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Due to the increasing prevalence of obesity and diabetes among children and adults worldwide, it is imperative to identify dietary strategies that promote weight control. One potential anti-obesity compound is conjugated linoleic acid (CLA), sold worldwide for weight loss. However, the safety and efficacy of this supplement remains questionable.

Current research shows that, in addition to reducing fat mass, trans-10,cis-12 (10,12) CLA supplementation in animals and some humans leads to insulin resistance, hyperlipidemia, or fatty liver. *In vitro* studies suggest that 10,12 CLA causes inflammation in primary cultures of human adipocytes by increasing mitogen activated extracellular kinase/extracellular signal-related kinase (MEK/ERK) and nuclear factor kappa B (NFκB) signaling, which impairs glucose and fatty acid uptake and utilization. However, the link between inflammatory signaling in primary cultures of human adipocytes and CLA-mediated delipidation has not been fully characterized. Additionally, the particular cell type (i.e., preadipoyctes vs. adipocytes) responsible for 10,12 CLA-mediated inflammation and insulin resistance in white adipose tissue is unknown. Therefore, the objective of this work was to determine the cell type responsible for 10,12 CLA-mediated inflammation and insulin resistance in cultures of newly differentiated human adipocytes (Aim 1), and to identify the specific upstream

mechanisms involved (Aim 2). To examine Aim 1, inflammatory gene expression and protein secretion, prostaglandin secretion, or mitogen-activated protein kinase (MAPK) and cJun phosphorylation was measured in adipocytes vs. preadipocytes in four distinct cell culture models. To examine Aim 2, chemical inhibitors were employed to determine the role of protein kinases, including the MAPK cJun N-terminal kinase (JNK) and diacylglycerol kinases (DGKs) in 10,12 CLA-mediated inflammatory signaling and insulin resistance in newly differentiated primary human adipocytes. Collectively, results from this project reveal that 10,12 CLA instigates release of inflammatory signals from adipocytes that subsequently activate adjacent preadipocytes. Mechanisms of 10,12 CLA-mediated inflammatory signaling and insulin resistance involve activation of the protein kinases JNK and DGKs. These findings are expected to contribute critical insights for the development of safe and effective therapeutic strategies for weight control.

CONJUGATED LINOLEIC ACID PROMOTES INFLAMMATION TO A GREATER EXTENT IN HUMAN ADIPOCYTES COMPARED TO PREADIPOCYTES

by

Kristina Martinez

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Approved by

_____
Committee Chair

APPROVAL PAGE

This dissertation has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

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Date of Acceptance by Committee

_____
Date of Final Oral Examination

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CHAPTER I

INTRODUCTION

Overview

Obesity is one of the most prevalent nutrition-related diseases worldwide and in the U.S. Recently, it has been reported that worldwide, 1.5 billion are overweight and 500 million are obese (1). In the U.S., 68% are overweight or obese and 34% of adults are obese (2). This level of prevalence results in great economic burden, as the total economic cost of overweight and obesity was ~300 billion in the U.S. and Canada in 2009 (3). Despite efforts to reduce the prevalence of obesity, this epidemic continues to rise, as the percent of obese adults has risen from ~ 14% percent in 1980 to 34% percent in 2008 (2,4). Obesity, characterized by an expansion of white adipose tissue (WAT), is linked to chronic diseases such as type 2 diabetes, hypertension, and cardiovascular disease. Thus, maintaining ideal body weight will decrease the incidence of obesity-related disorders and health care costs associated with obesity.

One method to decrease body weight and fat mass is consumption of the commercially-available weight loss supplement conjugated linoleic acid (CLA). CLA, found naturally in ruminant meats and dairy products, refers to a group of conjugated octadecadienoic acid isomers that contain two double bonds separated by a single bond. Many isomers of CLA exist, but the two main isomers found to have biological activity

include the cis-9, trans-11 (9,11) isomer and the trans-10, cis-12 (10,12) isomer. Consuming a mixture of these isomers has been shown to decrease adiposity in animals and some humans. Our lab group and others have shown that of the two major isomers, the 10,12 isomer is responsible for CLA's anti-obesity properties. For example, we have demonstrated that 10,12 CLA, but not 9,11 CLA, decreases fatty acid and glucose uptake and triglyceride (TG) content of newly differentiated primary human adipocytes (5,6). However, 10,12 CLA increases inflammatory cytokine and prostaglandin secretion from adipocytes, resulting in adverse metabolic consequences such as insulin resistance (7,8,9).

