanisms required to maintain body temperature, for example shivering, were energetically more costly and prevented significant weight gain. Conversely, the transgenic overexpression of UCP-1 in white adipose tissue, driven by the aP2 promoter, protected genetically obese mice against obesity (75). The prevention of obesity by the overexpression of UCP-1 was later determined to, in fact, be due to increased mitochondrial uncoupling (76). This group also demonstrated that only small amounts of ectopic expression of UCP-1 in white adipose tissue was required to have significant effects on mitochondrial uncoupling and the reduction of adipose mass (76). Several groups have demonstrated that UCP-1 expression is ectopically induced in white adipose tissue by stimulation of \( \beta_3 \)-adrenergic receptor (\( \beta_3 \)AR) signaling with a pharmacological agent, CL-316,243, and the increased expression is concomitant with reduced body weight and adipose mass (77-81). Inokuma et al (80) demonstrated that UCP-1 knockout mice treated with CL-316,243 were resistant to its adipose lowering effect suggesting
β3AR-dependent depletion of adipose was due to UCP-1 function. Furthermore, with β3AR stimulation the, usually unilocular, white adipose tissue developed a more multilocular morphology (77-79; 82; 83) and had increased mitochondrial biogenesis (82; 83)—traits generally indicative of brown adipose tissue. The adipose depletion effect of UCP-1 is not limited to increased uncoupling. Stimulation of β3AR and subsequently increased UCP-1 may also affect lipid accumulation in white adipose tissue by impairing adipogenesis through depression of PPARγ and aP2 (84). Interestingly, Unger’s group reported that adenovirus-induced hyperleptinemia in rats not only had reduced adipose mass, but the adipocytes in the white adipose tissue were shrunken and laden with mitochondria. They found evidence of increased fatty acid oxidation couple with increased expression UCP-1 and PGC-1α, a coactivator involved in the upregulation of UCP-1 and mitochondrial biogenesis (85). The induction of UCP-1 by leptin in white adipose tissue was later found to be dependent on β3AR signaling most likely activated by the sympathetic nervous system (86). In addition to the stimulation of β3AR signaling by the sympathetic nervous system, UCP-1 is directly activated by fatty acids; however, the mechanisms by which this occurs are unclear. One proposal is that fatty acids, themselves, might cause non-specific mitochondrial uncoupling (87). Others suggest that UCP-1 is a fatty acid cyclcer, returning anionic fatty acids to the intermembrane space of the mitochondria (88). Regardless, the relevance of these finding is questionable in humans. Although humans have an abundance of brown adipose at birth for temperature regulation, only relatively low levels are present in adults (89) translating into low detectable levels of UCP-1 expression in lean individuals and even lower levels in obese
people (90). Nevertheless, as mentioned before, several groups have suggested that human adult white adipocytes can be converted to brown adipose and express UCP-1 both \textit{in vitro} (91; 92) and \textit{in vivo} (93).

\textbf{Regulation of UCP-1:} UCP-1 is regulated primarily by activation of $\beta_3$-adrenergic signaling by norepinephrine released from the sympathetic nervous system in response to stimuli such as cold exposure or diet (94-96) (Fig 2.4). This activation initiates a cascade of events beginning with the production of cAMP and subsequent activation of protein kinase A (PKA). PKA phosphorylates both CREB and p38 mitogen-activated protein kinase (MAPK). Recently, Collins group (97; 98) demonstrated that phosphorylated-p38 MAPK (P-p38 MAPK) phosphorylates both ATF2 and PGC-1$\alpha$ and is a critical step in this signal transduction. The transcriptional regulation of UCP-1 is coordinated through several regulatory sites on the UCP-1 promoter and enhancer regions. Interestingly, these regulatory elements are also critical in adipogenesis. A highly conserved PPRE in the distal UCP-1 enhancer forms a complex with the PPAR$\gamma_2$-RXR heterodimer and the PGC-1$\alpha$ coactivator (99; 100). In the proximal promoter and distal enhancer regions, one or more of several cAMP response elements (CRE) interacts with cAMP response binding protein (CREB) and ATF2 (101; 102). Importantly, ATF2 also increases transcription of PGC-1$\alpha$ (97). $\beta$-adrenergic activation of PKA also stimulates lipolysis and the release of free fatty acids to serve as substrate and allosteric activators of UCP-1 (103-105).
Figure 2.4. $\beta_3$-adrenergic signaling of UCP-1 transcription (97).
**ii. UCP-2**

UCP-2 is a 33 kDa protein, ubiquitously expressed in many tissues, and shares 56% homology with UCP-1 (106; 107). Initially, like UCP-1, UCP-2 was thought to have a role in mediating energy expenditure possibly through diet-induced thermogenesis (106). Collins’ group (106) observed that A/J mice had upregulated mRNA expression of UCP-2 and were resistant to diet induced obesity. Conversely, B6 mice developed obesity with no change in UCP expression. However, no research exists that establishes a definitive link between UCP-2 and energy homeostasis (reviewed in (69)); although several studies do support an inverse relationship between UCP-2 expression in white adipose tissue and the accumulation of fat (90; 106; 108). Additionally, several groups have suggested UCP-2 regulates insulin secretion (109-111). Chan et al (109) first showed that overexpression of UCP-2 increased glucose-induced insulin secretion in vitro. Zhang and colleagues (111) observed that UCP-2 deficient mice secreted more insulin in response to glucose and that ablation of UCP-2 in ob/ob mice impaired the development of insulin resistance. Additionally, Tsuboyama-Kasaoka et al (15) proposed that a decrease in ATP due to UCP-2 induction by TNFα may contribute to global loss of cell membrane integrity and cell death by necrosis and/or apoptosis.
2.5 Conjugated linoleic acid

2.5.1 Structure and synthesis

i. Structure

Conjugated linoleic acid (CLA) refers to a group of positional and geometric conjugated dienoic isomers of octadecadienoic acid [18:2(n-6)], or linoleic acid. Sixteen naturally occurring isomers have been identified with conjugated double bonds of varying combinations of cis and trans configurations found ranging from the Δ^7,9 to the Δ^11,13 positions. Double bonds in the majority of polyunsaturated fatty acids (PUFAs) are usually separated by a methylene group (CH₂). However, conjugated double bonds are not separated by a methylene group, giving the isomers of CLA unique spatial structures, which may translate into distinctive biological functions. The cis-9, trans-11 (c9t11) and trans-10, cis-12 (t10c12) isomers (Fig. 2.5) are perhaps the most well-studied CLA isomers because of their unique biological functions.
Figure 2.5. Structures of CLA. Structures of t10c12-CLA (upper), c9t11-CLA (middle) and linoleic acid (lower) (112).
ii. Synthesis

**Biosynthesis:** Naturally occurring CLA is predominantly found in products derived from ruminants, such as beef, lamb, and dairy products. As such, CLA is an intermediate product of biohydrogenation of linoleic [18:2 (n-6)] or linolenic [18:2 (n-3)] acids by anaerobic, ruminant bacteria, in particular *Butyrivibrio fibrisolvens*. *B. fibrisolvens* possesses linoleate isomerase, a unique enzyme that can isomerize linoleic acid to c9t11-CLA (113), the predominant isomer (80-90%) in ruminant products (ref parodi 1977) and often designated rumenic acid (114). However, a variety of bacteria and bacterial isomerases are proposed to be involved in the formation of the extensive range of CLA isomers. Diet, particularly seasonal fluctuation of the diet of bovines, can influence the amount of CLA—producing more in summer months when on pasture and less when fed concentrates in the winter. Unbeknownst at the time, researchers observed this in 1932 when this novel fatty acid was first discovered (115).

**Endogenous synthesis:** Although CLA is thought to be derived primarily by absorption of the intermediate products of microbial biohydrogenation, it can also be synthesized from the delta-9 desaturation of *trans*-11 octadecenoic acid, also called *trans* vaccenic acid (TVA). Two independent studies were published at nearly the same time in 1980 describing the desaturation of TVA to isomers of 18:2 conjugated denoic acids by delta-9 desaturation in microsomal preparations of rat liver (116; 117). Nearly 2 decades later, Santora et al (118) reported that feeding TVA to mice increased the amount of CLA in the carcass. They attributed this to endogenous synthesis in the adipose tissue as the
resulting CLA was found in the triglycerol, but not the phospholipid fraction. Later, the same year, Bauman’s group (119) reported that feeding TVA to dairy cows increased levels of CLA in milk fat, and the addition of a delta-9 desaturase inhibitor significantly blunted that effect. They estimated 64% of the CLA in milk fat was derived from endogenous synthesis, but later reported 91% of CLA is due to endogenous synthesis in pasture-fed cows (120). Endogenous synthesis of CLA from delta-9 desaturation of TVA has also been reported in rats (121) and humans (122).

**Chemical synthesis:** The derivation of CLA from linoleic acid by alkali isomerization initially produced a poorly defined blend of isomers. However, the trends in CLA research and production of CLA as a human supplement have demanded essentially pure preparations of c9t11-CLA, t10c12-CLA, or an equal mixture of both. By using oils with high concentrations of linoleic acid, such as sunflower and safflower oil, and by modulating the kinetics of the reactions involved with the synthesis of CLA, preparations of specific isomers are available virtually devoid of contaminating isomers (123).

**2.5.2 Metabolism**

In 1995, Banni and colleagues detected not only the absorption and assimilation of CLA in liver tissues of rats, but also the accumulation of conjugated linolenic (18:3) and eicosatrienoic (20:3) acids. They suggested that these isomers were likely derived from the desaturation and elongation of CLA (124). Continued work by Banni
demonstrated CLA was preferentially incorporated into the neutral lipid fraction—more similar to oleic acid (18:1) than linoleic acid, which usually integrates into phospholipids (125). This occurs perhaps because the conjugated dienoic structure of CLA more closely resembles oleic acid (126). The actual metabolism of CLA, however, is comparable to linoleic acid, undergoing a similar pattern of δ6 desaturation, elongation, and δ5 desaturation, while maintaining the conjugated dienoic structure (Fig. 2.6). Unlike linoleic acid, though, the conjugated dienoic (CD) metabolites, CD 18:2, CD 18:3, and CD 20:3, accumulated in the neutral fraction (125). Conversely, the presence of CD 16:2 and CD 16:3 suggested that CLA and the metabolites of CLA were able to undergo partial β-oxidation (127).

Figure 2.6. Metabolism of t10c12- and c9t11-CLA (126)
2.5.3 Physiological Effects

While CLA was first discovered in the early 1930’s (115), it was not until the late 1980’s that CLA received attention for being more than a structurally novel fatty acid. In 1987, Ha and colleagues isolated the fraction of fried ground beef that inhibited mutagenesis in bacteria. The fraction contained 4 isomers of CLA that subsequently reduced tumor incidence in mice chemically treated to induce epidermal neoplasia (128). This seminal discovery catalyzed a profusion of research, which has produced well over 1300 research articles in the last two decades (129). Since then a number of physiological effects have been attributed to CLA including: the reduction or inhibition of carcinogenesis and atherosclerosis and the modulation of immune function, body composition—in particular adiposity, and factors associated with diabetes (130; 131). This review will focus on adiposity and diabetes.

i. Adiposity

Animal studies: Park et al first reported dietary CLA altered body composition in 1997. Dietary supplementation of 0.5% mixed isomer CLA (MI-CLA), containing approximately a 50:50 ratio of c9t11 and t10c12, significantly reduced body fat mass and increased lean body mass in ICR mice. They attributed this alteration to enhanced fatty acid oxidation in adipose and skeletal muscles and increased lipolysis in adipocytes (12). Since then, CLA has been reported to reduce adiposity in a variety of different animal models including mice, rats, pigs, chickens, and hamsters (132). Nearly all mouse models lose adipose mass in response to supplementation with CLA, including mice that
develop diet induced obesity (primarily C57BL/J (133)) and mice that are genetically obese such as the ob/ob mouse (13; 134). Conversely, while reductions of adipose mass have been observed in Zucker Diabetic Fatty (ZDF) (10), lean Zucker (135), and Sprague Dawley (136) rats, studies in Wistar (137) and obese Zucker rats (135) have reported no change or an increase in adiposity. Furthermore, changes in adipose mass in mice seem to be the most dramatic—with reductions up to 70% compared to ~25% in rats.

While the reduction of adipose occurs with supplementation of MI-CLA, several studies have demonstrated the t10c12 isomer is responsible for this effect. Shortly after their observation that CLA altered body composition, Park et al (9), using single isomer preparations of either c9t11 or t10c12, showed that the reduction of adipose was associated with the t10c12 isomer. Similarly, Ryder et al (138) corroborated this finding in ZDF rats, reporting that rats fed a MI-CLA diet, but not a c9t11 diet, had significantly reduced adiposity. They also demonstrated that the reduction of adipose mass was independent of alterations of food intake by using a group of pair-fed rats. Others confirmed the t10c12 isomer is responsible for adipose depletion (139), with both in vivo (140; 141) and supporting in vitro work (142-144), and that this effect is likely independent of food intake (17).

**Human studies:** The weight reducing effects of CLA in humans are not as dramatic or consistent as rodent studies. Most short-term studies report no changes in body weight or fat mass, if measured (145). Several studies do, however, report differences in body composition: MI-CLA supplementation ranging from 1.8 – 6.8 g/day
decreased fat mass, but did not change body weight in healthy exercising humans (146) or overweight subjects (147). Another study also reported no change in body weight, but did observe reductions in sagittal abdominal diameter in obese men (148). Long-term studies have reported higher lean body masses in 12 months (149) and decreases in fat mass maintained over 24 months (150). As aforementioned, the depletion of body fat in humans is less dramatic (~40-50% less body weight loss) than in mice (145). This discrepancy may be because mice receive experimental diets with almost five times more CLA than humans would (per kilogram body weight) (151). Most rodent feeding studies also occur in the growth stage rather than mature adults suggesting the ability of CLA to reduce adipose mass may be dependent on level of maturity. Additionally, ~28% of fat stores of mice are used for basal metabolism, whereas it is much lower for humans. Although the data supporting CLA induces weight reduction in humans are less than convincing, MI-CLA is marketed as a widely-available, weight-loss supplement (e.g. Tonalin™).

**ii. Diabetes**

*Animal studies:* In 1998, Belury’s group (10) first reported 14-days of 1.5% MI-CLA dietary supplementation normalized impaired glucose tolerance and improved hyperinsulinemia and elevated free fatty acids in ZDF (fa/fa) rats, a model for type 2 diabetes. These results were comparable to ZDF rats treated with troglitazone, an insulin-sensitizing thiazolidinedione, in the same study. Several studies thereafter also demonstrated CLA improves glucose tolerance and insulin sensitivity, as well as fasted
glucose and insulin levels in rat models of obesity and diabetes (138; 140; 152-156). A subsequent study by Ryder et al (138) demonstrated that, like adiposity, improved glucose tolerance, fasting glucose, and hyperinsulinemia could be attributed to the t10c12-CLA isomer. Nagao and colleagues (153) first observed that plasma adiponectin levels increased parallel to improved hyperinsulinemia in ZDF rats.

Conversely, the effects of CLA on indicators of type 2 diabetes in mice are quite the opposite of those seen in rats. In 1999, just one year after Belury’s group identified the potentially anti-diabetic property of CLA, Delany et al (17) observed that AKR/J mice fed varying levels of MI-CLA had a dose-dependent increase in insulin levels, with the highest dose (1.0%) being significantly higher than control. This trend accompanied trends of reduced body and adipose masses and also decreased plasma leptin. Tsuboyama-Kasaoka et al (15) subsequently reported that C57BL/6J mice also fed 1.0% MI-CLA developed characteristics resembling lipodystrophy. Mice lost significant adipose mass accompanied by hyperinsulinemia, insulin resistance, hepatic steatosis, and depletion of the adipokine leptin. They further demonstrated that administering exogenous leptin to mice the last 12 days of an 8 month-long feeding period, ameliorated hyperinsulinemia and hepatic steatosis as observed by histology. Tsuboyama-Kasaoka and colleagues (157) later showed that by increasing the fat level in the diet, CLA-fed C57BL/6J mice retained adipose mass, but did not have significantly reduced levels of leptin nor increased insulin. Despite the significant adiposity of ob/ob mice, CLA still worsens insulin resistance in this model of obesity and diabetes (13). However, Wargent et al (134) later reported that CLA initially decreased, but later, after 10 weeks of
supplementation, actually improved insulin sensitivity. Subsequent studies confirmed that CLA induced hyperinsulinemia and insulin resistance in mice (13-17). Several studies also support the associated depletion of the adipokines leptin and adiponectin and the incidence of hepatic steatosis with the development of insulin resistance (158). Poirier et al (18) showed the significant depletion of adipokines occurred rapidly—within two days of CLA treatment, and preceded significant alterations in body weight, adipose mass, hepatic steatosis, and insulin levels. They also reported pancreatic β-cell hyperplasia occurred in these C57BL/6J mice fed CLA for four weeks, accounting for the increased insulin production. An ensuing study also conducted by Poirier et al (159) demonstrated that inflammation, marked by increased mRNA levels of TNFα and IL-6, and macrophage infiltration, indicated by mRNA expression of MCP-1, CD68, and F4/80, occurred simultaneously with the depletion of adipokines and development of hyperinsulinemia. Poirier and colleagues (158) proposed the following progression of events in the development of insulin resistance and lipodystrophy (Fig. 2.7). The decrease in adipose by CLA depletes adipokines resulting in insulin resistance and compensatory β-cell hyperplasia. Hyperinsulinemia ensues, increasing lipid accretion in the liver resulting in hepatic steatosis. Paradoxically, the attenuation of insulin resistance in rats may result from a modest decrease in adiposity, but the occurrence of insulin resistance in mice is likely a consequence of dramatic depletion of adipose mass and the subsequent development of lipodystrophy.
Figure 2.7. Working model of chronology of lipodystrophy induced by CLA in mice. 1) decrease in fat stores; 2) adipokine-mediated insulin resistance; 3) beta-cell proliferation; 4) hyperinsulinemia; 5) liver steatosis (158).
**Human studies:** The effects of CLA on insulin resistance and associated factors are similarly controversial in humans. In 2001, Riserus et al reported obese men treated with 4.2 g/day MI-CLA for four weeks had reduced sagittal diameter, but did not altered fasting glucose or insulin levels (148). The following year they reported obese men treated for 12 weeks with 3.4 g/day t10c12-CLA, but not MI-CLA, had increased insulin resistance and fasting glucose levels (160) demonstrating an t10c12 isomer-specific effect. However, a subsequent study in obese men demonstrated that 3 g/day c9t11 for 3 months reduced insulin sensitivity (161). Other studies have reported no changes in insulin or glycemia (162), including two long-term studies lasting 12 (149) and 24 (150) months. Conversely, in young, sedentary subjects, 8 weeks treatment of 4 g/day MI-CLA increased insulin sensitivity; however the authors noted high variability among subjects (163).