Results from our lab suggest that 10,12 CLA-mediated delipidation is linked to inflammatory signaling and insulin resistance. For example, we have demonstrated that 10,12 CLA induces inflammation and insulin resistance via elevation of intracellular calcium levels (8), activation of extracellular signal-regulated kinase (ERK) (7), and nuclear factor kappa B (NFκB) (9), which upregulate inflammatory proteins such as tumor necrosis factor alpha (TNFα) and interleukin (IL)-6. Secretion of these cytokines attenuates the activation of transcription factors, such as peroxisome proliferator-activated receptor (PPAR)γ and sterol regulated enhancer binding protein (SREBP)-1c, that promote glucose and fatty acid uptake, fatty acid synthesis, and TG storage in adipocytes. However, the cell type in our cultures of newly differentiated primary human adipocytes responsible for initiating inflammatory signaling is unknown. Furthermore, the upstream signaling mechanisms activated by 10,12 CLA that trigger intracellular calcium accumulation, inflammatory protein activation (i.e., ERK and NFκB), and result

in insulin resistance and delipidation are unknown. Elucidating the signaling pathways activated by 10,12 CLA will provide valuable information on the efficacy, specificity, and potential side effects of CLA consumption for use as a weight loss agent. Lack of such knowledge is an important problem, because until it becomes available, CLA's potential use as a safe weight loss supplement cannot be evaluated. Therefore, the focus of this dissertation was to determine the cell type (i.e., preadipocyte or the adipocyte) responsible for 10,12 CLA-mediated inflammation and the mechanisms involved.

Central Hypothesis and Specific Objectives

The *central hypothesis* for the proposed research is that adipocytes are the major mediators of 10,12 CLA-induced inflammation, which in turn stimulate neighboring preadipocytes to produce inflammatory cytokines. These inflammatory agents antagonize PPARγ, leading to decreased lipogenesis and increased lipolysis in adipocytes.

In order to test this hypothesis, two specific aims were investigated using primary cultures of newly differentiated human adipocytes: **Aim 1**) Determine whether the preadipocyte or the adipocyte is responsible for 10,12 CLA–mediated inflammation, insulin resistance, and delipidation, and **Aim 2**) Identify the mechanism by which 10,12 CLA induces inflammation, insulin resistance, and delipidation.

CHAPTER II

REVIEW OF LITERATURE

Background and Significance

Obesity and White Adipose Tissue Inflammation

The incidence of obesity and diabetes has risen substantially over the past thirty years. Since 1976, the number of obese adults in America has approximately doubled (10). This modern rise in obesity often results from increased energy intake and decreased energy expenditure, leading to the storage of excess energy as triglycerides in white adipose tissue (WAT). Thus, obesity is characterized by the expansion of WAT which acts as an endocrine organ, secreting an array of adipokines that regulate a number of physiological processes including immunological responses, glucose homeostasis, appetite regulation, angiogenesis, and growth. Not surprisingly, over-expansion and dysregulation of WAT results in the development of serious metabolic disorders including hypertension, atherosclerosis, insulin resistance and type 2 diabetes. Notably, 80% of diabetic adults are also overweight or obese. This relationship between obesity and insulin resistance has been attributed to inflammation largely from WAT, but also from other tissues such as the liver and muscle.

One of the first reports of adipose-derived inflammation was in 1993, when Hotamisligil and Spiegelman reported that obese mice overexpressed tumor necrosis factor-α (TNF-α) in WAT (11). Based on these results, Kern et al. (12) showed that elevated TNFα gene and protein expression in WAT of obese humans significantly decreased after weight loss. Later in 1997, Uysal et al. (13) demonstrated that mice lacking TNFα were protected from insulin resistance, providing a link between inflammation in WAT and metabolic disease. Since these findings, a multitude of adipokines have been identified such as leptin, interleukin (IL)-8, IL-6, IL-1β, and monocyte chemoattractant protein (MCP)-1, all of which have been linked to insulin resistance (14). Thus, WAT, through secreting inflammatory adipokines, orchestrates a state of chronic, low-grade inflammation leading to the development of insulin resistance and diabetes.