2.5.4 Mechanisms of action

i. Anti-diabetic mechanisms

   The reduction in adiposity by CLA is not as impressive in rats as it is in mice, yet the modest decrease in adipose tissue likely contributes to improved glucose tolerance and insulin sensitivity. The increased insulin sensitivity results in increased glucose transport into the muscle. Ryder et al (138) first reported insulin-stimulated glucose uptake could be enhanced by MI-CLA in skeletal muscle. They did not, however, find any changes in phosphatidylinositol 3-kinase activity or protein kinase B (PKB; also known as Akt) activity in muscle, which are associated with insulin signaling.
Subsequent reports showed that insulin-stimulated glucose uptake was increased by CLA in both type I and type II muscle fibers (140) and was associated with lower triglyceride levels in muscle (140; 152). The increase in glucose uptake by rats fed CLA may be a result of increased levels of the glucose transporter GLUT-4 (155). Recently, Noto et al (155) suggested CLA supplementation preserved pancreatic function in rats, as evident by reduced pancreatic islet size in fa/фа Zucker rats, which was associated with reduced insulin and improved glucose tolerance.

Nagao and colleagues showed that, contrary to mice, CLA increased both adipose mRNA expression and circulating levels of the insulin-sensitizing adipokine adiponectin (153; 164). However, other studies report that another insulin-sensitizing adipokine, leptin, is decreased by CLA in rats (136; 141; 153; 165). Reduced inflammation by CLA in rats may also contribute to improved insulin sensitivity. Koba et al (166) reported a significant correlation between reduced adipose mass and reduced TNFα levels in Sprague-Dawley rats fed CLA. Additional reports support an association among reduced adipose, improved insulin sensitivity, and a reduction in inflammation, particularly TNFα (136; 155; 156; 167).

**ii. Delipidation**

The reduction of adipose by CLA most likely occurs through a combination of mechanisms in the adipose tissue including: 1. decreased proliferation and differentiation
of preadipocytes; 2. apoptosis; 3. increased lipolysis; 4. decreased lipogenesis; 5. increased fatty acid oxidation and 6. increased energy expenditure.

**Proliferation and differentiation of preadipocytes:** In 1999, Satory et al (168) first demonstrated CLA dose-dependently inhibited proliferation in 3T3-L1 preadipocytes, a commonly used cell model that can be induced to differentiate into lipid accumulating adipocytes. Unexpectedly, they also showed that CLA promoted lipogenesis and the accumulation of lipid in mature adipocytes, suggesting CLA decreased adipose mass by inhibiting proliferation of preadipocytes. Shortly after, Brodie and colleagues (169) reported that CLA also dose-dependently inhibited differentiation of 3T3-L1 which was marked by the reduction of lipid accumulation and a decrease in mRNA expression of peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer binding protein alpha (C/EBPα), and adipocyte specific protein 2 [aP2; also known as fatty acid binding protein 4 (FABP4)]. These proteins are involved in the differentiation of preadipocytes and are used as markers specific to differentiated adipocytes. Numerous studies have confirmed that CLA inhibits differentiation associated with decreased lipid accumulation and decreased expression of PPARγ expression both in vitro (143; 170-173) and in mice (15; 159; 174-177) and rats (178). In addition to suppressed expression of PPARγ, McIntosh’s group (143) demonstrated that both the t10c12 and c9t11 isomers of CLA inhibited ligand dependent activation of PPARγ. The group further demonstrated the suppression of PPARγ was dependent on the activation of NFκB and subsequent induction of IL-6 by CLA in human adipocytes (179).
However, as with adiposity and insulin resistance, the modulation of PPARγ by CLA is contradictory and appears to be specific to species and even tissue type. In 1998, Belury’s group (10) showed that CLA significantly increased aP2 mRNA expression in ZDF rats concomitant with a reduction in epididymal adipose mass. They also showed that 100 µM MI-CLA activated PPARγ in CV-1 cells as effectively as a thiazolidinedione, a known PPARγ agonist (180). The activation of PPARγ by as little as 50 µM CLA (specifically t10c12) was confirmed in COS-7 cells (134). A study in porcine SV cells showed CLA did not inhibit proliferation or differentiation, and no change in PPARγ was detected (181). Treatment of human preadipocytes with either the t10c12 or c9t11 isomer resulted in apparent decrease in proliferation but an increase in differentiation (182). Choi et al (183) reported differentiating 3T3-L1 cells treated with t10c12-CLA exhibited smaller lipid droplets but without any effects on adipogenic factors, specifically PPARγ. It should also be noted that CLA increases PPARγ expression in several different cell/tissue types including: colon (184); muscle (185); macrophage (186); and as will be discussed later, liver (14). While reporter assays suggest CLA is an agonist of PPARγ (10; 134), the differential modulation of PPARγ by CLA suggests there are precursory effects of CLA that may determine how PPARγ is regulated (such as CLA-induced activation of NFkB and increased IL-6 (179)).

**Apoptosis:** In 2000, two independent groups demonstrated CLA induced apoptosis in adipose (15; 187). McIntosh’s group showed CLA, specifically t10c12, induced apoptosis in 3T3-L1 preadipocytes. Tsuboyama-Kasaoka et al (15) reported the
reduction of adipose mass in C57BL/6J mice was associated with a significant increase in apoptosis in both white and brown adipose tissues. Liu and colleagues (133) found CLA increased the ratio of Bax/Bcl2 expression, which is indicative of increased apoptosis. Additional in vivo studies confirm the association of reduced fat mass and the induction of apoptosis in adipose tissue (176; 188; 189). However, subsequent work done by McIntosh’s group in human preadipocytes suggests CLA does not induce apoptosis (143; 171).

**Lipolysis:** In 1997, Park and colleagues (12) first reported that CLA increased lipolysis in 3T3-L1 adipocytes, which was later supported by additional studies in 3T3-L1 cells (9; 144; 183; 190). However, there is little evidence that CLA increases lipolysis in vivo. In fact, in vivo research suggested that CLA did not change or decreased lipolysis (177; 191; 192). Microarray analysis of white adipose tissue from mice fed t10c12-CLA showed decreased expression of genes involved with lipolysis such as, hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), lipoprotein lipase (LPL), and monoglyceride lipase (177). No change in lipolysis was detected in human SV cells with treatment of either c9t11 or t10c12 (193).

**Lipogenesis:** Park et al (12) were also the first to show that CLA dose-dependently decreased LPL activity in 3T3-L1 cells, suggesting CLA may subsequently inhibit lipogenesis in adipose. Numerous studies have reported decreased activity or expression of LPL (9; 15; 133; 143; 171; 192-195) associated with the reduction of other
markers of lipogenesis. Tsuboyama-Kasaoka et al (15) demonstrated that, in addition to LPL, CLA decreased adipose mRNA expression of sterol regulatory binding protein-1 (SREBP-1), its downstream target fatty acid synthase (FAS), and acetyl-coA carboxylase (ACC). While the dramatic depletion of adipose is observed in mice, a decrease in lipogenesis, as marked by depressed SREBP-1 and FAS mRNA expression, also occurs in rats (141). Roche et al (13) reported t10c12-CLA did not have an affect on SREBP-1 mRNA expression in ob/ob mice, but c9t11-CLA increased expression. Additional reports corroborate with decreased levels of FAS (172; 177) and ACC (177; 196). Microarray analyses show that all reported genes associated with lipogenesis were decreased in mice, of which ACC expression was strongly reduced (177). Several groups have also reported inhibition of (197) or reduced mRNA levels (183) of stearoyl-coA desaturase-1 (SCD-1) levels in 3T3-L1 cells. However, Kang et al (174) showed that CLA decreased adipose mass in SCD-1 null mice similar to wild-type and concluded the reduction of adiposity by CLA is not due to alterations in SCD-1. Conversely, lipogenesis was stimulated in the adipose of Wistar rats without altering body weight (198).

The effect of CLA on lipogenesis in the liver seems to be species-specific. In mice, which have dramatic ablation of adipose tissue and develop hepatic steatosis, markers of lipogenesis, such as SREBP-1 and SCD-1, tend to increase, possibly to due the mobilization of lipid from adipose (14; 199). Conversely, CLA decreases markers of lipogenesis in rats, which do not have as significant weight loss as mice (137; 141).
**Fatty acid oxidation:** CLA may also mediate delipidation by increasing fatty acid oxidation in adipose. Park et al (12) first demonstrated decreased adipose mass was associated with increased carnitine palmitoyltransferase-1 (CPT-1) activity in the adipose tissue. Both the c9t11 and t10c12-CLA isomers were shown to increase CPT-1 activity in the adipose of rats (165; 200). Another study, using radiolabeled fatty acids, showed that differentiating 3T3-L1 cells treated with t10c12-CLA, but not c9t11 or LA, had increased levels of oxidation (144). However, this same group later found that t10c12-CLA reduced fatty acid oxidation in human stromal vascular (SV) cells (143). A microarray analysis of adipose of obese mice showed that CPT-1a and CPT-1b were both increased by t10c12-CLA treatment (176). A separate microarray confirmed and further demonstrated that, of the genes involved with fatty acid oxidation reported, only CPT-1a and CPT-1b were increased with CLA treatment in mice (177).

CLA may contribute to the reduction of adipose mass by increasing markers of fatty acid oxidation in the liver and muscle. Park et al, (12) showed that, along with adipose, CPT activity increased in skeletal muscle. Rahman and colleagues (165) reported that CLA increased CPT activity in adipose, muscles, and livers of Otsuka Long-Evans Tokushima Fatty (OLETF) rats concomitant with reductions in adipose mass. Additional reports also found increased CPT activity in livers of mice and rats fed CLA (167; 175; 190; 201). Degrace et al (201) reported that hepatic acyl-coA oxidase (ACO) mRNA expression and associated peroxisomal oxidation activities were also increased by CLA in mice. Importantly, they concluded that CLA-induced hepatic steatosis was not
due to suppressed fatty acid oxidation in the liver. Others have also reported increased markers or activity of peroxisomal β-oxidation in the liver (137; 175; 202; 203), which may be a result of activation of PPARα by CLA (203).

**Energy expenditure:** In addition to oxidative phosphorylation, the energy created from the oxidation of fatty acids may be dissipated as heat. In 1998, West and colleagues (204) observed mice fed CLA had significantly increased metabolic rates. They later found that the increased energy expenditure was not likely through increased expression of uncoupling proteins (UCPs) (16). Conversely, Ealey et al (205) reported that CLA did not affect the expression of UCP in most tissues of rats but showed increased UCP-2 in most tissues of mice fed CLA. They concluded that increased UCP expression may contribute to the adiposity lowering effect of CLA in mice, but not likely in rats. Numerous studies have reported increased expression of UCP-2 in white adipose tissue (15; 138; 176; 177; 205; 206) associated with reduced fat mass. Interestingly, Peters and colleagues (206) found that CLA increased white adipose mRNA expression of UCP-1, which is usually exclusively expressed in brown adipose tissue. Two independent microarray analyses supported that CLA increased UCP-1 (and UCP-2) expression in white adipose of mice. Additionally, both reported UCP-1 as one of the greatest differentially expressed genes (176; 177). Ohnuki et al (207) observed that noradrenalin and adrenalin were increased in CLA-treated mice, simultaneous with increased energy expenditure, suggesting that CLA was mediating its effects through stimulation of sympathetic nervous activity, a major regulatory pathway for UCP expression.
t10c12-conjugated linoleic acid

Figure 2.8. Proposed model of adipose depletion by CLA (151).
iii. Lipodystrophy

The dramatic depletion of adipose tissue in mice fed CLA likely contributes to a lipodystrophic-like syndrome. Poirier and colleagues (158) proposed the following progression of events in the development of insulin resistance and lipodystrophy (Fig. 2.3). CLA-induced inflammation increases apoptosis and suppresses PPARγ and consequently adipogenesis. The decrease in adipose by CLA depletes adipokines resulting in insulin resistance and compensatory β-cell hyperplasia. Hyperinsulinemia ensues, increasing lipid accretion in the liver resulting in hepatic steatosis.

Inflammation: Pariza et al (208) first proposed that TNFα, an inflammatory cytokine, may be a key mediator in the effects of CLA. Tsuboyama-Kasaoka et al (15) showed that TNFα mRNA expression transiently increased in adipose tissue of mice fed CLA concomitant with the development of lipodystrophic-like effects. Others have also reported the simultaneous increase in inflammatory cytokines, such as TNFα and IL-6, with the development of lipodystrophy in mice (157; 159; 177). In cultures containing both human differentiated adipocytes and SV cells, Brown and colleagues (209) demonstrated that t10c12-CLA promotes NFκB activation subsequently increasing IL-6 and TNFα expression, which leads to the inhibition of PPARγ expression and consequently, adipogenesis. Induction of TNFα contributes to increased apoptosis. This overall results in the depletion of adipose mass.
**Adipokines:** CLA dramatically reduces levels of the adipokines leptin and adiponectin within days of treatment and is associated with increased insulin resistance, increased inflammation, and increased steatosis (18; 159). Several studies have manipulated the levels of these adipokines, either by model selection, diet adjustment, or exogenous supplementation to elucidate what roles leptin and adiponectin have in the development of insulin resistance and subsequent effects. Tsuboyama-Kasaoka et al (15) demonstrated that infusion of exogenous leptin supplementation in the last 12 days of an 8 month feeding study resulted in a decrease in plasma insulin levels and the attenuation of hepatic lipid accumulation. They later showed that by feeding high fat diet in combination with CLA, adipose and subsequent leptin levels were maintained resulting in normalized insulin levels and attenuation of liver weight gain (157). These two studies suggested an important role for leptin in the development of lipodystrophic-like insulin resistance and hepatic steatosis. Contrary to the addition of leptin, Roche et al (13) observed in an ob/ob mouse model, which lack functioning leptin, that mice fed t10c12-CLA still developed increased fasting glucose and insulin levels, but with no alteration in TNFα in white adipose tissue. Another study found similar results in ob/ob mice—fasting glucose, plasma insulin, and glucose tolerance were worsened after 2 weeks of CLA treatment (134). In contrast to the findings of Tsuboyama-Kasaoka et al (15; 157), these two studies demonstrated that CLA induced hyperinsulinemia and hyperglycemia in the absence of leptin, suggesting that leptin is not a crucial mediator in the development of lipodystrophic-like insulin resistance. Purushotham and colleagues showed that upon removal of CLA from the diet after 4 weeks of feeding, leptin and adiponectin serum
concentrations elevated and the mice became more insulin sensitive compared to when they were on a CLA diet (210). The second study in the same paper administered rosiglitazone (ROSI), a thiazolidinedione which is known to induce adiponectin, to mice fed a CLA diet. They found that CLA with ROSI supplementation increased adiponectin levels, did not worsen insulin resistance, and decreased hepatic steatosis (210). These studies suggested adiponectin may have a pivotal role in the lipodystrophic like effects mediated by CLA. Nevertheless, the depletion of adiponectin and leptin by CLA contribute to the development of insulin resistance.

**Hyperinsulinemia:** Poirier et al (18) reported glucose stimulated insulin secretion in pancreatic islets was in increased in mice fed CLA. They subsequently showed pancreatic β-cell mass and numbers were increased, indicative of β-cell hyperplasia. They postulated (158) that β-cell hyperplasia may be a compensatory response to increased insulin resistance, as has been shown in other models of insulin resistance (211; 212). In addition, CLA may be interfering with the PPARγ signaling pathway, thus inducing β-cell hyperplasia and subsequent hyperinsulinemia.

**Hepatic steatosis:** The development of hepatomegaly and/or hepatic steatosis has been observed simultaneously with increased insulin resistance in several studies (14; 15; 18; 133; 157; 210; 213). However, there is no evidence of localized inflammation as measured by histological analysis (15; 17) The promotion of lipid accretion in the liver may stem from ectopic induction of PPARγ and its target genes such as, aP2 and fatty
acid transporter (FAT/CD36), which are normally expressed in white adipose tissue (14). This phenomenon also occurs in fat-less A-ZIP/F-1 mice, a model of lipodystrophy that develops hepatic steatosis (214). CLA can also promote fat deposition in the liver by induction of lipogenesis. The increase in insulin by β-cell hyperplasia contributes to increased hepatic lipogenesis through induction of SREBP-1 (215). Several studies have not only showed CLA-induced SREBP-1 expression, but also increased activity and expression of its downstream target, FAS, and other enzymes involved in lipogenesis, such as ACC and stearoyl-coA desaturase-1 (SCD-1) (14; 15; 133; 199). Concomitantly, CLA induces both mitochondrial (199-201) and peroxisomal (216; 217) fatty acid oxidation in the liver. However, Takahashi et al (199) demonstrated that CLA significantly increased hepatic lipid levels simultaneously with increased expression of markers of both lipogenesis and fatty acid oxidation, indicating that the increased lipid accumulation is likely due to increased lipogenesis. Additionally, while both c9t11 and t10c12-CLA isomers are potent activators of PPARα (203), a transcription factor involved in the regulation of enzymes associated with hepatic oxidation, CLA-induced fatty liver is not dependent on PPARα activation (206). Furthermore, the depletion of adipokines may contribute to decreased fatty acid oxidation and increased lipogenesis. A recent study showed when rosiglitazone was administered to CLA-fed to induce adiponectin levels, hepatic steatosis was ameliorated. This effect could also be mediated by the attenuation of insulin resistance seen in these mice (210).
CHAPTER 3

EFFECTS OF CONJUGATED LINOLEIC ACID AND TROGLITAZONE ON LIPID ACCUMULATION AND COMPOSITION IN LEAN AND ZUCKER DIABETIC FATTY (fa/fa) RATS

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3.1 ABSTRACT

The dietary fat conjugated linoleic acid (CLA) has been shown to enhance glucose tolerance in several animal models, but induces insulin resistance and lipodystrophy in mice. In this study, the effects of two weeks diet supplementation with either 1.5% CLA or 0.2% troglitazone (TZD), an insulin sensitizing thiazolidinedione, on glucose tolerance and lipid accumulation and composition of both lean and Zucker diabetic fatty (fa/fa; ZDF) rats were examined. Compared with lean rats, which maintained normal glucose tolerances after two weeks of feeding regardless of diet, ZDF rats fed a control diet (CON) had significantly worsened glucose tolerance. ZDF rats fed CLA and TZD diets, however, maintained normal glucose tolerances. In contrast to significantly elevated lipid levels in ZDF rats fed CON diet, concentrations of plasma free fatty acids and triglycerides in ZDF rats fed CLA and TZD diets were normalized. A similar trend of the reduction of plasma lipid levels was observed in lean rats fed CLA and TZD compared to lean rats fed the CON diet. While ZDF CON rats developed significant hepatic steatosis, both CLA- and TZD-fed rats had hepatic triglyceride levels similar to those of lean rats. Both lean and ZDF rats fed CLA diet had reduced adipose mass compared to respective genotype controls; however, TZD had no effect. Ratios of 16:1/16:0 and 18:1/18:0 fatty acids, surrogate markers for stearoyl-CoA desaturase-1 (SCD-1) activity, were reduced in livers of ZDF rats fed CLA and TZD diets. These results show that, like TZD, CLA
normalizes glucose tolerance and plasma lipids, and also improves hepatic steatosis and fatty acid composition in ZDF rats. The effects of CLA and TZD on hepatic lipid composition suggest that the effects of these two agents on glucose tolerance may be associated with a reduction in SCD-1.
3.2 INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of polyunsaturated fatty acids that exist as positional and stereo-isomers of octadecadienoic acid (18:2). CLA is found naturally in foods derived from ruminants, such as beef, lamb, and dairy products and in supplement form (eg. Tonalin™). Due to the potential health benefits of CLA, a great deal of research has been devoted to this group of polyunsaturated fatty acids. CLA acts as an anticarcinogenic (128) and anti-atherosclerogenic agent, modulator of immune function and body composition, and as a possible anti-diabetic agent (130). In the treatment of diabetes, CLA improves glucose tolerance and insulin sensitivity, as well as fasted glucose and insulin levels in several rodent models of obesity and diabetes (10; 13; 138; 140; 152). Further, CLA reduces plasma FFA (10; 13; 138; 152; 165) and plasma (13; 138; 152; 165) and hepatic (167) triglyceride concentrations, which are thought to contribute to insulin resistance and subsequent increased hepatic glucose production (218). The decrease in hepatic triglyceride and altered lipid metabolism by CLA may, in part, be attributed to the reduction of stearoyl-CoA desaturase-1 (SCD-1), a rate-limiting enzyme in the desaturation of palmitoyl- (16:0) and stearoyl-CoA (18:0) fatty acids and essential to triglyceride formation. Previous research has shown that CLA reduces SCD-1 mRNA expression and activity in several different in vitro and in vivo models (183; 219-221). Nevertheless, the mechanism and the relative efficacy of CLA to improve
glucose tolerance, insulin sensitivity, and the associated lipid metabolism have not been fully elucidated.

Similarly, treatment with thiazolidinediones, insulin-sensitizing agents used in type 2 diabetes therapy, lowers fasting glucose and insulin concentrations, corrects plasma lipid levels (222), and reduces hepatic triglyceride levels (223). Thiazolidinediones have also been shown to decrease SCD-1 expression and activity (224; 225), which may contribute to the reductions in lipid levels. Additionally, thiazolidinediones function as a high affinity ligand for the nuclear receptor peroxisome proliferator activated receptor-γ (PPARγ). PPARγ, in turn, stimulates the differentiation, proliferation, and lipid accumulation of adipocytes, thereby increasing adiposity (226). The induction of adipocyte differentiation promotes lipid to accumulate in adipose tissue and prevents lipid accumulation in peripheral tissues such as liver, muscle, and pancreas. The ability of thiazolidinediones to prevent steatosis in non-adipose tissues, as well as other indirect mechanisms, may contribute to the activity of thiazolidinediones to act as insulin sensitizers (227).

Our group has previously reported that following 11 days on experimental diets, ZDF rats fed diets supplemented with either CLA or a thiazolidinedione had similar fasting glucose levels as lean rats; whereas, ZDF rats fed a control diet had elevated fasting glucose levels (10). Because glucose metabolism and insulin sensitivity are associated with reduced steatosis (228) and decreased SCD-1 activity (229; 230), the objective of this
study was to compare the effects of CLA and a thiazolidinedione on lipid composition and metabolism in the liver. The impact of CLA on glucose tolerance, plasma lipid levels, and hepatic lipid accumulation, composition, and SCD-1 activity was compared to this known type 2 diabetes agent in both lean and ZDF rats, as many of the anti-diabetic effects of CLA and thiazolidinediones are similar.
3.3 MATERIALS AND METHODS

Materials. Diet components were obtained from Dyets, Inc. (Bethlehem, PA). The CLA oil (Pharmanutrients, Inc., Lake Bluff, IL) was ~90% CLA with the following composition: 42% c9, t11- and t9, c11-CLA; 43.5% t10, c12-CLA; 1% c9, c11-CLA; 1% c10, c12-CLA; 1.5% t9, t11- and t10, t12-CLA; 0.5% linoleate; 5.5% oleate; and 5% unidentified. The usage of this mixed isomer form of CLA is most relevant regarding the impact of CLA on the management of type 2 diabetes as this source of CLA is currently available for commercial usage in humans (e.g. Tonalin™). The thiazolidinedione, troglitazone, was obtained from Parke-Davis, Ann Arbor, Michigan.

Animals and experimental diets. 6-week old, male, obese, Zucker diabetic fatty (ZDF/Gmi-fa/fa) rats (n=24) and lean littermates (ZDF/Gmi-+/?; n=12) were purchased from Genetic Models, Inc. (Indianapolis, IN). Prior to beginning experimental diets, rats were determined to be normoglycemic by a fasting glucose measurement. Rats were then randomized by genotype and body weight and assigned to one of three isocaloric, modified AIN-76 diets containing 6.5% fat for 14 days (from 8 to 10 weeks of age). Corn oil (5%) was used in all diets, and research grade, tocopherol-striped lard was used to balance caloric density so that diets contained either 5% corn oil / 1.5% lard / no CLA (CON); 5% corn oil / 1.5% CLA (CLA); or 5% corn oil / 1.5% lard / 0.2% troglitazone.
(TZD). Each diet group contained n=8 ZDF rats and n=4 lean rats. All procedures were in accordance with institution guidelines and approved by the Purdue Animal Care and Use Committee. A glucose tolerance test was conducted on day 11 of dietary intervention after an overnight (16 hr) fast as previously reported (10). Glucose areas under the curves (AUCs) are expressed as positive incremental AUCs calculated using the trapezoidal rule (231). After 14 days on experimental diets, rats were euthanized by CO\textsubscript{2} and cervical dislocation. Blood was collected immediately in heparinized test tubes for plasma analyses as described below. Liver and adipose tissues were harvested, weighed, and immediately frozen and stored at -80\textdegree C.

**Analysis of triglycerides and free fatty acids.** Plasma FFAs were determined using a colorimetric kit (NEFA C, Wako Chemicals, Richmond, VA). Lipids were extracted from tissues with chloroform: methanol (2:1, v/v) and 0.2 vol. 0.88% KCl. To measure triglyceride concentrations, lipid extracts were further processed as previously described by Denton and Randle (232) with alterations by Frayn and Maycock (233). In brief, phospholipids were removed with silicic acid, and the remaining lipids were saponified with 80% KOH: ethanol (5:95, v/v). Addition of 0.15 M MgSO\textsubscript{4} stopped the saponification and after centrifugation, the supernatants, as well as plasma, were analyzed for triglycerides by colorimetric enzymatic hydrolysis (Triglyceride, GPO-Trinder Reagents, Sigma, St. Louis, MO).
**Fatty acid analyses.** Lipid extracts were separated into neutral and phospholipids by the silica column procedure described by Hamilton and Comai (234). In brief, neutral and phospholipids were separated by PrepSep -Si columns (Fisher Scientific, Fair Lawn, NJ) by eluting extracts with methyl tert-butyl ether: acetic acid (100:0.2, v/v) and methyl tert-butyl ether: methanol: ammonium acetate (pH 8.6) (5:8:2, v/v/v), respectively. Fatty acid methyl esters of the fractions were prepared by incubating the fractions with 1,1,3,3-tetramethylguanidine at 100°C and analyzed by gas chromatography using a 30-m Omegawax 320 capillary column. Helium flow rate was 30 ml/min and oven temperature was programmed to start at 175°C for 4 min and increase to 220°C at a rate of 3°C/min.

**Statistical analyses.** Data are expressed as least square mean (LSM) ± standard error (SE). Effects of genotype (Lean and ZDF) and diet (CON, CLA, and TZD) were analyzed by two-way ANOVA using the MIXED procedure of Statistical Analysis System (SAS V8; Cary, NC). Differences of P<0.05 were considered significant.
3.4 RESULTS

**Glucose tolerance.** A glucose tolerance test was administered after 11 days on experimental diets. There were no differences in the AUC among any of the lean diet groups. While ZDF rats fed the CON diet had significantly worsened glucose tolerance as indicated by elevated AUC, ZDF rats fed CLA or TZD diets maintained an AUC similar to lean rats (Fig. 3.1).
Fig. 3.1. Effect of dietary CLA and TZD on glucose tolerance test in lean and ZDF rats. Rats were fed either a control (CON; open bars), 1.5% CLA (CLA; hatched bars), or 0.2% troglitazone (TZD; closed bars) diet for 14 days. A glucose tolerance test was administered on day 11 of experimental diets after a 16 hr fast as described in Materials and Methods. Values represent LSM ± SE. Values with different superscripts are significantly different (P<0.05).
**Plasma free fatty acid and triglyceride levels.** Plasma FFA concentrations were reduced by CLA and TZD treatment in both lean and ZDF rats. Plasma FFA levels were similar between lean and ZDF rats fed the CON diets, but were significantly reduced in lean TZD and ZDF CLA and TZD rats. The plasma FFA concentration in lean rats fed CLA was similar to the reduced levels, but was not statistically lower compared to lean CON (Fig. 3.2A). Plasma triglyceride levels did not differ among diet groups of lean rats (Fig. 3.2B). While the plasma triglyceride concentrations of ZDF rats fed the CON diet were significantly elevated compared to lean rats fed the CON diet, both CLA and TZD treatments in ZDF rats reduced plasma triglycerides. Additionally, lean and ZDF rats fed the TZD diet had significantly lower plasma triglyceride levels than ZDF rats fed the CLA diet.
Fig. 3.2. Effect of dietary CLA and TZD on plasma A) FFA and B) triglyceride levels in lean and ZDF rats. Rats were fed either a control (CON; open bars), 1.5% CLA (CLA; hatched bars), or 0.2% troglitazone (TZD; closed bars) diet for 14 days. FFA and triglyceride levels were measured as described in Materials and Methods. Values represent LSM ± SE. Values with different superscripts are significantly different (P<0.05).
*Epididymal fat mass and liver triglyceride concentrations.* Regardless of dietary group, ZDF rats had significantly more epididymal fat mass than all the lean diet groups following 14 days on experimental diets (Fig. 3.3A). Rats fed the CLA diet, whether the lean or ZDF group, had lower epididymal fat mass than the CON and TZD-fed rats within their respective genotype group. Liver triglyceride concentrations were not altered by diet in lean rats (Fig. 3.3B). ZDF rats fed the CON diet had significantly elevated levels of triglyceride in their livers, but CLA and TZD treatment maintained triglyceride levels in ZDF rats similar to those of lean rats.
Fig. 3.3. Effect of dietary CLA and TZD on A) epididymal fat mass and B) liver triglyceride levels in lean and ZDF rats. Rats were fed either a control (CON; open bars), 1.5% CLA (CLA; hatched bars), or 0.2% troglitazone (TZD; closed bars) diet for 14 days. Values represent LSM ± SE. Values with different superscripts are significantly different (P<0.05).
Liver fatty acid composition. As expected, compared to rats fed the CON and TZD diets, rats fed the CLA diet, regardless of genotype, accumulated greater amounts of both isomers in both the neutral lipid and phospholipid fractions of the liver (Tables 3.1-2). In both lipid fractions of the liver, all ZDF rats had significantly reduced levels of arachidonic acid (20:4) compared to lean animals. While dietary treatment had no effect on arachidonic acid levels in lean rats, arachidonic acid levels were significantly higher in ZDF rats fed CLA and TZD (Tables 3.1-2). Ratios of palmitoleate (16:1) / palmitate (16:0) and oleate (18:1) / stearate (18:0) were used as an index of SCD activity. Compared to lean rats, all ZDF rats had elevated SCD indices in both lipid fractions of the liver; however, the increase was partially rescued by both CLA and TZD treatment in the ZDF rats (Tables 3.1-2).
<table>
<thead>
<tr>
<th>Fatty acid (%)</th>
<th>Lean</th>
<th>ZDF</th>
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<tbody>
<tr>
<td></td>
<td>CON</td>
<td>CLA</td>
</tr>
<tr>
<td>14:0</td>
<td>0.28 ± 0.05 a</td>
<td>0.32 ± 0.06 a</td>
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<td>19.88 ± 0.60 a</td>
<td>22.40 ± 0.70 b</td>
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<td>18:0</td>
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<td>20.52 ± 0.59 c</td>
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<td>18:1 n-9</td>
<td>7.95 ± 0.84 a</td>
<td>8.82 ± 0.97 a</td>
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<tr>
<td>18:1 n-7</td>
<td>3.51 ± 0.11 a</td>
<td>2.77 ± 0.13 b</td>
</tr>
<tr>
<td>18:2</td>
<td>18.95 ± 0.67 d</td>
<td>15.58 ± 0.77 e</td>
</tr>
<tr>
<td>18:3</td>
<td>0.15 ± 0.04 a</td>
<td>0.21 ± 0.05 b</td>
</tr>
<tr>
<td>e9t11 CLA</td>
<td>0.00 ± 0.14 a</td>
<td>0.72 ± 0.17 b</td>
</tr>
<tr>
<td>t10c12 CLA</td>
<td>0.00 ± 0.09 a</td>
<td>0.40 ± 0.11 b</td>
</tr>
<tr>
<td>20:1</td>
<td>0.21 ± 0.02 b</td>
<td>0.12 ± 0.02 a</td>
</tr>
<tr>
<td>20:4</td>
<td>26.95 ± 1.02 c</td>
<td>26.79 ± 1.18 c</td>
</tr>
<tr>
<td>16:1/16:0</td>
<td>0.06 ± 0.01 a</td>
<td>0.06 ± 0.01 a</td>
</tr>
<tr>
<td>18:1/18:0</td>
<td>0.38 ± 0.19 a</td>
<td>0.43 ± 0.22 a</td>
</tr>
</tbody>
</table>

*Fatty acids expressed as a percentage of total reported fatty acids. Values are LSM ± SE. Different superscripts within row indicate significant differences (P<0.05).

Table 3.1. Effects of Dietary CLA and TZD on Fatty Acid Composition in the Neutral Lipid Fraction of the Livers of Lean and ZDF Rats.
<table>
<thead>
<tr>
<th>Fatty acid (%)</th>
<th>Lean CON</th>
<th>Lean CLA</th>
<th>Lean TZD</th>
<th>ZDF CON</th>
<th>ZDF CLA</th>
<th>ZDF TZD</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.25 ± 0.05 ab</td>
<td>0.23 ± 0.06 ab</td>
<td>0.14 ± 0.05 a</td>
<td>0.70 ± 0.05 c</td>
<td>0.74 ± 0.05 c</td>
<td>0.37 ± 0.05 b</td>
</tr>
<tr>
<td>16:0</td>
<td>26.16 ± 1.30 a</td>
<td>26.30 ± 1.50 a</td>
<td>26.06 ± 1.30 a</td>
<td>35.60 ± 1.30 b</td>
<td>35.60 ± 1.50 b</td>
<td>35.70 ± 1.30 b</td>
</tr>
<tr>
<td>16:1</td>
<td>0.60 ± 0.28 a</td>
<td>0.65 ± 0.32 a</td>
<td>0.80 ± 0.28 a</td>
<td>4.13 ± 0.28 c</td>
<td>3.31 ± 0.28 bc</td>
<td>2.69 ± 0.28 b</td>
</tr>
<tr>
<td>18:0</td>
<td>24.82 ± 1.56 ab</td>
<td>25.20 ± 1.80 ab</td>
<td>23.17 ± 1.56 ab</td>
<td>22.65 ± 1.56 ab</td>
<td>25.49 ± 1.56 b</td>
<td>20.21 ± 1.56 a</td>
</tr>
<tr>
<td>18:1 n-9</td>
<td>4.90 ± 0.88 a</td>
<td>4.96 ± 1.02 a</td>
<td>7.52 ± 0.88 a</td>
<td>13.92 ± 0.88 e</td>
<td>12.81 ± 0.88 b</td>
<td>10.16 ± 0.88 b</td>
</tr>
<tr>
<td>18:1 n-7</td>
<td>3.21 ± 0.25 bc</td>
<td>2.50 ± 0.29 a</td>
<td>2.59 ± 0.25 ab</td>
<td>2.95 ± 0.25 c</td>
<td>3.38 ± 0.25 c</td>
<td>2.30 ± 0.25 ab</td>
</tr>
<tr>
<td>18:2</td>
<td>14.29 ± 0.73 c</td>
<td>11.53 ± 0.84 b</td>
<td>11.19 ± 0.73 b</td>
<td>8.05 ± 0.73 a</td>
<td>7.76 ± 0.73 a</td>
<td>7.71 ± 0.73 a</td>
</tr>
<tr>
<td>18:3</td>
<td>0.20 ± 0.04 a</td>
<td>0.14 ± 0.05 a</td>
<td>0.34 ± 0.04 b</td>
<td>0.30 ± 0.04 ab</td>
<td>0.32 ± 0.04 ab</td>
<td>0.73 ± 0.04 c</td>
</tr>
<tr>
<td>c9t11 CLA</td>
<td>0.28 ± 0.05 bc</td>
<td>0.61 ± 0.06 c</td>
<td>0.31 ± 0.05 b</td>
<td>0.00 ± 0.05 a</td>
<td>0.29 ± 0.05 b</td>
<td>0.08 ± 0.05 a</td>
</tr>
<tr>
<td>t10c12 CLA</td>
<td>0.43 ± 0.05 c</td>
<td>0.75 ± 0.06 d</td>
<td>0.51 ± 0.05 c</td>
<td>0.00 ± 0.05 a</td>
<td>0.26 ± 0.05 b</td>
<td>0.00 ± 0.05 a</td>
</tr>
<tr>
<td>20:1</td>
<td>0.31 ± 0.04 bc</td>
<td>0.28 ± 0.05 b</td>
<td>0.45 ± 0.04 c</td>
<td>0.22 ± 0.04 ab</td>
<td>0.22 ± 0.04 ab</td>
<td>0.15 ± 0.04 a</td>
</tr>
<tr>
<td>20:2</td>
<td>24.56 ± 1.23 c</td>
<td>26.85 ± 1.42 c</td>
<td>26.93 ± 1.23 c</td>
<td>11.49 ± 1.23 a</td>
<td>18.73 ± 1.23 b</td>
<td>19.90 ± 1.23 b</td>
</tr>
<tr>
<td>16:1/16:0</td>
<td>0.02 ± 0.01 a</td>
<td>0.02 ± 0.01 a</td>
<td>0.03 ± 0.01 a</td>
<td>0.12 ± 0.01 c</td>
<td>0.08 ± 0.01 b</td>
<td>0.08 ± 0.01 b</td>
</tr>
<tr>
<td>18:1/18:0</td>
<td>0.20 ± 0.04 a</td>
<td>0.20 ± 0.05 ab</td>
<td>0.33 ± 0.04 b</td>
<td>0.62 ± 0.04 d</td>
<td>0.51 ± 0.04 ed</td>
<td>0.50 ± 0.04 c</td>
</tr>
</tbody>
</table>

*Fatty acids expressed as a percentage of total reported fatty acids. Values are LSM ± SE. Different superscripts within row indicate significant differences (P<0.05).