One potential obesity treatment is conjugated linoleic acid (CLA), which has been shown to decrease body fat mass in animals and some humans. Initially identified as a potential anti-carcinogen, CLA has been reported to prevent obesity, diabetes, or atherosclerosis in different animal and cell models, depending on the doses, isomers, and models used. Potential mechanisms for preventing these diseases include inducing cancer cell apoptosis, increasing energy expenditure and delipidating adipocytes, increasing insulin sensitivity, or reducing aortic lesions, respectively. Ironically, potential side effects of CLA supplementation include chronic inflammation, particularly in adipose tissue, insulin resistance, and lipodystrophy (7, 9, 33, 53, 71, 93, 94, 95, 98). Results from our lab implicate a relationship between inflammatory signaling and delipidation in

cultures of newly differentiated primary human adipocytes. However, the exact mechanisms underlying this relationship have not been fully elucidated. Additionally, the cell type in cultures of newly differentiated adipocytes responsible for initiating the inflammatory response to CLA is unknown. Furthermore, more mechanistic studies are needed to better understand the true potential health benefits vs. risks of consuming CLA isomers, and their mechanisms of action.

Chemistry and synthesis of CLA

Natural Synthesis of CLA Isomers

CLA isomers are produced naturally in the rumen of ruminant animals by fermentative bacteria *Butyrovibrio fibrisolvens,* which isomerize linoleic acid into CLA isomers (**Fig. 2.1**). A second pathway of CLA synthesis in ruminants is in the mammary gland via delta 9-desaturation of trans-11, octadecanoic acid (15). Thus, natural food sources of CLA are dairy products including milk, cheese, butter, yogurt, and ice cream and ruminant meats such as beef, veal, lamb, and goat meat (**Table 2.1**). The cis-9, trans-10 (9,11) isomer (i.e., rumenic acid) is the predominate CLA isomer in these products (~80%), whereas the trans-10, cis-12 (10,12) isomer represents about 10%. Although several other isoforms of CLA have been identified, the 9,11 and 10,12 isomers appear to be the most biologically active (16). Levels of CLA isomers in ruminant meats or milk can be augmented by dietary manipulation, including feeding cattle on fresh pasture (17) or by adding oils rich in linoleic acid (e.g., safflower oil) or ingredients that alter biohydrogenation of linoleic acid (e.g., ionophores) to their diet (18).

Chemical Synthesis of CLA Isomers

Because of the relatively low levels of CLA isomers in naturally occurring foods that are high in fat content, the chemical synthesis of CLA has been developed for making supplements and for fortifying foods. CLA can be synthesized from linoleic acid found in safflower or sunflower oils under alkaline conditions, yielding a CLA mixture containing approximately 40% of the 9,11 isomer and 44% of the 10,12 isomer (Reviewed in 19). Commercial preparations also contain approximately 4 - 10% trans-9, trans-11 CLA and trans-10, trans-12 CLA, as well as trace amounts of other isomers.

Pharmacokinetics and Efficacy of CLA

Human and Animal Studies

As with other long chain unsaturated fatty acids (FA)s, CLA is absorbed primarily in the small intestine, packaged into chylomicrons, and distributed to extra-hepatic tissues having lipoprotein lipase (LPL) activity or returned to the liver via chylomicron remnants or other lipoproteins. The average daily intake of CLA is approximately 152 - 212 mg for non-vegetarian women and men, respectively (20), and human serum levels range from 10-70 µmol/L (21, 22).

One major discrepancy between animal and human studies is the dose of CLA administered (i.e., equal levels of 9,11 and 10,12 isomers- referred to as a CLA mixture), when expressed per unit body weight. For example, most adult human studies provide 3-6 g/day of a CLA mixture, whereas rodent studies provide 0.5-1.5% of a CLA mixture (w/w) in the diet. When expressed per unit of body weight, humans receive

approximately 0.05 g CLA/kg body weight, whereas mice received 1.07 g CLA/kg body weight, which is 20 times the human dose based on body weight. Thus, part of the discrepancy in results obtained from human and animal studies is likely due to this large difference in the dose of CLA administered. Supplementing humans with higher, or animals with lower, doses of CLA would address this issue. Other discrepancies in experimental designs include using CLA isomer mixtures vs. single isomers, duration of CLA supplementation, and the age, weight, gender, and metabolic status of the subjects or animals.

Cell Studies

In vitro studies have been conducted in a variety of cells types, primarily using an equal mixture of 9,11 and 10,12 CLA, or each isomer individually. Doses used in cell studies generally range between 1-100 µM, reflecting the concentration found in human subjects following supplementation. Results from these studies suggest that these isomers are readily taken up by the cultures. For example, we found that 10,12 CLA is readily incorporated into neutral- and phospholipids fractions of the primary human adipocyte cultures, and reduced lipid and glucose metabolism (6). Similar to *in vivo* studies, 9,11 CLA acted more like the linoleic acid controls.

Anti-Obesity Actions of CLA

Due to the substantial rise in obesity over the past 30 years, there is a great deal of interest in CLA as a weight loss treatment, as it has been shown to decrease body weight

and body fat mass (BFM). For example, supplementation with a CLA mixture (i.e., 10,12 + 9,11 isomers in equal concentrations) or the 10,12 isomer alone decreases BFM in many animal and some human studies (Reviewed in 23 and 24). Of the two major isomers of CLA, the 10,12 isomer is responsible for the anti-obesity properties (5, 25, 26, 27, 28).

CLA Decreases Body Weight and BFM

Park et al. (29) were one of the first groups to demonstrate that CLA modulated body composition. Compared to controls, male and female mice supplemented with a 0.5% (w/w) CLA mixture had 57 and 60% less BFM, respectively. Since these findings, researchers have demonstrated that CLA supplementation consistently reduces BFM in mice, rats, and pigs (30, 31, 32, 33). For example, dietary supplementation with 1% (w/w) CLA mixture for 28 days decreased body weight and peri-uteral WAT mass in C57BL/6J mice (33).

In humans, some studies show that CLA decreases BFM and increases lean body mass (LBM), whereas others show no such effects. For example, supplementation of 3- 4 g/day of a CLA mixture for 24 weeks decreased BFM and increased LBM in overweight and obese people (34). On the other hand, supplementation of 3.76 g/day of a CLA mixture in yogurt for 14 weeks in healthy adults had no effect on body composition (35). Supplementation with 3.2 g/day of a CLA mixture decreased total BFM and trunk fat compared to placebo in overweight subjects, but not obese subjects (36). These contradictory findings among human studies may be due to the following differences in

experimental design 1) mixed vs. individual CLA isomers, 2) CLA dose and duration of treatment, and 3) gender, weight, age and metabolic status of the subjects.

These anti-obesity effects of CLA do not appear to be solely due to reductions in food intake in animals (37, 38, 39) or humans (40, 41). Several mechanisms by which CLA decreases BFM will now be examined.

CLA Increases LBM

A recent meta-analysis of 18 human, placebo-controlled CLA studies found that consuming the CLA mixture increased fat-free mass (FFM) by 0.3 kg, regardless of the duration or dose (42). When these same 18 studies were examined for reductions in BFM, it was shown that CLA supplementation decreased BFM by 0.05 kg/week for up to 1 year (23). The average CLA mixture dose for these studies was 3.2 g/day. Collectively, these meta-analyses studies suggest that CLA supplementation of humans results in a rather small but rapid increase in FFM or LBM, and a much larger decrease in BFM over an extended period of time. The effects of CLA on FFM or LBM in humans may vary depending on baseline body mass index, gender, age, and exercise status of the subjects.

Two proposed mechanisms by which CLA increases LBM are via increasing bone or muscle mass. 10,12 CLA supplementation for 10 weeks with a 0.5% (w/w) CLA mixture increased bone mineral density (BMD) and muscle mass in C57BL/6 female mice (43). CLA supplementation has been proposed to increase BMD via increasing osteogenic gene expression and decreasing osteoclast activity (43, 44). Furthermore, CLA supplementation alone or with exercise increased BMD compared to control mice

(45). An alternative mechanism could be that CLA decreases adipogenesis of pluripotent mesenchymal stem cells (MSC) in bone marrow, and instead promotes their commitment to become bone cells. Indeed, 10,12 CLA has been shown to decrease the differentiation of MSC into adipocytes and increase calcium deposition and markers of osteoblasts (46). In contrast, 9,11 CLA increased adipocyte differentiation and decreased osteoblast differentiation. Consistent with these *in vitro* data, CLA mixture supplementation of rats treated with corticosteroids prevented reductions in LBM, BMD, and bone mineral content (47). Increasing LBM is directly linked to an increase in basal metabolic rate (BMR).