Table 3.2. Effects of Dietary CLA and TZD on Fatty Acid Composition in the Phospholipid Fraction of the Livers of Lean and ZDF Rats.
3.5 DISCUSSION

Of the prospective health benefits of CLA, the potential anti-diabetic effects may be the most controversial due to conflicting data. This report agrees with others that have shown CLA attenuates insulin resistance and glucose intolerance in diabetic or obese rat models (10; 138; 140; 152; 153). However, other groups have shown that CLA induced insulin resistance and hyperglycemia primarily in mouse models (13; 15-17). In mice, insulin resistance developed in parallel with severe lipoatrophy and hepatic steatosis (15). Consequently, the ablation of adipose tissue caused by CLA resulted in lipid accumulation in peripheral tissues, such as liver, as well as decreases in adipocytokines, such as leptin and adiponectin (15; 18; 235), all of which may contribute to insulin resistance. Tsuboyama-Kasaoka et al (157) demonstrated that feeding CLA with a high-fat diet partially preserved adipose mass and prevented hyperinsulinemia and hepatomegaly. These results suggest that the ability of CLA to enhance insulin sensitivity is dependent on maintaining a certain level of adipose mass and therefore preventing a marked reduction in adipocytokines and lipid accumulation in peripheral tissues, especially the liver.

In the present study, the effects of dietary CLA and TZD were compared in obese (Gmi-\textit{fa/fa}) and lean (Gmi-+/?\textit{?}) ZDF rats. In contrast to lean rats that are non-obese and
otherwise phenotypically 'normal', ZDF rats have a mutated, nonfunctional leptin receptor (236). ZDF rats are therefore leptin resistant, obese, and develop hyperlipidemia, hyperleptinemia, insulin resistance, and hyperglycemia similar to type 2 diabetes (237; 238). As in mice and other animal models (11; 12), both lean and ZDF rats fed CLA had significantly reduced adipose mass. However, the reduction in adipose in either genotype was not sufficient enough to induce lipoatrophy and subsequent hepatic steatosis as observed in mice (29, 34). While liver triglycerides were significantly elevated in ZDF CON rats, liver triglycerides of ZDF rats fed CLA were similar to lean rats. TZD, a PPARγ agonist, did not affect adipose mass in this 2 week study, but consistent with another report (223), reduced liver triglycerides. Notably, liver triglyceride levels were not affected by either treatment in lean rats. These results support previous reports that CLA reduced adipose mass and hepatic steatosis in ZDF and Zucker rats (140; 167; 239). Furthermore, CLA was as effective as TZD in lowering liver triglycerides and did not induce hepatic steatosis in lean rats. The prevention and reduction of hepatic steatosis is important for the ability of CLA to improve glucose tolerance as hepatic steatosis contributes to insulin resistance and increased hepatic glucose production. Additionally, despite ZDF rats being leptin resistant, a condition that contributes to hepatic steatosis (240), CLA was able to prevent hepatic steatosis. Nagao et al (167) showed that CLA increased adiponectin, an insulin sensitizing adipocytokine, and suggests that the increase may have prevented the development of hepatic steatosis. While adiponectin levels were not measured in this study, others have shown that CLA enhances adiponectin levels in ZDF rats (153).
The decrease in liver triglycerides by CLA treatment in ZDF rats may also partially be attributed to the reduction of SCD-1. SCD-1 is important in lipid metabolism as it catalyzes the delta-9 desaturation predominantly of palmitoyl- (16:0) and stearoyl-CoA (18:0) to palmitoleoyl- (16:1) and oleoyl-CoA (18:1), respectively. These monounsaturated fatty acids are integral components of phospholipids, triglycerides, wax esters, and cholesterol esters (241). Therefore, the regulation of SCD-1 is crucial in the formation of these lipid fractions and essential for triglyceride synthesis. Decreases in SCD-1 expression or activity have been associated with reduced triglyceride levels in plasma and liver (242-244), as well as increased insulin sensitivity (229; 230). These effects may also be due to increased beta oxidation, which also occurs with reduced SCD-1 expression (229). Both CLA (183; 219-221) and thiazolidinediones (224; 225) have been reported to reduce SCD-1 expression and activity or alter fatty acid composition, specifically decreasing monounsaturated fatty acids, indicating a reduction of SCD-1 activity. In this study, CLA and TZD treatment decreased the SCD-1 indices in neutral and phospholipid fractions in livers of ZDF rats suggesting a reduction of SCD-1 activity. Although ZDF rats lack responsiveness to leptin, a regulator of SCD-1(245), CLA and TZD reduced SCD-1 indices, indicating these effects are independent of a leptin: leptin receptor mediated mechanism. However, perhaps due to the unresponsiveness to leptin, ZDF rats, regardless of diet, had significantly higher SCD-1 indices than lean rats. Conversely, lean rats respond to leptin and had relatively low SCD-1 indices, which were not further reduced by either diet. This suggests that despite the presence of down-
regulators of SCD-1, such as leptin, CLA, or TZD, other regulatory mechanisms maintain a basal level of SCD-1 activity. Hepatic triglyceride and cholesterol ester synthesis is dependent on SCD-1 activity, and therefore maintenance of the activity of this enzyme is crucial to lipid homeostasis (246).

Impairments in insulin sensitivity have been closely linked to increases in plasma free fatty acid and triglyceride concentrations. Studies in which both glucose metabolism and lipid accumulation were measured suggest that a reduction in plasma, as well as tissue triglycerides correlate with improved glucose tolerance and insulin sensitivity (20). Thiazolidinediones act, in part, by promoting uptake of circulating free fatty acids and storage in the adipose tissue, thus lowering plasma free fatty acids allowing for improved insulin sensitivity. Previous research shows that CLA reduced plasma triglyceride and free fatty acid levels in both rats and mice (13; 138; 140; 152; 165; 247). The exact mechanism by which CLA is able to reduce plasma triglyceride and FFA is largely unknown, but the enhanced uptake of circulating free fatty acids due to increased lipid oxidation has been suggested as a possible mechanism (165; 206). The present study shows that CLA decreased plasma FFA and triglycerides in both lean and ZDF rats and to the same degree as TZD (except ZDF-CLA plasma triglyceride). Other studies have reported that CLA improved fasting glucose and hyperinsulinemia in obese or diabetic rats (10; 138; 140). Accordingly, we report that CLA normalized glucose tolerance in ZDF rats. Importantly, in this study, CLA was as effective as TZD in improving glucose tolerance, and CLA did not affect glucose tolerance in non-obese, lean rats.

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In summary, this study shows that CLA normalized glucose tolerance and plasma lipids and improved hepatic steatosis and fatty acid composition in ZDF rats with the same efficacy as a common diabetic treatment, a thiazolidinedione. Hepatic lipid composition suggests that these effects may be partially attributed to a reduction in SCD-1. Furthermore, CLA did not affect glucose tolerance or hepatic steatosis in non-obese lean rats.
3.6 ACKNOWLEDGEMENTS

We thank Dr. Carolyn Gunther for her critical feedback regarding the content of this paper. We also wish to thank Kai-Li Liu, Kwangok Nickel and Silvia Moya-Camarena for their technical assistance and the National Cattleman’s Beef Association for supporting part of this study.
CHAPTER 4

CONJUGATED LINOLEIC ACID FAILS TO WORSEN INSULIN RESISTANCE
BUT INDUCES HEPATIC STEATOSIS IN THE PRESENCE OF LEPTIN IN
OB/OB MICE

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Department of Human Nutrition, The Ohio State University, Columbus, OH 43210
4.1 ABSTRACT

Conjugated linoleic acid (CLA) induces insulin resistance preceded by rapid depletion of the adipokines leptin and adiponectin, increased inflammation, and hepatic steatosis in mice. To determine the role of leptin in CLA-mediated insulin resistance and inflammation, recombinant leptin was co-administered with dietary CLA in ob/ob mice to control leptin levels and to, in effect, negate the leptin depletion effect of CLA. In a 2x2 factorial design, 6-week old, male ob/ob mice were fed either a control diet or a diet supplemented with CLA and received daily, intraperitoneal injections of either leptin or vehicle for 4 weeks. In the absence of leptin, CLA significantly depleted adiponectin and induced insulin resistance, but did not increase hepatic triglyceride concentrations or adipose inflammation, marked by IL-6 and TNFα mRNA expression. Insulin resistance was, however, accompanied by increased macrophage infiltration (F4/80 mRNA) in adipose tissue. In the presence of leptin, CLA depleted adiponectin but did not induce insulin resistance or macrophage infiltration. Despite this, CLA induced hepatic steatosis. In summary, CLA worsened insulin resistance without evidence of inflammation or hepatic steatosis in mice after 4 weeks. In the presence of leptin, CLA failed to worsen insulin resistance, but induced hepatic steatosis in ob/ob mice.
4.2 INTRODUCTION

Obesity contributes to the etiologies of a variety of co-morbid conditions, such as cardiovascular disease, hypertension, and type-2 diabetes. In addition to storing lipid for energy, adipose secretes a variety of adipokines, many of which affect metabolism and inflammation in adipose and non-adipose tissues. Modulation of the endocrine functions of adipose tissue can contribute to a chronic state of inflammation, which leads to the pathogenesis of associated disorders, specifically insulin resistance (4). Conjugated linoleic acid (CLA) is a group of dietary fatty acids that modulate adiposity and adipokine levels (15; 18; 134; 210). CLA exists as positional and stereo-isomers of octadecadienoic acid (18:2) and is found naturally in foods derived from ruminants, such as beef, lamb, and dairy products. Commercially, mixed isomer CLA is marketed as a weight loss supplement (e.g. Tonalin™). Different isomers of CLA have varied biological functions such as reducing carcinogenesis, decreasing adipose mass, and modulating immune function and type 2 diabetes (130). While CLA, specifically the trans-10, cis-12 (t10,c12) isomer (9), significantly decreases body weight primarily through a reduction of adipose tissue in a variety of species (10-12), CLA also induces hyperinsulinemia and insulin resistance, primarily in mice (13-17).
In mice, insulin resistance induced by CLA develops in parallel with lipodystrophy, i.e. decreased adipose mass, significant and rapid depletion of the adipokines leptin and adiponectin, and increased hepatic steatosis (15; 18). However, the mechanism by which CLA causes lipodystrophy in mice and the reason this effect is species-specific is not completely understood. Results from several studies emphasize the importance of leptin and adiponectin in the development of CLA-induced insulin resistance: Leptin levels and adipose mass were partially preserved when CLA was fed as part of a high-fat diet in C57BL/6J mice. The preservation of leptin may have contributed to improvements in plasma insulin and liver weight also observed in these mice (157). In lean mice supplemented with CLA, adipokines decreased rapidly and prior to a significant reduction of adipose mass. Further, hyperinsulinemia and increased hepatic lipid concentration accompanied the time-dependent depletion of adiponectin and leptin (18). These findings indicate that the initial reduction of adipokines by CLA may be independent of reduced adipose mass. In a subsequent study, the reduction of leptin and adiponectin induced by CLA coincided with a proinflammatory state marked by increased expression of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and macrophage infiltration in adipose tissue, likely contributing to increased insulinemia (159). These results suggest that the deleterious effects of CLA on insulin sensitivity and hyperinsulinemia may be dependent on the alteration of adipokines and induction of inflammation in adipose tissue.
Recent studies show that CLA worsens hyperinsulinemia and insulin resistance in ob/ob mice, which have non-functional leptin due to a gene mutation (13; 134). Consequently, insulin resistance induced by CLA cannot solely be attributed to the depletion of leptin. However, because both CLA-fed and ob/ob mice are leptin deficient, insulin resistance and the other effects of CLA may not necessarily be completely independent of leptin depletion. In this study, we controlled the level of leptin in the ob/ob mouse model with chronic administration of recombinant leptin. By controlling leptin levels and, in effect, negating the leptin depletion effect of feeding CLA, we aimed to determine whether effect of CLA on insulin resistance, hepatic steatosis, and inflammation occur in a leptin-dependent manner.
4.3 RESEARCH DESIGN AND METHODS

*Experimental animals and design.* 6-week old, male B6.V-Lep\(^{ob}\)/OlaHsd (ob/ob) mice were obtained through Harlan (Indianapolis, IN) and housed 4/cage at 22°C ± 0.5°C on a 12-hour light/dark cycle. Mice were maintained on isocaloric, modified AIN-93G powdered diets (Bio-Serv, Frenchtown, NJ) containing 6.5% fat by weight. Diets contained either 6.5% soybean oil (CON) or 5% soybean oil and 1.5% CLA mixed triglycerides (CLA). CLA mixed triglycerides (Tonalin™ TG 80, Cognis Corp., Cincinnati, OH) were ~80% CLA composed of 39.2% c9t11- and 38.5% t10c12-CLA isomers. In a 2x2 factorial design, mice were randomized by body weight and fed either the CON or CLA diet and received intraperitoneal injections of either 1 mg/kg BW recombinant mouse leptin (R&D Systems, Minneapolis, MN) (CON + or CLA +) or a similar volume of the vehicle (PBS) (CON - or CLA -) for 4 weeks (n=8 mice per diet +/- leptin group). Mice were injected every day, two hours before the onset of the dark cycle. The leptin dose was based on the lowest dose that induced a reduction in body weight gain and fat and rescued serum insulin levels in ob/ob mice (52). 4 weeks was chosen as the end point because this duration allows time for the development of insulin resistance and hepatic steatosis induced by CLA supplementation in mice (13; 14; 248) and independently, the correction of metabolic abnormalities of ob/ob mice by leptin (51; 52). Body weights and food intake were measured every other day. There were no
detectable differences in food intake. At 4 weeks, after an overnight (12 hr) fast, mice were anesthetized with isoflurane and blood was collected via cardiac puncture. After clotting, blood was centrifuged at 1500 x g for 20 min, and sera were used for analyses. Tissues were quickly harvested, weighed, snap-frozen with liquid nitrogen, and stored at -80°C until analyses. All procedures were in accordance with institution guidelines and approved by the Institutional Animal Care and Use Committee of The Ohio State University.

**Fasting glucose and insulin tolerance test.** Glucose levels were measured after an overnight, 12 h fast immediately before (baseline) and at 2 and 4 weeks of experimental treatments via tail vein blood using a One Touch Basic glucometer (Lifescan, Milpitas, CA). An insulin tolerance test was conducted 3 days before necropsy. After an overnight, 12 h fast, mice were injected intraperitoneally with 1.5 U/kg BW insulin (Humulin® R, Eli Lilly and Co., Indianapolis, IN). Tail vein blood was used to measure glucose immediately prior to injection (time 0) and at 15, 30, 45, 60, 90, and 120 minutes following the injection. Area under the curve (AUC) was calculated as the net area contained between individual baselines (set by the glucose value at time 0) and curves using the trapezoidal rule (231). HOMA (homeostasis model assessment) values were calculated according to Matthews et al. (249) as insulin (mU/L)/(22.5e-ln glucose (mM)).

**Serum hormones.** Fasted serum levels of insulin, IL-6, and TNF-α were measured by the LINCOplex Mouse Serum Adipokine Immunoassay kit (Linco Research, Inc., St.
Charles, MO). Adiponectin and resistin serum concentrations were determined by ELISA (Linco Research, Inc.) according to manufacturer’s directions.

**Analysis of hepatic triglycerides.** Lipids were extracted from a section of liver with 2:1 (v/v) chloroform and methanol. Final extracts were solublized in 3:1:1 (v/v/v) tert-butanol, methanol, Triton X-100 (250). Tissue lipid extracts were analyzed for triglycerides by colorimetric enzymatic hydrolysis (Triglyceride, Free-Glycerol reagents, Sigma, St. Louis, MO). Data are expressed as equivalent triolein concentrations.

**Real-time RT-PCR.** RNA was extracted from epididymal adipose tissue using the RNeasy® Lipid Tissue Mini kit (Qiagen, Valencia, CA) and from liver using Trizol (Invitrogen, Carlsbad, CA) according to manufacturers’ protocols. 3 ng RNA was reverse transcribed with High Capacity cDNA Archive Kit (ABI, Foster City, CA) according to directions. 5-10 ng cDNA was amplified by real-time PCR in a total reaction volume of 25 µl with TaqMan Gene Expression Assays (ABI, Foster City, CA) using pre-designed and validated primers (FAM probes) from ABI under universal cycling conditions defined by ABI. Target gene expression was normalized to the endogenous control 18s (VIC probe) amplified in the same reaction and expressed as $2^{-\Delta\Delta\text{ct}}$ relative to the CON-group (251).

**Statistical analyses.** Data are expressed as least square mean (LSM) ± standard error (SE). The main and interaction effects of diet (CON or CLA) and treatment (leptin or
vehicle) were analyzed by two-way ANOVA using a complete model in the GLM procedure of Statistical Analysis System (SAS v9.1; SAS Institute Inc., Cary, NC). T-tests adjusted for multiple comparisons were used for post-hoc analyses. Body weights over time, fasting glucoses, and ITT curves were analyzed as repeated measures. Differences of P<0.05 were considered significant.
4.4 RESULTS

Effects of CLA and leptin on body and tissue weights. Prior to beginning experimental diets and treatments, all groups had similar average body weights (32.4-32.7 g). After 4 weeks on experiment diets and treatments, leptin and CLA, both in the absence and presence of leptin, significantly reduced body weights compared to CON diet alone (Table 4.1). The reduction in body weight by CLA was significantly more than that of leptin alone; however, there was not a significant additive or synergistic effect of CLA and leptin on body weight. While leptin alone decreased weight gain, CLA, regardless of leptin, induced significant weight loss from initial body weights. Body weights over time are shown in Fig. 4.1. Significant differences in weight between mice fed CLA, regardless of leptin treatment, and vehicle-treated mice fed CON were first observed on day 9. On day 15, weights of CLA-fed mice were first significantly different from leptin-treated mice fed CON. A significant difference in weight between vehicle-treated and leptin-treated mice fed CON was first detected on day 17. All weights among aforementioned comparisons remained significantly different through the end of the study. CLA-fed groups, whether in the absence or presence of leptin, never significantly differed from each other over the duration of the study. Differences in body weight were reflected by differences in epididymal adipose mass (Table 4.1). Leptin and CLA, in the absence and presence of leptin, significantly reduced epididymal adipose mass compared
to CON diet alone, and the reduction of epididymal adipose mass by CLA was significantly greater than that of leptin alone. Leptin treatment, regardless of diet, significantly decreased liver weights, whereas CLA did not have a significant effect.
Factors include the main effects of diet and leptin treatment (Trt), and the interaction (Int) between diet and leptin treatment.

Table 4.1. Effects of CLA and leptin on body and tissue weights.

<table>
<thead>
<tr>
<th>Weights (g)</th>
<th>Diet ± leptin</th>
<th>Factors, (P) (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON -</td>
<td>CLA -</td>
</tr>
<tr>
<td>Final body weight</td>
<td>42.20 ± 0.97(^c)</td>
<td>28.98 ± 0.97(^a)</td>
</tr>
<tr>
<td>Change in body weight</td>
<td>9.74 ± 0.56(^c)</td>
<td>-3.54 ± 0.56(^a)</td>
</tr>
<tr>
<td>Epididymal adipose</td>
<td>2.96 ± 0.14(^c)</td>
<td>1.71 ± 0.14(^a)</td>
</tr>
<tr>
<td>Liver</td>
<td>2.76 ± 0.17(^b)</td>
<td>3.18 ± 0.17(^b)</td>
</tr>
</tbody>
</table>

\(^a\)\(^b\)\(^c\)Values represent LSM ± SE with significant differences (P<0.05) within row denoted by different superscripts.

\(^1\)Factors include the main effects of diet and leptin treatment (Trt), and the interaction (Int) between diet and leptin treatment.
**Fig. 4.1.** Effects of CLA and leptin on body weights over time. Mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either leptin (+) or vehicle (-) by IP injection daily. Body weights were measured every other day. Values represent LSM of n=8 mice/group. * P<0.05 compared to CON-; # P<0.05 compared to CON+. *, # indicate first day significant difference was observed—differences maintained through completion of study.
**Effects of CLA and leptin on serum analytes and insulin tolerance.** After two weeks of diets and treatments, CLA significantly elevated fasting glucose levels in the absence of leptin (Table 4.2). Elevated fasting glucose levels were maintained, but not increased, by CLA after four weeks of diets and treatments. In the presence of leptin, CLA did not significantly increase fasting glucose levels at 2 or 4 weeks. Leptin also prevented an increase in fasting glucose in mice fed the CON diet by 4 weeks. In the absence of leptin, CLA significantly increased fasting insulin levels compared to CON, while leptin significantly reduced fasting insulin levels in both diet groups (Table 4.2).
Factors include the main effects of diet and leptin treatment (Trt), and the interaction (Int) between diet and leptin treatment. Fasting glucoses were analyzed by repeated measures ANOVA; only differences within diet ± leptin group and within time are shown.

Values represent LSM ± SE with significant differences (P<0.05) from baseline within diet ± leptin group denoted by #; there were no significant differences (P<0.05) between 2 weeks and 4 weeks within any diet ± leptin group.

Table 4.2. Effects of CLA and leptin on fasted serum analytes.

<table>
<thead>
<tr>
<th></th>
<th>Diet ± leptin</th>
<th>Factors, (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON -</td>
<td>CLA -</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>441.24 ± 49.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>771.05 ± 58.51&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>9.37 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.91 ± 0.57&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Resistin (ng/ml)</td>
<td>22.13 ± 2.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.67 ± 2.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>25.89 ± 5.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.22 ± 5.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>2.01 ± 0.99</td>
<td>0.20 ± 0.99</td>
</tr>
</tbody>
</table>

Fasting glucose (mg/dl)<sup>2</sup>

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>2 weeks</th>
<th>4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>115.12 ± 10.38</td>
<td>101.25 ± 10.38</td>
<td>117.00 ± 10.38</td>
</tr>
<tr>
<td></td>
<td>130.25 ± 10.38&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>161.59 ± 12.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>106.25 ± 10.38&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>122.87 ± 10.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>153.63 ± 10.38&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>89.50 ± 10.38&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

superscripts.  

<sup>1</sup>Factors include the main effects of diet and leptin treatment (Trt), and the interaction (Int) between diet and leptin treatment. 

<sup>2</sup>Fasting glucoses were analyzed by repeated measures ANOVA; only differences within diet ± leptin group and within time are shown.

<sup>a</sup>Values represent LSM ± SE with significant differences (P<0.05) from baseline within diet ± leptin group denoted by #; there were no significant differences (P<0.05) between 2 weeks and 4 weeks within any diet ± leptin group.
After 4 weeks of diets and treatments, an ITT was conducted to assess response to insulin. In the absence of leptin, glucose levels in mice fed CLA did not decrease at any time point after the administration of insulin, indicating unresponsiveness to insulin (Fig. 4.2A). In the presence of leptin, glucose levels in mice fed CLA significantly decreased after insulin injection, as they did in mice fed the CON diet. In figure 4.2B, the net areas contained within the ITT curves were quantified. As implied by the positive area, in the absence of leptin, mice fed CLA were unresponsive to insulin, and was significantly different from other groups. There were no significant differences among the other groups, and the net areas were negative, indicating insulin responsiveness. A second estimation of insulin resistance by HOMA shows that in the absence of leptin, CLA worsened insulin resistance. Leptin decreased insulin resistance in both diet groups (Fig. 4.2C). Together, the ITT and HOMA data show that in the absence of leptin CLA worsens insulin resistance, but does not in the presence of leptin.
Fig. 4.2. Effects of CLA and leptin on insulin tolerance. (A.) Insulin tolerance test. After 4 weeks, mice received 1.5 U/kg insulin by IP injection after an overnight fast (12 hr). Blood glucose was measured at intervals indicated over 2 hrs. Statistical differences at each time point were omitted for clarity. (B.) Net area contained within curve of insulin tolerance test. (C.) HOMA estimates of insulin resistance measured as: insulin (mU/L)/(22.5e-ln glucose (mM)). Mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either leptin (+) or vehicle (-) by IP injection daily. Values represent LSM ± SE of n=8 mice/group with significant differences (P<0.05) denoted by different superscripts.
Effects of CLA and leptin on adipokines. Serum adiponectin levels were significantly reduced by CLA in both the absence and presence of leptin (Table 4.2). Leptin had no effect on serum adiponectin levels in either respective diet group. Serum resistin, however, was significantly decreased by leptin and CLA, in the absence and presence of leptin. Similar to serum adiponectin, CLA, in the absence and presence of leptin, significantly reduced epididymal white adipose tissue (WAT) adiponectin mRNA expression compared to respective CON diet groups (Fig. 4.3A). Unlike serum levels, however, leptin significantly increased WAT adiponectin mRNA expression in CON-fed mice. Both leptin and CLA significantly decreased WAT resistin mRNA expression; however, the reduction induced by CLA, regardless of leptin treatment, was significantly greater than leptin alone (Fig. 4.3B).
**Fig. 4.3.** Effects of CLA and leptin on WAT gene expression of adipokines. (A.) Adiponectin. (B.) Resistin. mRNA expression is expressed as $2^{-\Delta\Delta C_t}$ relative to the 18s endogenous control and the CON- group. Mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either leptin (+) or vehicle (-) by IP injection daily for 4 weeks. Values represent LSM ± SE of n=6 to 8 mice/group with significant differences (P<0.05) denoted by different superscripts.
**Effects of CLA and leptin on hepatic lipid metabolism.** CLA had no effect on hepatic triglyceride concentrations in the absence of leptin (Fig. 4.4A). However, in the presence of leptin, CLA increased hepatic TG. Leptin significantly reduced hepatic triglyceride levels in both diet groups; however leptin only partially reduced hepatic triglycerides in mice fed CLA compared to CON. Hepatic mRNA expression of markers of lipogenesis, sterol regulatory element binding protein-1 (SREBP-1) and fatty acid synthase (FAS), and lipid transport, fatty acid transporter (FAT/CD36), were not altered by CLA (Fig. 4.4B), but were reduced by leptin in both respective diet groups. In the absence of leptin, CLA did not alter markers of fatty acid oxidation, peroxisome proliferator-activated receptor-α (PPARα), carnitine palmitoyltransferase 1α (CPT-1α), fatty acid binding protein 1 (FABP1), and acyl-coenzyme A oxidase 1 (ACOX1) compared to vehicle-treated mice fed the CON diet. These markers were increased by leptin in CON-fed mice. In the presence of leptin, CLA decreased these markers to levels of vehicle-treated mice in either diet group.
**Fig. 4.4.** Effects of CLA and leptin on hepatic lipid metabolism. (A.) Hepatic triglyceride levels. (B.) Hepatic mRNA expression of markers of lipid metabolism. mRNA expression is expressed as $2^{-\Delta\Delta Ct}$ relative to the 18s endogenous control and the CON- group. Mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either leptin (+) or vehicle (-) by IP injection daily for 4 weeks. Values represent LSM ± SE of n=6 to 8 mice/group with significant differences (P<0.05) denoted by different superscripts.
Effects of CLA and leptin on markers of inflammation and macrophage infiltration.

Both CLA and leptin significantly reduced serum IL-6 compared to CON- mice (Table 4.2). Serum TNF-α did not significantly change with any diet or treatment. Contrary to the trend in serum IL-6, only leptin significantly decreased WAT IL-6 mRNA expression compared to vehicle-treated mice respective of diet (Fig. 4.5A). Likewise, only leptin significantly decreased WAT TNF-α mRNA expression compared to vehicle-treated mice (Fig. 4.5B). CC chemokine ligand 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1), a macrophage recruiter, was significantly decreased by leptin treatment, regardless of diet, but was not altered by CLA (Fig. 4.5C). In the absence of leptin, CLA significantly increased F4/80 mRNA expression, a macrophage specific marker, nearly 2-fold compared to all other groups (Fig. 4.5D).
Fig. 4.5. Effects of CLA and leptin on WAT gene expression of markers of inflammation and macrophage infiltration. (A.) IL-6; (B.) TNF-α; (C.) CCL2 and (D.) F4/80 mRNA expression was measured in epididymal adipose tissue. mRNA expression is expressed as $2^{-\Delta\Delta C_{t}}$ relative to the 18s endogenous control and the CON- group. Mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either leptin (+) or vehicle (-) by IP injection daily for 4 weeks. Values represent LSM ± SE of n=6 to 8 mice/group with significant differences (P<0.05) denoted by different superscripts.
4.5 DISCUSSION

Adipokines, particularly leptin and adiponectin, produced by adipose tissue are recognized as key mediators in both insulin sensitivity and inflammation and provide an important link of communication among tissues. Rapid depletion of both adiponectin and leptin coincide with increased inflammation and subsequent insulin resistance and hepatic steatosis with CLA supplementation in mice (18; 159). In the present study, recombinant leptin was chronically administered to ob/ob mice to determine the effects of dietary CLA in the absence or presence of leptin on insulin resistance, hepatic steatosis, and inflammation. This study demonstrates that in the absence of leptin, CLA augments insulin resistance without evidence of increased hepatic steatosis or inflammation in serum or WAT. When co-administered with leptin, CLA fails to worsen insulin resistance, but increases hepatic steatosis.

CLA induces hyperinsulinemia and hepatic steatosis preceded by the depletion of adipokines, specifically leptin and adiponectin, in mice (18). Tsuboyama-Kasaoka et al. (15) reported that 12 days of leptin infusion decreased non-fasted plasma insulin and hepatic steatosis in mice that had been fed CLA for 8 months prior. However, the effect of leptin infusion on adiponectin concentrations was not reported nor was it compared to a control-fed group. Leptin treatment has been shown to increase plasma adiponectin
(252), therefore, although speculative, insulin concentrations and hepatic steatosis may have been attenuated by a leptin-induced increase in adiponectin. In the present study, induction of hyperinsulinemia and insulin resistance in ob/ob mice by CLA paralleled the depletion of adiponectin. In the presence of leptin, CLA supplementation did not worsen insulin resistance, but serum adiponectin levels were still reduced. Although CLA did not significantly increase serum insulin levels in the presence of leptin, insulin levels of these mice were double those of the CON-fed group treated with leptin. This finding, CLA induces hyperinsulinemia without worsening insulin resistance in the presence of leptin, suggests that there may be direct, opposing effects of CLA and leptin on insulin secretion. Hyperinsulinemia induced by CLA has been shown to be associated with pancreatic beta cell hyperplasia and increased insulin secretion (18). Leptin, conversely, reduces insulin release from pancreatic beta cells (253). The incomplete attenuation of CLA-induced hyperinsulinemia with leptin treatment may also suggest that these effects of CLA may be dependent on adiponectin, or perhaps a basal level of both leptin and adiponectin, as adiponectin was suppressed by CLA regardless of leptin treatment. Our group has previously reported that while CLA rapidly depleted adiponectin levels in mice, upon removal of CLA from the diet, adiponectin and leptin levels significantly increased and insulin sensitivity improved (210). Yamauchi et al (254) reported that only a combination of adiponectin and leptin could correct insulin resistance in lipodystrophic mice—leptin or adiponectin alone only partially reversed insulin resistance. Increased levels of resistin, another adipokine, have been associated with insulin resistance, but the data are contradictory (255-257). Here, in the absence of leptin, CLA decreases serum and WAT
expression of resistin despite increased insulin resistance. In the presence of leptin, which also decreases resistin (258), and with improved insulin sensitivity, CLA still reduces WAT expression of resistin. These data support previous reports that CLA decreases resistin levels (159; 176; 248), and also supports a disconnect between resistin levels and insulin resistance. These data also suggest that leptin status does not influence the effect of CLA on WAT resistin expression.

Hepatic steatosis and hepatomegaly generally develop with CLA-induced insulin resistance (14; 15; 213). In this study, we did not observe a significant increase in liver weight or hepatic steatosis by CLA in the absence of leptin in ob/ob mice. While hepatic steatosis in non-alcoholic fatty liver disease is defined as accumulation of lipid of 5-10% of the liver by weight (259), livers of both the CON and CLA groups in vehicle-treated mice were 17.3% triglycerides. It is therefore possible that CLA did not increase hepatic lipid levels in livers of ob/ob mice because they were already grossly steatotic. When leptin is present, livers of CON-fed mice were 4.8% lipid and not steatotic, but CLA increased hepatic lipid concentration to 10.2%. These differences in hepatic triglyceride levels may be attributed to changes in insulin levels and adipokines. Insulin contributes to increased hepatic lipogenesis through induction of SREBP-1 (215). Appropriately, hyperinsulinemia in vehicle-treated mice fed the CON diet was accompanied by hepatic steatosis; however, the increase in insulin by CLA did not contribute to greater levels of hepatic triglycerides or markers of lipogenesis, SREBP-1 or FAS. Roche et al. (13) also reported that the t10c12-CLA isomer did not alter hepatic mRNA expression of SREBP-
decreased despite hyperinsulinemia in ob/ob mice, but hepatic triglyceride or adipokine levels were not reported. In addition to insulin, both leptin and adiponectin contribute to hepatic lipid metabolism to modulate triglyceride levels. Adenovirus-induced hyperleptinemia reduced hepatic mRNA expression of SREBP-1c and the lipogenic enzymes FAS and acetyl coenzymeA carboxylase (ACC) in lean (+/+ ) ZDF rats (260). Leptin increases fatty acid oxidation by upregulating PPARα and its target genes (261). Adiponectin promotes fatty acid oxidation through activation of AMPK (262) and increased expression of PPARα and target genes involved in fatty acid oxidation (263). In this study, leptin lessened hepatic steatosis and liver mass in both diet groups. Interestingly, however, CLA only increased hepatic triglycerides in the presence of leptin. The decrease in hepatic triglycerides by leptin seemed to be through both the reduction of lipogenesis and increased lipid oxidation. CLA, however, blunted the increase in lipid oxidation by leptin. This lack of increase in lipid oxidation by leptin in CLA-fed mice may be attributed to the depletion of adiponectin, again suggesting a basal level of both leptin and adiponectin may be critical to maintain energy homeostasis.

Chronic inflammation is a major factor in obesity-driven insulin resistance. Reports in both animal models of obesity and insulin resistance and in humans show strong relationships between expression of TNF-α and IL-6, as well macrophage infiltration into adipose tissue with increased adiposity and insulin resistance (57; 67; 264). Despite these and previous reports of CLA-mediated increased inflammation (159; 177; 265), in this study, two prominent markers of inflammation, serum and WAT mRNA expression of
TNF-α and IL-6 were not increased by CLA. Leptin reduced WAT expression of both TNF-α and IL-6 regardless of diet. The reduction in WAT inflammation by leptin may have contributed to the increased insulin sensitivity in CLA-fed mice. It may have also been a factor in the reductions of fasting insulin and glucose, as well as hepatic steatosis in both CON- and CLA-fed mice. Similarly, CLA did not have an effect on WAT mRNA expression of CCL2, a macrophage recruiter, but increased WAT F4/80 mRNA expression, a macrophage specific marker. Leptin reduced expression of CCL2, which may have prevented the increase in F4/80 expression in WAT of mice fed CLA, but did not have an effect on the level of macrophage infiltration in WAT of CON-fed mice.

After 4 weeks, induction of insulin resistance by CLA coincided with macrophage infiltration, but without increased levels of WAT IL-6 and TNF-α. A previous study showed that 3 and 7 days of treatment with CLA significantly increased WAT mRNA expression of markers of inflammation, IL-6 and TNF-α, and macrophage infiltration, F4/80, CD68, and MCP-1. These changes occurred simultaneous with depletion of adiponectin and leptin and an increase in insulin levels (159). Tsuboyama-Kasaoka et al. (15) reported that the maximum increase in TNF-α mRNA expression in WAT by CLA in C57BL/6J mice occurred at 4 days, but subsequently declined. Wargent et al. (134) showed that after initially inducing impaired glucose tolerance and hyperinsulinemia, prolonged supplementation (10 weeks) with CLA actually improved glucose tolerance and insulinemia in ob/ob mice, regardless of reduced adiponectin concentrations. Markers of WAT inflammation were not reported in that study. This study demonstrates
that after 4 weeks of CLA-supplementation in ob/ob mice, the mice are hyperinsulinemic and insulin resistant without overt evidence of increased inflammation compared to the control. Increased F4/80, but not CCL2 mRNA expression in the WAT of CLA-fed mice suggests that there was still evidence of macrophage infiltration in WAT, which is associated with inflammation (264), but that macrophage recruitment had perhaps subsided. Taken together, results from these studies suggest that some events of CLA mediated inflammation (such as changes in WAT TNF-α and IL-6 and macrophage infiltration) and the possible, subsequent effects on insulin resistance may be transient.