In addition to its effects on BMD, recent evidence supports a role of CLA in increasing endurance and muscle strength. For example, maximum swimming time until fatigue was higher in CLA fed vs. control mice (48). Aging mice supplemented with a CLA mixture and 10,12 CLA had higher muscle weight compared to 9,11 CLA and corn oil controls (49). In addition, CLA isomers increased levels of antioxidant enzyme activity, ATP, and enhanced mitochondrial potential, indicating a protective effect against age-associated muscle loss (49). In humans, CLA increased bench press strength in males supplemented with 5 g/day for 7 weeks who underwent resistance training 3 days per week (50). Furthermore, supplementation with CLA combined with creatine monohydrate (C) and whey protein (P) led to greater increases in bench-press and leg-press strength than supplementation with C+P or P alone (51). Although preliminary, these data suggest that CLA may enhance exercise-induced muscle strength or prevent sarcopenia or age-related muscle loss.

CLA Increases Energy Expenditure

CLA has been proposed to reduce adiposity by elevating energy expenditure via increasing BMR, thermogenesis, or lipid oxidation in animals (25, 39, 52). In BALB/c male mice fed mixed isomers of CLA for 6 weeks, body fat was decreased by 50% and was accompanied by increased BMR compared to controls (39). Enhanced thermogenesis may be associated with increased uncoupling of mitochondria via uncoupling protein (UCP)s, which facilitate proton transport over the inner mitochondrial membrane thereby leading to dissipation of energy as heat instead of ATP synthesis. UCP1 is highly expressed in brown adipose tissue (BAT), and in white adipose tissue (WAT) at lower levels. UCP3 is expressed in muscle and a number of other tissues, whereas UCP2 is the form expressed at the highest level across most tissues. Supplementation with a CLA mixture or 10,12 CLA in rodents induced UCP2 mRNA expression in WAT (26, 53). Recently it was demonstrated that CLA increased mRNA and protein expression of UCP1 in WAT (54). Similarly, CLA supplementation induced UCP gene expression and elevated beta oxidation in muscle and liver (55, 56, 57, 58, 59).

CLA Increases Fat Oxidation

CLA has been shown to regulate the gene expression or activity of proteins associated with FA oxidation in adipose tissue, muscle, and the liver. For example, CLA induced the expression of carnitine palmitoyl transferase 1 (CPT1) in WAT of obese Zucker fa/fa rats (60). Additionally, 10,12 CLA increased the expression of peroxisome proliferator-activated receptor (PPAR)γ coactivator-1α (PGC-1α) in WAT of mice (54).

Consistent with these *in vivo* findings, 10,12 CLA increased β-oxidation in differentiating 3T3-L1 preadipocytes (61). Furthermore, 10,12 CLA treatment increased AMP kinase (AMPK) activity and increased phospho- acetylCoA carboxylase (ACC) levels in 3T3-L1 adipocytes, suggesting an increase in FA oxidation and a decrease in FA esterification to triglyceride (TG) (62).

In muscle, 10,12 CLA increased CPT1 expression in hamsters fed an atherogenic diet (57). Supplementation of a CLA mixture in high fat fed hamsters led to increased CPT1 activity in muscle (63). A CLA mixture increased CPT1b, UCP3, acetyl CoA oxidase (ACO) 2, and PPARα mRNA levels in skeletal muscle of Zucker rats (64). Consistent with these data, 10,12 CLA increased mRNA levels (60) and activity (65) of CPT1 in the liver. Additionally, 10,12 CLA increased hepatic peroxisomal fatty ACO activity (65), suggesting increased peroxisomal beta oxidation in addition to mitochondrial oxidation. These findings suggest CLA may reduce adiposity through increased energy expenditure via increased mitochondrial uncoupling and FA oxidation in WAT, muscle, and liver.