In summary, this study demonstrates that insulin resistance induced by CLA in ob/ob mice was prevented by leptin without an increase in adiponectin. However, the ability of CLA to increase hyperinsulinemia and hepatic steatosis in the presence of leptin suggests these effects may be dependent on other factors such as adiponectin, a basal level of both leptin and adiponectin, and/or other factors not yet identified. Further, IL-6 and TNFα, markers of inflammation frequently associated with obesity and insulin resistance, were not increased after 4-weeks supplementation with CLA. Future studies are needed to determine the mechanistic roles of adiponectin and/or markers of inflammation in lipodystrophic-like insulin resistance and hepatic steatosis induced by CLA.
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CHAPTER 5

INDUCTION OF UNCOUPLING PROTEIN-1 IN WHITE ADIPOSE FROM OB/OB MICE FED CONJUGATED LINOLEIC ACID

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5.1 ABSTRACT

Conjugated linoleic acid (CLA) reduces body weight and adipose mass in a variety of species. However, the mechanisms by which CLA depletes adipose mass are unclear. Therefore, the objective of this study is to elucidate mechanisms by which CLA reduces adipose mass. 6-week old, male ob/ob mice were fed either a control diet (CON) or a diet supplemented with 1.5% mixed isomer CLA (CLA) for 4 weeks. A third group of mice (LEPTIN) were fed the control diet and received daily, intraperitoneal injections of 1 mg/kg BW recombinant leptin as a positive control for adipose depletion in ob/ob mice. CLA significantly reduced body weight and epididymal white adipose tissue (WAT) mass compared to CON and LEPTIN. The mRNA levels of several markers of lipid oxidation were not altered by CLA or LEPTIN in WAT. The mRNA level of carnitine palmitoyltransferase-1b (CPT-1b) was significantly increased by CLA. Interestingly, CLA increased mRNA expression of two genes normally exclusively expressed in brown adipose tissue (BAT): uncoupling protein-1 (UCP-1) and Cidea. Concomitantly, CLA and LEPTIN increased mRNA expression of PPAR gamma coactivator-1α (PGC-1α), but did not affect mRNA expression of downstream targets nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (mtTFA), transcription factors involved in mitochondrial biogenesis. β3-adrenoceptor mRNA and phosphorylated-p38 mitogen activated protein kinase (MAPK) protein levels were not affected by CLA, but were
upregulated by LEPTIN. These data suggest the acquirement of BAT-like characteristics, such as increased CPT-1b, PGC-1α, and UCP-1, in WAT by CLA may facilitate the reduction of adipose mass by increasing mitochondrial oxidation and energy dissipation. Furthermore, CLA does not appear to increase UCP-1 mRNA through β3AR signaling.
Conjugated linoleic acid (CLA) is a group of dietary fatty acids that exist as positional and geometric isomers of linoleic acid [18:2 (n-6)]. CLA is found naturally in products derived from ruminants, such as dairy and beef, but is also commercially available as a mixed isomer, weight-loss supplement. A mixture of CLA isomers (cis-9, trans-11 and trans-10, cis-12), but specifically t10c12-CLA significantly reduces adipose tissue in a variety of species (9-12). The reduction of adipose by CLA is most likely through a combination of mechanisms including apoptosis, decreased preadipocyte differentiation and lipogenesis, and increased fatty acid oxidation and energy expenditure (151).

Increased energy expenditure by CLA is of particular interest, specifically through increased expression of uncoupling proteins (UCPs). UCPs are a family of mitochondrial proteins that share considerable homology with UCP-1. UCP-1, uncouples respiration, producing heat instead of ATP, thereby defining its role in thermogenesis. UCP-1, which is expressed almost exclusively in brown adipose tissue (BAT), has an implicated role in preventing increased adiposity, as mice with genetic ablation of BAT become obese (72) and stimulation of UCP-1 prevents or reduces adiposity (75; 77-81). The functions of UCP-2 and UCP-3 are less clear, although both have been shown to uncouple respiration and have been associated with reduction of lipid accumulation.
Several studies report CLA decreases adipose mass concomitant with increased UCP-1 and/or -2 expression in WAT (15; 138; 176; 177; 205; 206). However, the data are inconsistent as some studies show either no change (16; 266) or a decrease (175) in expression of UCPs simultaneous with reduced WAT mass. Nevertheless, two independent microarray analyses of WAT from mice fed CLA report that UCP-1 and -2 were among genes most differentially expressed (176; 177). An increase in UCPs in WAT may have considerable roles, along with the aforementioned mechanisms, in the CLA-mediated reduction of adiposity.

Leptin, an adipokine produced by WAT, also induces delipidation through WAT apoptosis, increased fatty acid oxidation, and decreased lipogenesis. Supplementation with exogenous leptin increases energy expenditure through induction of UCP-1 and PGC-1α expression in WAT, likely via a β-3 adrenoceptor mediated pathway. In the present study, we use leptin as a positive control to further decipher mechanisms by which CLA reduces WAT mass. Genetically obese, leptin-deficient ob/ob mice were used to control for leptin levels as CLA dramatically decreases leptin concentrations within days of supplementation.
5.3 RESEARCH DESIGN AND METHODS

Experimental design. 6-week old, male B6.V-Lepob/OlaHsd (ob/ob) mice (Harlan, Indianapolis, IN) were housed 4/cage at 22°C ± 0.5°C on a 12-hour light/dark cycle. Diets were isocaloric, modified AIN-93G diets (Bio-Serv, Frenchtown, NJ) containing 6.5% fat by weight. Mice received either a control diet that contained 6.5% soybean oil (CON; n=8) or a CLA-supplemented diet containing 5% soybean oil and 1.5% CLA mixed triglycerides (CLA; n=8). CLA mixed triglycerides (Tonalin TG 80, Cognis Corp., Cincinnati, OH) were ~80% CLA composed of 39.2% c9t11- and 38.5% t10c12-CLA isomers. A third group of mice received the control diet and an intraperitoneal injection of 1 mg/kg BW recombinant mouse leptin (R&D Systems, Minneapolis, MN) (LEPTIN; n=8). CON and CLA mice received injections of similar volumes of the vehicle (PBS). Mice were injected daily, two hours before the onset of the dark cycle. At 4 weeks, after an overnight (12 hr) fast, mice were anesthetized with isoflurane and blood was collected via cardiac puncture. Livers, epididymal adipose, and gastrocnemius muscles were quickly harvested, weighed, snap-frozen with liquid nitrogen, and stored at -80°C until analyses. All procedures were in accordance with institution guidelines and approved by the Institutional Animal Care and Use Committee of The Ohio State University.
**Analysis of triglycerides and free fatty acids.** Serum free fatty acids were determined using a colorimetric kit (NEFA C, Wako Chemicals, Richmond, VA). Lipids were extracted from tissues with 2:1 (v/v) chloroform and methanol. Final extracts were solublized in 3:1:1 (v/v/v) tert-butanol, methanol, Triton X-100 (250). Tissue lipid extracts and serum were analyzed for triglycerides or glycerol by colorimetric enzymatic hydrolysis of triglycerides to glycerol (Triglyceride, Free-Glycerol reagents, Sigma, St. Louis, MO). Data are reported as glycerol equivalents.

**Real-time RT-PCR.** mRNA was extracted from epididymal adipose tissue using the RNeasy® Lipid Tissue Mini kit (Qiagen, Valencia, CA) according to manufacturers’ protocols. mRNA was reverse transcribed with High Capacity cDNA Archive Kit (ABI, Foster City, CA) according to directions and then amplified by real-time PCR using pre-designed and validated primers (FAM probes) under universal cycling conditions defined by ABI (TaqMan Gene Expression Assays, ABI, Foster City, CA). Target gene expression was normalized to the endogenous control 18s (VIC probe) amplified in the same reaction and expressed as $2^{-\Delta\Delta ct}$ relative to the CON group (251).

**Western blot analysis.** Adipose tissue was homogenized in 3 volumes of ice cold lysis buffer (20 mM Trizma base, 50mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM Na4P2O7 . 10H2O, 1% Triton-X100, and protease inhibitors). Protein concentrations were measured by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Protein (40 µg) was mixed and boiled for 5 min with 4X loading buffer (125 mM Tris HCl at pH
6.8, 50% glycerol, 4% SDS, 0.02% Bromophenol Blue) and β-mercaptoethanol and subjected to SDS-PAGE using 10% gels for 1 h. Protein was then transferred to 0.45 µm nitrocellulose membranes for 1 h at 120 V on ice. Membranes were probed for P-AMPK or P-p38 (Cell Signaling Technology, Inc., Danvers, MA) according to manufacturer’s protocol. Membranes were incubated in SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) for 5 min. Densities of bands were detected and measured by Kodak ImageStation 2000RT using 1D Kodak software. Membranes were stripped with Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL) for 30 min at RT and reprobed with total-AMPK or –p38 (Cell Signaling).

**Statistical analyses.** Data are expressed as least square mean (LSM) ± standard error (SE). Data were analyzed by one-way ANOVA using the GLM procedure of Statistical Analysis System (SAS v9.1; SAS Institute Inc., Cary, NC). Differences of P<0.05 were considered significant.
5.4 RESULTS

*CLA decreased body weight and adipose mass, but did not induce ectopic lipid accumulation.* Prior to beginning experimental diets, all groups had similar average body weights (32.4-32.5 g). After 4 weeks, CON and LEPTIN groups significantly gained body weight from beginning weights (P<0.0001 and P=0.012, respectively, paired t-tests); whereas CLA significantly reduced body weights of mice (P<0.0001, paired t-test). Final body weights (Table 5.1) were significantly different among all groups. CLA significantly reduced body weights compared to both the CON and LEPTIN groups, while LEPTIN significantly decreased body weights compared to CON. Epididymal adipose masses paralleled body weights (Table 5.1). CLA did not affect liver or gastrocnemius muscle weights. Lipid concentrations in the liver and muscle also did not significantly change with CLA (Table 5.1). LEPTIN, however, decreased liver mass, reflected by a reduction in hepatic triglyceride levels, and increased gastrocnemius muscle mass but decreased muscle triglycerides (Table 5.1).
Table 5.1. Effects of CLA on tissue weights and triglyceride concentrations.

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<th>CON</th>
<th>LEPTIN</th>
<th>CLA</th>
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<tr>
<td>Final body weight (g)*</td>
<td>42.20 ± 0.99&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34.79 ± 0.99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.98 ± 0.99&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epididymal adipose (g)*</td>
<td>2.96 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.16 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.71 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Liver (g)*</td>
<td>2.76 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.29 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.18 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver triglycerides (mg TG/ g tissue)*</td>
<td>172.91 ± 13.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.03 ± 14.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>173.31 ± 13.61&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Gastrocnemius muscle (g)</td>
<td>81.40 ± 8.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>112.09 ± 8.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.08 ± 8.61&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Muscle triglycerides (mg TG/ g tissue)</td>
<td>52.52 ± 5.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.51 ± 5.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.40 ± 5.87&lt;sup&gt;ab&lt;/sup&gt;</td>
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<sup>a</sup>Also reported in Chapter 4, Table 4.1 and Fig. 4.4A.<br/>
<sup>abc</sup> Values represent LSM ± SE (n=7-8) with significant differences (p<0.05) within row denoted by different superscripts.
**CLA did not alter markers of lipolysis.** Two markers of lipolysis from adipose are elevated levels of non-esterified fatty acids (NEFA) and glycerol in the serum. These products of lipolysis and serum triglycerides were not altered by CLA or LEPTIN after 4 weeks of treatments (Table 5.2). Expression of adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL), two prominent lipases involved in lipolysis in adipose tissue, was measured in WAT. CLA did not change ATGL but decreased HSL mRNA expression compared to CON (Table 5.2). LEPTIN did not change HSL but increased ATGL mRNA expression compared to both CON and CLA.
Table 5.2. Effects of CLA on markers of lipolysis.

<table>
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<tr>
<th></th>
<th>CON</th>
<th>LEPTIN</th>
<th>CLA</th>
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<tr>
<td>Serum triglyceride (mg/dl)</td>
<td>88.13 ± 16.16</td>
<td>122.94 ± 14.00</td>
<td>131.45 ± 16.16</td>
</tr>
<tr>
<td>Serum NEFA (mEq/L)</td>
<td>0.90 ± 0.09</td>
<td>0.83 ± 0.09</td>
<td>1.03 ± 0.09</td>
</tr>
<tr>
<td>Serum glycerol (mg/dl)</td>
<td>56.95 ± 8.32</td>
<td>55.98 ± 7.21</td>
<td>36.88 ± 10.19</td>
</tr>
<tr>
<td>WAT ATGL mRNA*</td>
<td>1.05 ± 0.09a</td>
<td>1.39 ± 0.09b</td>
<td>1.03 ± 0.09a</td>
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<tr>
<td>WAT HSL mRNA*</td>
<td>1.07 ± 0.09b</td>
<td>1.18 ± 0.09b</td>
<td>0.60 ± 0.09a</td>
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</table>

* Relative expression
abc Values represent LSM ± SE (n=4-8) with significant differences (p<0.05) within row denoted by different superscripts.
**CLA increased mRNA expression of markers of fatty acid uptake (CD36), β-oxidation (Cpt-1b).** Fig. 5.1A shows that lipoprotein lipase (LPL) is significantly decreased by both CLA and LEPTIN, whereas, fatty acid transporter (FAT/CD36) mRNA, a marker of fatty acid uptake, is significantly increased by CLA and LEPTIN. Both CLA and LEPTIN decreased mRNA of sterol receptor element binding protein-1 (SREBP-1), a marker of lipogenesis, but only LEPTIN decreased mRNA of its downstream target, fatty acid synthetase (FASN). The mRNA levels of several markers of fatty acid oxidation, acetyl coA oxidase-1 (ACOX-1), carnitine palmitoyltransferase-1α (CPT-1α), and peroxisome proliferator activated receptor-α (PPARα), were not significantly altered by CLA or LEPTIN (Fig 5.1A). However, CPT-1β mRNA, a marker of mitochondrial β-oxidation, is significantly increased by CLA nearly 25-fold over CON and 20-fold over LEPTIN (Fig. 5.1B).
Fig. 5.1. Effects of CLA on markers of lipid metabolism in WAT. (A) mRNA expression of markers of LPL, lipid uptake (CD36), lipogenesis (SREBP-1 and FASN), and lipid oxidation (ACOX-1, CPT-1a, and PPARα). (B) CPT-1b mRNA expression, a marker of mitochondrial β-oxidation. Mice were fed a control diet and treated with vehicle (CON) or leptin (LEPTIN) or fed a CLA-supplemented diet and treated with vehicle (CLA). After 4 weeks, mRNA was measured from WAT of fasted mice by real-time RT-PCR. Data are expressed as $2^{-\Delta\Deltact}$ relative to the 18s endogenous control and the CON group. Values represent LSM ± SE (n=8) with significant differences (p<0.05) denoted by different superscripts.
To determine the involvement of upstream effectors of β-oxidation, we examined levels of phospho- (P-) and total-AMPK in WAT. AMPK has an essential role in energy homeostasis, particularly in fatty acid metabolism. When phosphorylated, activated AMPK drives β-oxidation and inhibits lipogenesis, primarily through phosphorylation and inactivation of ACC. CLA and LEPTIN increased the ratio of phosphorylated-AMPK relative to total-AMPK compared to CON (Fig. 5.2); however, these increases were not significant. Further, the downstream target of AMPK P-ACC / total-ACC was not significantly increased by either CLA or LEPTIN (data not shown).
**Fig. 5.2.** Effect of CLA on activation of AMPK in WAT. Mice were fed a control diet and treated with vehicle (CON) or leptin (LEPTIN) or fed a CLA-supplemented diet and treated with vehicle (CLA). After 4 weeks, expression of P-AMPK and total-AMPK were measured from WAT of fasted mice by SDS-PAGE and Western blot analysis. A representative blot is shown. Values represent LSM ± SE (n=8) with significant differences (p<0.05) denoted by different superscripts.
CLA increased UCP-1 and UCP-2 mRNA expression in WAT. After 4 weeks, UCP-1 mRNA expression was induced nearly 64-fold over CON by CLA (Fig. 5.3A). As expected, LEPTIN also increased UCP-1 expression, ~43-fold over CON. CLA increased UCP-2 mRNA expression compared to both CON and LEPTIN (Fig. 5.3B). CLA, however, did not alter levels of UCP-3 mRNA expression (Fig. 5.3C), a UCP homolog predominantly found in muscle, whereas LEPTIN increased UCP-3 expression ~2.6-fold.
Fig. 5.3: Effect of CLA on UCP expression in WAT. (A) UCP-1; (B) UCP-2; and (C) UCP-3 mRNA were measured from WAT of fasted mice by real time RT-PCR. Data are expressed as $2^{-\Delta\Delta ct}$ relative to the 18s endogenous control and the CON group. Values represent LSM ± SE (n=7-8) with significant differences (p<0.05) denoted by different superscripts.
Effects of CLA on markers associated with BAT and mitochondrial biogenesis.

Because UCP-1 is normally only expressed in BAT, but was induced by CLA in WAT, we assessed other markers associated with BAT and mitochondrial biogenesis. Cidea (cell-death-inducing DFF45-like effector) mRNA is expressed in several tissues at low levels; however, both Cidea mRNA and protein is expressed at high levels in BAT and is therefore considered a BAT-specific marker (267). Concomitant with an increase in UCP-1, CLA significantly increased Cidea mRNA expression (Fig. 5.4A). Although LEPTIN also increased UCP-1, it did not affect levels of Cidea. Parallel to increases in UCP-1, both CLA and LEPTIN increased PGC-1α mRNA expression (Fig. 5.4B). Despite increased PGC-1α expression, CLA and LEPTIN did not alter mRNA expression of either nuclear respiratory factor-1 (NRF-1) (Fig. 5.4C) or mitochondrial transcription factor A (mtTFA) (Fig. 5.4D).
Fig 5.4: Effects of CLA on markers associated with BAT and mitochondrial biogenesis. (A) Cidea; (B) PGC-1α; (C) mtTFA; and (D) NRF-1 mRNA were measured from WAT of fasted mice by real time RT-PCR. Data are expressed as $2^{-\Delta\Delta Ct}$ relative to the 18s endogenous control and the CON group. Values represent LSM ± SE (n=7-8) with significant differences (p<0.05) denoted by different superscripts.
Effects of CLA on markers of β-3 adrenergic signaling. Increased UCP-1 transcription may be mediated through β3 adrenergic signaling. We, therefore, measured transcript levels of β3 adrenoceptor (β3AR). CLA did not alter levels of β3AR (Fig. 5.5A), but LEPTIN increased β3AR mRNA expression ~4.3-fold over CON. Phosphorylation of p38 mitogen activated protein kinase (MAPK) is a crucial step in β3 adrenergic induction of PGC-1α and, in turn, UCP-1. Similar to the trend in β3AR expression, LEPTIN increased P-p38 / total-p38 MAPK protein expression compared to CON and CLA (Fig. 5.5B). The increase was only significant compared to CLA.
Fig. 5.5: Effects of CLA on markers of β-3 adrenergic signaling. (A) β-3 adrenoceptor mRNA expression was measured from WAT of fasted mice by real time RT-PCR. Data are expressed as $2^{-\Delta \Delta CT}$ relative to the 18s endogenous control and the CON group. (B) P-p38 protein expression. Expression of P-p38 and total-p38 were measured from WAT of fasted mice by SDS-PAGE and Western blot analysis. A representative blot is shown. Values represent LSM ± SE (n=7-8) with significant differences (p<0.05) denoted by different superscripts.
5.5 DISCUSSION

The ability of CLA to significantly reduce adipose mass in a variety of species has received considerable attention. However, the mechanisms by which CLA induces WAT loss remain unclear. In the present study, we report that CLA increased markers associated with the functions of BAT in WAT, particularly those involved in the uncoupling of oxidative phosphorylation.

CLA rapidly and significantly reduces body weight and adipose mass in as little as 6 days in C57Bl/6J mice (18; 159). However, hepatomegaly and hepatic lipid accumulation (14; 15; 213) may also occur with CLA supplementation. This redistribution of lipid, coupled with increased lipolysis, as reported in several in vitro studies (12; 144; 193) may partially account for the loss of WAT mass. Here, we report CLA decreased body weight paralleled by a reduction in epididymal WAT. These changes were not accompanied by ectopic lipid accumulation in the liver or muscle or with increased serum NEFA or glycerol concentrations. This suggests that, after 4 weeks of CLA supplementation, lipids were not being mobilized from the adipose by increased lipolysis and accumulating or being oxidized in other tissues, as markers of fatty acid oxidation were not increased in these other tissues (data not shown). Additionally, mRNA expression of HSL and ATGL were not increased in WAT, which further supports previous in vivo data suggesting CLA
does not mediate adipose loss by increased lipolysis (191; 192). CLA decreased LPL, an enzyme produced by adipose that catalyzes the degradation of circulating triglycerides into fatty acids, which can then be transported into tissues via diffusion or fatty acid transporters such as FAT/CD36 (268). The reduction of LPL by CLA supports previous findings (190; 192; 194). Interestingly, FAT/CD36 mRNA expression was significantly increased by CLA-supplementation. LPL and FAT/CD36 work in concert to increase fatty acid uptake into tissues, therefore the contradictory expression of LPL and FAT/CD36 in WAT by CLA is unclear, but this trend was also observed in ob/ob mice receiving daily leptin treatment.

Despite the decrease in LPL, CLA did not seem to decrease WAT lipogenesis in this study. CLA significantly reduced transcript levels of SREBP-1 but did not affect levels of its downstream target FASN. Further, CLA did not alter protein expression of P-ACC, the inactive form of ACC which would shift fatty acids towards β-oxidation rather than esterification. CLA generally reduces markers of lipogenesis, (15; 143; 176; 177; 195), however, the lack of change in markers of lipogenesis in WAT of this study suggests lipogenesis may not have an essential role in CLA depletion of adipose, especially in a system in which there does not seem to be significant ectopic lipid accumulation. This leads us to believe that CLA may be depleting adipose primarily through increased energy expenditure.
Previous studies demonstrated that CLA increases fatty acid oxidation in adipose in both *in vitro* and *in vivo* models, but the results are somewhat contradictory. McIntosh’s group reported that CLA increased fatty acid oxidation in 3T3-L1 preadipocytes (144) but later showed CLA decreased fatty acid oxidation in human adipocytes derived from primary stromal vascular cells (171). Park et al (12) demonstrated that CLA increased CPT activity in WAT of fed, but not fasted mice. Others (165; 200) have reported increased CPT activity in rats fed CLA. Analyses of differential gene expression in WAT of CLA-fed mice show a general increase in mRNA expression of genes involved in fatty acid oxidation, most notably, both CPT-1a and CPT-1b (176; 177). We did not find significant differences in either CPT-1a or Acox1, genes involved with mitochondrial and peroxisomal fatty acid oxidation, respectively, in adipose. However, CPT-1b expression was significantly increased by CLA but not by leptin treatment. CPT-1b, while expressed in WAT, is highly expressed in mitochondria-rich BAT (269), and transports fatty acids into the mitochondria for oxidation. The protons created from fatty acid oxidation could then either drive ATP generation, as in normal oxidative phosphorylation, or be dissipated by UCPs, producing heat instead of ATPs. Accordingly, as reported in previous studies, we observed increases in both UCP-2 (15; 138; 176; 177; 205; 206) and UCP-1 (176; 177; 206) mRNA expression in WAT of mice fed CLA. Leptin increased UCP-1, but not UCP-2 expression, supporting previous research (270). Uncoupled energy expenditure is generally used for non-shivering thermogenesis, however, the evidence for a role for UCPs in adipose depletion is mounting. While several groups have shown that UCP-1 deficient mice do not become obese (72-74), others show that either overexpression of
UCP-1 in WAT (75) or stimulation with β3-adrenergic agonists (77-81) prevent or reverse obesity in rodents. Stimulation of β3AR and subsequently increased UCP-1 may affect WAT lipid accumulation by impairing adipogenesis through depression of PPARγ and aP2 (84). As aforementioned, both leptin and CLA decreased mRNA levels of PPARγ as well as aP2 in WAT (data not shown). Furthermore, upon stimulation with β3AR agonists or with leptin, WAT adopts BAT-like characteristics, including increased UCP-1 expression (77-79; 83) increased mitochondrial biogenesis (82; 83) and a multilocular morphology (77-79; 82; 83). Interestingly, we show that Cidea mRNA expression, which is normally restricted to BAT (267), was increased several-fold by CLA. Leptin, conversely, did not increase Cidea expression despite increasing UCP-1 and PGC-1α, which confirms a previous report (271). The increased expression of CPT-1b, UCP-1, PGC-1α, and Cidea suggests that CLA may transform white adipose cells or influence preadipocytes in WAT into more oxidative brown adipocytes or that CLA induces hypertrophy of brown adipocytes within WAT. This conversion could contribute to the depletion of adipose by CLA.

PGC-1α is an important coactivator in both UCP-1 upregulation (79) and the increase of mitochondrial biogenesis (85). Despite increased transcript levels of PGC-1α, we did not see an increase in either NRF-1 or mtTFA mRNA expression, two markers of mitochondrial biogenesis. PGC-1α stimulates transcription and coactivates transcriptional function of NRF-1, a transcription factor which then activates the transcription of numerous genes involved in mitochondrial function and replication,
including mtTFA, which induces replication of mitochondrial DNA (85). The lack of changes in these markers by CLA and leptin suggests after 4 weeks of treatments, it is unlikely mitochondrial biogenesis was actively occurring.

\(\beta_3\)AR have important roles in the regulation of energy expenditure, particularly by inducing UCP-1. The \(\beta_3\)AR-mediated stimulation of UCP-1 is dependent on a cascade of events, notably activation of p38 MAPK (97; 98). Stimulation of the sympathetic nervous system by leptin likely increases transcription of UCP-1 through \(\beta_3\)AR. Appropriately, we report both \(\beta_3\)AR mRNA expression and P-p38 MAPK protein expression are increased in WAT of ob/ob mice, confirming the involvement of \(\beta_3\)AR signaling in the leptin-mediated increase in UCP-1. CLA, however, did not alter expression of either of these markers, suggesting \(\beta_3\)AR signaling may not be contributing to CLA-induced UCP-1 expression. The lack of effect after 4 weeks of CLA-supplementation does not rule out involvement of \(\beta_3\)AR in earlier events.

In summary, this study demonstrates that the depletion of adipose by CLA is accompanied by increased expression of several markers of uncoupled energy dissipation. The acquirement of brown adipose-like characteristics, such as increased CPT-1b, PGC-1\(\alpha\), and UCP-1, by WAT in CLA-fed mice may facilitate the reduction of adipose by increasing mitochondrial oxidation and energy dissipation. Furthermore, CLA does not appear to increase UCP-1 through \(\beta_3\)AR signaling, but perhaps by some other mechanisms, for which future studies are needed. The increased understanding of the
mechanisms by which unique fatty acids, such as CLA, can enhance energy expenditure in white adipose tissue may contribute to the development of therapeutic prevention and treatment of obesity and type 2 diabetes.
We thank all of the members of the Belury lab for their assistance with care and feeding of mice and discussions of the work in this manuscript. This work was supported by funds from the Carol S. Kennedy professorship, the Ohio Agriculture Research and Development Center (OARDC), USDA, scholarships from the College of Human Ecology, the Natural Health Research Institute Scholarship for Diabetes, The American Oil Chemists’ Society, and the J. Parker and Kathryn Webb Dinius Fellowship.
Conjugated linoleic acid (CLA) significantly decreases body weight primarily through a reduction of adipose tissue in a variety of species. CLA also improves glucose tolerance and hepatic steatosis in rat models of obesity and diabetes. However, CLA induces lipodystrophic-like insulin resistance in mice accompanied by decreased adipose mass, significant and rapid depletion of the adipokines leptin and adiponectin, and increased hepatic steatosis. The research presented investigated the roles of adipokines in CLA-mediated effects and the mechanisms by which CLA depletes adipose tissue.

The first study demonstrated that CLA improved hepatic steatosis and fatty acid composition associated with improved glucose tolerance. The changes in hepatic fatty acid composition suggested the improved hepatic steatosis may, in part, be due to decreased stearoyl-CoA desaturase-1 activity. We can also presume that the improvements in glucose tolerance and hepatic steatosis are independent of changes in leptin receptor mediated effects because ZDF rats lack functioning leptin receptors. How CLA improves insulin resistance, hyperinsulinemia, and hepatic steatosis in rats has yet
to be determined. While the modest reduction in adipose has been suggested as a contributing factor, our group has recently shown that CLA improves hepatic steatosis in Wistar rats without altering body weight, adiposity, insulin, or adipokine levels (137). This suggested CLA acts directly hepatic lipid metabolism to reduce lipid accumulation. While Peters et al (206) showed that the increase in markers of hepatic fatty acid oxidation are dependent on the activation of PPARα by CLA, future studies are needed to determine how CLA suppresses SCD-1 and other lipogenic genes. The suppression may be similar to other PUFAs which decrease SREBP-1 transcription by interfering with liver X receptor (LXR). It may also be through direct post-translation modification of SCD-1 by CLA, as Choi et al has suggested (360).

In the second study, CLA did not worsen insulin resistance or hyperinsulinemia in the presence of leptin but hepatic steatosis was increased. One would assume hyperinsulinemia induced by CLA may likely supersede any direct effects of CLA (as previously discussed) and induce hepatic lipid accumulation by inducing lipogenic factors such as SREBP-1. However, in our study, the increased insulinemia by CLA did not increase hepatic steatosis in the absence of leptin. In the presence of leptin, CLA induced hepatic steatosis despite not significantly increasing insulin levels. In both cases, neither SREBP-1 nor FAS mRNA expression was increased. It is important to note that livers were already grossly steatotic in the absence of leptin, which may have prevented more accumulation even in the absence of leptin. In the presence of leptin, the increase in insulin levels by CLA was not statistically significant, but the question remains if the
increased insulin levels were physiologically significant. Future studies are needed to determine the underlying cause of the development of hepatic steatosis by CLA.

Based on the results from the second study, the final study asked the question, how does CLA induce delipidation and reduce adipose mass? Results from this study, such as no changes in lipolysis and liver or muscle triglycerides, demonstrated that the lipid from adipose was not likely being mobilized and accumulating or being oxidized in other tissues. Increases in mRNA levels for carnitine palmitoyltransferase-1b (CPT-1b) and uncoupling protein-1 (UCP-1) in the white adipose mass suggested that the energy from lipids was being dissipated by uncoupled oxidative phosphorylation in the mitochondria. Further, because these gene products are usually considered markers specific to brown adipose tissue, it seems that increased energy expenditure in WAT may mimic that usually reserved for BAT and contribute to the depletion of adipose tissue by CLA. Results from this study present a multitude of questions to be answered, but the most pressing question is how and why is CLA increasing UCP-1. We provide evidence suggesting that CLA does not appear to be increasing UCP-1 expression through $\beta_3$AR signaling. The induction of expression of UCP-1 and Cidea in white adipose suggests that CLA may be facilitating the transformation of white adipose to brown adipose or preferentially inducing differentiation of brown adipose preadipocytes already present in the white adipose. Future research will be required to determine if the increased UCP-1 is consequence of simply more brown adipocytes, or if CLA is directly activating UCP-1 and the increase in Cidea is a response to the increased UCP-1. Future studies are also
needed to determine if CLA is modulating transcription factors, such as PPARγ, and co-modulators such as PGC-1α, or RIP140, involved in the transcription of UCP-1. Finally, further work will be required to determine why CLA induces these changes. It is possible that CLA induces some precursory event that releases an abundance of substrate (ie. lipid), increasing β-oxidation and eventually limiting ADP for ATP synthesis. Reduced ADP could upregulate UCP-1 as a compensatory mechanism to dissipate the proton gradient.


162. **Naumann E, Carpentier YA, Saebo A, Lassel TS, Chardigny JM, Sebedio JL and Mensink RP.** Cis-9, trans-11 and trans-10, cis-12 conjugated linoleic acid (CLA) do not affect the plasma lipoprotein profile in moderately overweight subjects with LDL phenotype B. *Atherosclerosis* 188: 167-174, 2006.


269. **Esser V, Brown NF, Cowan AT, Foster DW and McGarry JD.** Expression of a cDNA isolated from rat brown adipose tissue and heart identifies the product as the muscle isoform of carnitine palmitoyltransferase I (M-CPT I). M-CPT I is the predominant CPT I isomorph expressed in both white (epididymal) and brown adipocytes. *J Biol Chem* 271: 6972-6977, 1996.


APPENDIX A

ADDITIONAL DATA FROM THE OB/OB STUDY
Figure A.1. Effects of CLA and leptin on WAT gene expression of markers of adipose differentiation and glucose uptake. (A.) PPARγ. (B.) Pref-1 (preadipocyte factor 1). (C.) GLUT-4, glucose transporter. mRNA expression is expressed as $2^{-\Delta\Delta Ct}$ relative to the 18s endogenous control and the CON- group. Mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either leptin (+) or vehicle (-) by IP injection daily for 4 weeks. Values represent LSM ± SE of n=6 to 8 mice/group with significant differences (P<0.05) denoted by different superscripts.
Factors include the main effects of diet and leptin treatment (Trt), and the interaction (Int) between diet and leptin treatment.

Table A.1. Hepatic fatty acid composition.
Figure A.2. Effects of CLA and leptin on hepatic UCP-2 mRNA expression. mRNA expression is expressed as $2^{\Delta\Delta Ct}$ relative to the 18s endogenous control and the CON-group. Mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either leptin (+) or vehicle (-) by IP injection daily for 4 weeks. Values represent LSM ± SE of n=6 to 8 mice/group with significant differences (P<0.05) denoted by different superscripts.
Figure A.3. Effects of CLA and leptin on lipid metabolism in gastrocnemius muscle. mRNA expression is expressed as $2^{-\Delta\Delta Ct}$ relative to the 18s endogenous control and the CON- group. Mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either leptin (+) or vehicle (-) by IP injection daily for 4 weeks. Values represent LSM ± SE of n=6 to 8 mice/group with significant differences (P<0.05) denoted by different superscripts.
Figure A.4. Effects of CLA and leptin on PPAR mRNA expression in gastrocnemius muscle. (A.) PPARδ. (B.) PPARγ. mRNA expression is expressed as $2^{-\Delta \Delta Ct}$ relative to the 18s endogenous control and the CON- group. Mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either leptin (+) or vehicle (-) by IP injection daily for 4 weeks. Values represent LSM ± SE of n=6 to 8 mice/group with significant differences (P<0.05) denoted by different superscripts.
Figure A.5. Effects of CLA and leptin on GLUT-4 mRNA expression in gastrocnemius muscle. mRNA expression is expressed as $2^{-\Delta\Delta C_T}$ relative to the 18s endogenous control and the CON- group. Mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either leptin (+) or vehicle (-) by IP injection daily for 4 weeks. Values represent LSM ± SE of n=6 to 8 mice/group with significant differences (P<0.05) denoted by different superscripts.
Figure A.6. Effects of CLA and leptin on UCP mRNA expression in gastrocnemius muscle. (A.) UCP-2. (B.) UCP-3. mRNA expression is expressed as $2^{\Delta\Delta Ct}$ relative to the 18s endogenous control and the CON- group. Mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either leptin (+) or vehicle (-) by IP injection daily for 4 weeks. Values represent LSM ± SE of n=6 to 8 mice/group with significant differences (P<0.05) denoted by different superscripts.
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$^a,b,c$Values represent LSM ± SE with significant differences (P<0.05) within row denoted by different superscripts.

$^1$Factors include the main effects of diet and leptin treatment (Trt), and the interaction (Int) between diet and leptin treatment.

Table A.2. Gastrocnemius muscle fatty acid composition.
ADDITIONAL METHODS

**Fatty acid analyses.** Lipids were extracted from sections of either liver or muscle tissue with 2:1 (v/v) chloroform and methanol. Fatty acid methyl esters of the lipid fractions were prepared by incubating the fractions with 1,1,3,3-tetramethylguanidine at 100°C and analyzed by gas chromatography using a 30-m Omegawax 320 capillary column. Helium flow rate was 30 ml/min and oven temperature was programmed to start at 175°C for 4 min and increase to 220°C at a rate of 3°C/min. Fatty acids were identified using authentic standards (Matreya Inc, Pleasant Gap, PA). Fatty acids were quantified by determining areas under identified peaks (ChemStation Software; Packard Instrument Company, Meriden, CT) and were expressed as a percentage of the total area of identified peaks.

**RNA extraction modification for muscle.** RNA was extracted from gastrocnemius muscle using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer’s protocol with the following modification: A high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) was used in place of half of the isopropanol to solublize proteoglycans.
INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of polyunsaturated fatty acids that exist as positional and stereo-isomers of linoleic acid (18:2). CLA is in ruminant-derived foods such as beef, lamb, and dairy products, and in dietary supplements such as Tonalin™. Commercially, CLA is marketed as a weight loss supplement and studies indicate CLA may induce actions to reduce carcinogenesis as well as modulate immune function and type 2 diabetes. Due to potential health benefits of CLA, a great deal of research has been devoted to this dietary oil. A great deal of the health properties of CLA may be explained in part by the ability of CLA to lower body fat. CLA appears to reduces the ability of adipocytes to accumulate fat. In some animal models this improves insulin resistance and in others it worsens insulin sensitivity \{255, 145, 203, 47, 461\}, (13; 15-17). In mouse models where CLA causes insulin resistance, severe lipodystrophy, mobilization of lipid from adipose to non-adipose tissue, such as the liver, occurs (15; 18; 235). This accumulation of excess lipid in the liver, known as hepatic steatosis contributes to increased insulin resistance and is accompanied by hepatomegaly, or enlargement of the liver (14; 15; 157). The impact and mechanism of the hepatomegaly induced by CLA has not been explored.

Hepatomegaly, resulting from CLA supplementation, has casually been attributed to increased hepatic steatosis. However, we recently reported that in rat models for hepatic steatosis, CLA improved hepatic steatosis, but did not reduce liver weight as would be
expected (137; 180). In mice, others have reported CLA significantly increased liver mass relative to body weight after only two days of supplementation; however, a significant increase in hepatic triglycerides did not occur until day 10 of supplementation (18).

In addition to excessive lipid storage, hepatic fibrosis also contributes to hepatomegaly. Hepatic fibrosis, or scarring, occurs when the normal wound-healing response of the liver becomes dysregulated and there is an excessive and harmful accumulation of extracellular matrix (ECM). This excessive accumulation of ECM promotes further injury to the liver and eventually cirrhosis. While previous research has characterized some of the mechanisms contributing to CLA-induced insulin resistance and hepatic steatosis, to our knowledge the effects of CLA on hepatic fibrosis have not been reported. Therefore, the objective of this research was to determine the extent and mechanism that conjugated linoleic acid (CLA) induces hepatic repair and/or fibrosis.
RESULTS AND DISCUSSION

Additional analyses of the livers from the ob/ob mice in the study described in Chapters 4 and 5 suggest that CLA may induce hepatic repair as indicated by an accumulation of collagen in livers of obese mice fed CLA. Fig. B.1A shows that type 1(a) collagen (col1a1) mRNA expression, which is commonly used as a marker for hepatic fibrosis, is over 2.5 fold higher in mice fed CLA than in control-fed mice, in the absence of leptin. The increase in col1a1 was associated with enlarged livers induced by CLA (Table 4.1), yet these mice lacked increases in hepatic lipid concentration (Fig. 4.4A). In addition, adipose depots and adiponectin levels of mice fed CLA were significantly reduced (Tables 4.1 and 4.2, respectively), indicating possible lipodystrophy. A critical step in the development of hepatic fibrosis is the transdifferentiation and activation of hepatic stellate cells (HSC). Activation of HSC includes increased proliferation and a shift to a myofibroblastic phenotype. As myofibroblasts, HSC begin producing extracellular matrix, such as collagen, resulting in fibrosis of the liver. Activation of HSC is often indicated by the expression of smooth muscle α-actin (SMAα) and production of type 1a collagen (col1a1) (272). However, in this study, CLA did not induce mRNA expression of SMAα, either in the absence or presence of leptin (Fig. B.1B).

Cytokines such as TGF-β, TNF-α and the adipokine adiponectin, are three key factors that are postulated to modulate activation of satellite cells. TGF-β is an essential cytokine for tissue repair and transcriptional modulation of α-SMA and Col1a1.
Activation of HSC increases synthesis of TGF-β and thus perpetuates collagen production and fibrosis (273). In a study examining the effect of CLA on inflammatory bowel disease, CLA was shown to induce TGF-β (184). TNF-α has an important counter-regulatory role in hepatic fibrosis. TNF-α inhibits the expression of collagen by competitively binding to a TNF-α response element in the col1a1 promoter and repressing transcription. This element overlaps with the TGF-β responsive element and thus, if one transcription factor is bound to the promoter region, the other cannot—decreasing TGF-β-induced collagen production (274). TNF-α also induces apoptosis—programmed cell death crucial to destroying uncontrolled cells like activated HSC, which is dysregulated in fibrosis (275). Several studies report a reduction of TNF-α by CLA (184; 235). Here, we show in the absence of leptin, CLA increased TGF-β mRNA expression (Fig. B.1D), as well as, its upstream regulator SMAD-7 (Fig. B.1C).

Adiponectin, may also have a significant role in the prevention of hepatic fibrosis. Low concentrations of adiponectin are associated with hepatic fibrosis and overexpression of adiponectin seems to have a protective effect against fibrosis (276). The dramatic reduction of adipose mass and adiponectin by CLA, in turn, may abolish the protective effects of adiponectin against fibrosis.

Our preliminary data showing increased liver weight, col1a1, TGF-β, and SMAD-7 and decreased adipose mass and adiponectin by CLA, support the possibility that CLA may induce hepatic repair.
Figure B.1. Effects of CLA and leptin on gene expression of markers of hepatic repair. (A.) Type 1(a) collagen (col1a1). (B.) Smooth muscle α-actin (SMAα). (C.) SMAD-7. (D.) Tumor growth factor-β (TGF-β). mRNA expression is expressed as $2^{-\Delta\Delta Ct}$ relative to the 18s endogenous control and the CON- group. Mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either leptin (+) or vehicle (-) by IP injection daily for 4 weeks. Values represent LSM ± SE of n=6 to 8 mice/group with significant differences (P<0.05) denoted by different superscripts.
APPENDIX C

PROTOCOLS
**Tissue Lysis**

**Preparation:**
- Lysis buffer:

<table>
<thead>
<tr>
<th>Final C</th>
<th>Compound</th>
<th>FW</th>
<th>for 1 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM</td>
<td>Trizma base</td>
<td>121.14</td>
<td>2.42 g</td>
</tr>
<tr>
<td>1%</td>
<td>Triton-X100</td>
<td>-</td>
<td>10 ml</td>
</tr>
<tr>
<td>50 mM</td>
<td>NaCl</td>
<td>58.44</td>
<td>2.92 g</td>
</tr>
<tr>
<td>250 mM</td>
<td>Sucrose</td>
<td>342.3</td>
<td>85.58 g</td>
</tr>
<tr>
<td>50 mM</td>
<td>NaF</td>
<td>41.99</td>
<td>2.10 g</td>
</tr>
<tr>
<td>5 mM</td>
<td>Na₄P₂O₇·10H₂O</td>
<td>446.1</td>
<td>2.23 g</td>
</tr>
</tbody>
</table>

**Prior to use, dissolve 1 protease inhibitor tablet (#11836153, Roche) per 10 ml lysis buffer (or per 7 ml for samples with high proteolytic activity)—good for 1-2 weeks stored at 4°C**

**Procedure:**
* keep everything on ice; raw tissues on dry ice

1. Cut tissue—record weight
   a. most analyses require at least 10 mg tissue: figure amount needed for all analyses and add extra
      i. For example, if doing GC, TG, and protein analyses:
         1. need 40 mg tissue, but cut ~60+ mg
   b. cut all samples—store on dry ice or at -80°C
2. Homogenize in exactly 10x (v/w) ice cold lysis buffer in a plastic test tube
   a. eg. 52.5 mg tissue + 525 µl lysis buffer
   b. this is so 1 mg tissue = 10 ul homogenate so that assays can be normalized to tissue weight
3. Immediately transfer homogenate to 2.0 ml microcentrifuge tube
4. Incubate tubes on the rocker in cold room for ~1 hr
5. Vortex; aliquot homogenate in 2 ml microcentrifuge tubes:
   Amounts needed for various analyses:
   a. 100 µl → GC analysis
   b. 100 µl → TG analysis
   c. 200 µl → TG/DAG/MAG separation
   d. 200 µl → NL/PL separation
   e. 200+ µl → protein (usually use all of the homogenate remaining)
6. Can be stored at -80°C
7. Use protein samples for Western blotting and FA extraction samples for FA extraction, TG assay, methylation, and GC analysis etc
Protein Assay

Materials:
- BCA Protein Assay kit (23225, Pierce)

Preparation:
1. Centrifuge ‘protein’ aliquot at 16.1k x g for 15 min at 4°C
2. Transfer supernatant to new tube

- Make a Working Reagent stock: Reagent A / Reagent B (50:1, v/v)
- Make standards in microcentrifuge tubes (Can be used again; store @ RT)

<table>
<thead>
<tr>
<th>Standard</th>
<th>Concentration (µg/ml)</th>
<th>BSA (µl)</th>
<th>DI water (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>300</td>
</tr>
<tr>
<td>1</td>
<td>125</td>
<td>18.75</td>
<td>281.25</td>
</tr>
<tr>
<td>2</td>
<td>250</td>
<td>37.5</td>
<td>262.5</td>
</tr>
<tr>
<td>3</td>
<td>500</td>
<td>75</td>
<td>225</td>
</tr>
<tr>
<td>4</td>
<td>750</td>
<td>112.5</td>
<td>187.5</td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>6</td>
<td>1500</td>
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<td>75</td>
</tr>
<tr>
<td>7</td>
<td>2000</td>
<td>300</td>
<td>0</td>
</tr>
</tbody>
</table>

Procedure:
1. Add 10 µl of standard to wells of a 96-well plate
2. Add _ (2) µl of sample to well
   - May have to adjust according to amount of protein in sample
3. Add 200 µl Working Reagent
4. Incubate @ 37°C for 30 min.
5. Read OD at 562 nm
Fatty Acid Extraction

Preparation:
- Make 0.88% KCl

Procedure:
1. Add 0.4 ml methanol (MeOH); vortex
2. Add 0.8 ml chloroform (CHCl₃); vortex
3. Incubate at 4°C for at least 30 min (on shaker is better) or overnight (can store in refrigerator or -20°C)
   a. transfer to glass vial if storing longer than overnight—chloroform will react with the plastic
4. Add 0.24 ml 0.88% KCl; vortex (can use 0.25 ml if using repeater pipet)
5. Centrifuge @ 1000 x g for 15 min @ 4°C
6. Transfer bottom, CHCl₃ layer to new 2 ml microcentrifuge tube or a glass test tube for GC analysis
7. Add 0.8 ml CHCl₃ to remaining layer; vortex
8. Centrifuge @ 1000 x g for 15 min @ 4°C
9. Transfer bottom, CHCl₃ layer to tube in step 6
10. Dry combined CHCl₃ layers under N₂
11. For GC analysis:
   a. Add 300 µl hexane and vortex if need to store (at -20°C)
   b. Otherwise continue with methylation
12. For TG assay:
   a. Add 200 µl (amt. will vary depending on tissue) tert-butanol:methanol:Triton X-100 (3:1:1, v/v/v); vortex
   b. centrifuge @ max briefly (~5 sec. just to get all sample down)
   c. TG assay
   d. Can store samples @ -20°C
13. For TG/DAG/MAG separation:
   a. Add 0.2 ml hexane-methylene chloride-ethyl ether (94.5:5:0.5, v/v/v)
      i. Can be stored @ -20°C
14. For NL/PL separation
   a. Add 1 ml MTBE: acetic acid (100:0.2, v/v) to dried fatty acids
      i. Can be stored @ -20°C
**FA Methylation**

**Materials:**
- 1,1,1,1-tetramethylguanidine (TMG)
- GC vials
- GC vial inserts

**Preparation:**
- Set up 100°C (boiling) water bath
- Cool centrifuge to 4°C
- Make methanolysis reagent (1 part TMG : 4 parts methanol)

**Procedure:**
1. Dry sample under N₂ if not already done
2. Add 0.40 ml methanolysis reagent to dried sample; cap and vortex
3. Heat in a 100°C water bath for 20 min.
4. Add 1 ml hexane and 0.5 ml 0.88% KCl to each sample; invert to mix gently
5. Centrifuge @ 1000 g for 10 min @ 4°C
6. Transfer the top hexane phase into a new tube
7. Add 1 ml hexane to the remaining bottom aqueous phase
8. Centrifuge @ 1000 g for 10 min @ 4°C
9. Transfer top phase to tube in Step 7
10. Dry combined top phases at RT under nitrogen
11. Add 300 µl hexane to completely dried samples; vortex; transfer to vial for GC
12. Store at -20°C until GC analysis
Triglyceride Assay

Materials:
- Glycerol Standard 250 mg/dl (Sigma G1394)
- Free Glycerol Agent (Sigma F6428)
- Triglyceride Reagent (Sigma T2449)

Preparation:
- Reconstitute Free Glycerol Agent with 40 ml DI water
- Reconstitute Triglyceride Reagent with 10 ml DI water
- Make a Working Reagent stock: Free Glycerol Agent / Triglyceride Agent (4:1, v/v)
- Make standards in microcentrifuge tubes (Can be used again—refrigerate)

<table>
<thead>
<tr>
<th>Standard</th>
<th>Concentration (mg/dl)</th>
<th>Glycerol (µl)</th>
<th>DI water (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
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<tr>
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</tr>
<tr>
<td>4</td>
<td>200</td>
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</tr>
<tr>
<td>5</td>
<td>250</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

Procedure:
1. Add 2 µl of standard to well of a 96-well plate
2. Add _ (2) µl of sample to well
3. May have to adjust according to amount of lipid in sample
4. Add 200 µl Working Reagent
5. Incubate on rocker for 15 min.
6. Read OD at 540 nm
Determining Tissue TG/DG/MG

Materials:
- diol bonded-phase columns (#52751-U, Supelco via Sigma)

Preparation:
- Hexane : methylene chloride : ethyl ether (89:10:1, v/v/v)
- Hexane : ethyl acetate (85:15, v/v)
- tert-butanol : methanol : Triton X-100 (3:1:1, v/v/v)

Procedure:
1. After FA extraction:
2. Place columns on vacuum
3. Wash column with 4 ml hexane (turn off vacuum before all hexane is eluted to prevent column from drying out)
4. Vortex sample
5. Add sample to column
6. Add 4 ml hexane-methylene chloride-ethyl ether (89:10:1, v/v/v)
   a. Collect 1st fraction in glass tube => TAG
7. Add 4 ml hexane-ethyl acetate (85:15, v/v)
   a. Collect 2nd fraction in glass tube => DAG
8. Add 4 ml ethanol
   a. Collect 3rd fraction in glass tube => MAG
9. Wash columns with 3 ml acetone and dry under N₂**
10. Dry fractions under N₂ until ~1.5 ml solvent remains
11. Transfer to GC vial
12. Completely dry sample under N₂
13. Add 100 µl tert-butanol: methanol: Triton X-100
14. Vortex; centrifuge @ max briefly (~5 sec. to get sample down)
15. TG assay

**columns can be used up to six times

Separation of Neutral and Phospholipids

Materials:
- PrepSep -Si columns (P478, Fisher)
- MTBE (443808, Sigma)
- Ammonium hydroxide (A6899, Sigma)
- Acetic acid (A6283, Sigma)
- Hexane (H292, Fisher)
- Methanol (A412, Fisher)

Preparation:
- Hexane : MTBE (96:4, v/v)
- MTBE : acetic acid (100:0.2, v/v)
- MTBE : methanol : ammonium acetate (5:8:2, v/v/v)
- 1 mM ammonium acetate (AmmAc)
  - 1 mM ammonium hydroxide / 1 mM acetic acid (2:1, v/v)
  - adjust pH to 8.6
  - Using reagents listed above:
    - 7.0 µl ammonium hydroxide
    - 6.0 µl acetic acid
    - 300 ml dH₂O

Procedure:
1. Condition column with 2 ml hexane: MTBE (96:4, v/v) followed by 4 ml hexane
2. Add 1 ml MTBE: acetic acid (100:0.2, v/v) to dried fatty acids = sample
3. Load sample into column very slowly
4. Wash sample vial with 1 ml MTBE: acetic acid (100:0.2, v/v) again to ensure all sample is out; load into column
5. Wash with 2 ml MTBE: acetic acid (100:0.2, v/v) to remove nonpolar lipids and fatty acids.
6. Collect all wash from steps 3 - 5 (4 ml total) in a test tube = neutral lipids
7. Wash with 4 ml MTBE: methanol: ammonium acetate (pH 8.6) (5:8:2, v/v/v) to remove polar lipids.
8. Collect wash in a test tube = phospholipids
9. Dry neutral lipids and phospholipids under N₂
   a. Store in hexane @ -20°C until ready for TMG methylation for GC analysis
   b. Otherwise continue with methylation

References:

APPENDIX D

T10C12-CLA INHIBITS DIFFERENTIATION, BUT NOT PROLIFERATION OF C2C12 CELLS
METHODS

**Cell culture.** C2C12 cells were obtained from American Type Culture Collection (Manassas, VA). At day 0, cells were plated at 2000 cells/cm³ in high-glucose (20 mM) DMEM + 10% FBS. The next day, media was changed to DMEM + 10% FBS with 25μM BSA and either 100 μM c9t11, t10c12-CLA, linoleic acid, or an equivalent volume (0.1%) of the vehicle DMSO. On day 3, media was switched to DMEM + 2% horse serum to induce differentiation.

**DAPI staining.** Cells were cultured in 12-well plates as described above. On the indicated day, cells were washed twice with cold, sterile PBS and then incubated in cold methanol for 10 minutes at room temperature. After 2 more washes with PBS, cells were incubated in 0.5 ml/well, 5 μg/ml 4’,6-diamidino-2-phenylindole (DAPI) for 15 minutes at 37°C. Cells were washed 2 more times with PBS and finally covered with 1 ml PBS. Cell morphology and nuclei staining were visualized at 200X magnification using an Olympus IX50 inverted fluorescent microscope equipped with a Pixera Pro 150ES camera.

**Cell proliferation.** Cells were cultured in 96-well plates as described above. To assess cell number, media was refreshed and 20 μl of CellTiter 96® AQeous One Solution Cell Proliferation Assay (Promega, Madison, WI) was added to each well. After incubation at 37°C for 60 minutes, the absorbance at 490 nm was measured. Absorbances were
corrected by subtracting the blank from each day. Absorbances from each day were normalized to day 1, and data were collected from triplicate wells/treatment/day and are expressed as viable cells relative to day 1.
Figure D.1. Effects of CLA on differentiation of C2C12 cells. Cells were treated with 100 µM of c9t11-CLA, t10c12-CLA, LA, or an equivalent volume of the vehicle, DMSO as described in Methods. On the specified day, cells were stained with DAPI and representative pictures were taken at 200X magnification under brightfield or fluorescence to visualize cell morphology or nuclei staining, respectively.
Figure D.2. Effects of CLA on proliferation of C2C12 cells. Cells were treated with 100µM of c9t11-CLA, t10c12-CLA, LA, or an equivalent volume of the vehicle, DMSO. Cell number was assessed each day in 3 wells/treatment as described in Methods. Arrow indicates day C2C12 were induced to differentiate.
APPENDIX E

LIPID LOADING C2C12 MYOCYTES
METHODS

Cell culture. C2C12 cells were obtained from American Type Culture Collection (Manassas, VA). At day 0, cells were plated at 2000 cells/cm\(^3\) in 6-well plates with high-glucose (20 mM) DMEM + 10% FBS. On day 3, media was switched to DMEM + 2% horse serum to induce differentiation. Media was refreshed every other day. On day 8, cells were fully differentiated and induced to accumulate lipid. Cells were treated with or without 1 µM insulin and with increasing concentrations of 200, 500, 750, 1000, or 2000 µM palmitic acid (PA) conjugated to BSA (4:1) in high glucose DMEM + 2% horse serum or with an equivalent volume of the vehicle DMSO for 12 hours. Cells were washed twice with cold PBS and harvested with 100 µl RIPA buffer with protease inhibitors. After freezing and thawing, cells were scraped into microcentrifuge tubes and centrifuged at 12,000 x g for 10 minutes. Protein concentrations were measured by BCA protein assay (Pierce). Lipids were extracted as described in APPENDIX C and triglycerides were measured by an enzymatic, colorimetric triglyceride assay (Sigma).
Figure E.1. Effects of insulin and palmitic acid on lipid accumulation in C2C12 myocytes. C2C12 myocytes were treated with or without 1µM insulin and with 0, 200, 500, 750, 1000, or 2000 µM palmitic acid for 12 h. Lipids were extracted and triglycerides were measured as described in Methods.
Figure E.2. Effects of insulin and palmitic acid on protein levels of C2C12 myocytes. C2C12 myocytes were treated with or without 1µM insulin and with 0, 200, 500, 750, 1000, or 2000 µM palmitic acid for 12 h. Protein was measured as described in Methods.