At least one report demonstrates that CLA increases FA oxidation in human subjects (66). In this study, overweight adults supplemented with 4 g/day of a CLA mixture for 6 months had a lower respiratory quotient (RQ), indicating an increase in FA oxidation compared to placebo controls. However, others have shown no effect of CLA on energy expenditure or fat oxidation in humans (67, 68). These discrepancies may be due to the length of treatment, time period of measurement, and time at which measurements are taken. For instance, CLA treatment for 4-8 weeks had no effect on

energy expenditure or FA oxidation, based on a 20 minute measurement during resting and walking (67). In contrast, the study by Close et al. (66) gave CLA for 6 months and measured FA oxidation over a 24 hour period. Thus, discrepancies in this area may be due to insufficient duration of CLA treatment or measurements of energy expenditure or FA oxidation.

CLA Decreases Adipocyte Size

Lipolysis is the process by which stored TG is mobilized, releasing free FAs and glycerol for use by metabolically-active tissues. C57BL/6J mice fed 10,12 CLA for 3 days had increased mRNA levels of hormone sensitive lipase (HSL), a key enzyme for TG hydrolysis (53). Consistent with these data, acute treatment with CLA mixture or 10,12 CLA alone increased lipolysis in 3T3-L1 (29, 69) and newly differentiated human adipocytes (9). *In vitro*, a CLA mixture and to a greater extent 10,12 CLA decreased TG content, adipocyte size, and lipid locule size in adipocytes (70). Similarly, mice fed 1% CLA displayed increased numbers of small adipocytes with a reduction in the number of large adipocytes (71). Furthermore, a CLA mixture reduced adipocyte size rather than cell number in Sprague Dawley (37) and fa/fa Zucker rats (72). Thus, CLA may reduce adipocyte size by increasing lipolysis.

CLA Decreases Adipocyte Number

Apoptosis is another mechanism by which CLA may reduce BFM. Apoptosis can occur through activation of the death receptor pathway, endoplasmic reticulum (ER)

stress, or the mitochondrial pathway. A number of *in vivo* and *in vitro* studies have reported apoptosis in adipocytes supplemented with a CLA mixture or 10,12 CLA alone (53, 61, 73, 74). For example, supplementation of C57BL/6J mice with 1% (w/w) CLA mixture reduced BFM and increased apoptosis in WAT (71). Mice fed a high-fat diet containing 1.5% (w/w) CLA mixture had an increased ratio of BAX, an inducer of apoptosis relative to Bcl2, a suppressor of apoptosis (75). Reported mechanisms by which CLA reduces adiposity are shown in **Fig. 2.2.**

CLA Decreases Adipocyte Differentiation

The conversion of preadipocytes to adipocytes involves the activation of key transcription factors such as PPARγ and CAAT/ enhancer binding proteins (C/EBPs). There is much evidence showing that CLA suppresses preadipocyte differentiation in animal (76, 77, 78) and human (6, 7) preadipocytes treated with a CLA mixture or 10,12 CLA alone. 10,12 CLA treatment has been reported to decrease the expression of PPARγ, C/EBPβ, sterol regulatory element binding protein 1c (SREBP-1c), liver X receptor (LXRα), and adipocyte fatty acid binding protein (aP2), thereby reducing adipogenesis and lipogenesis (6, 26, 78).

In rodents, supplementation of 10,12 CLA decreased the expression of PPARγ and its target genes (47, 78, 79). In contrast, humans supplemented with a CLA mixture had higher mRNA levels of PPARγ in WAT, but no difference in body weight or BFM (35). In mature, *in vitro*-differentiated primary human adipocytes or in mature 3T3-L1 adipocytes, 10,12 CLA treatment leads to a substantial decrease in the expression and

activity of PPARγ (80, 81), and a decrease in PPARγ target genes and lipid content (7). This shows that 10,12 is not only able to inhibit, but also to reverse the adipogenic process and indicates that this may be mediated by suppression of PPARγ activity. In addition to its effect on PPARγ, 10,12 CLA may also directly impact the activity of other transcription factors involved in adipogenesis and lipogenesis (i.e., LXRα, C/EBPs, SREBP-1c), which could contribute to CLA's anti-obesity actions.

CLA Decreases Glucose and FA Uptake and TG Synthesis

Conversion of glucose and FAs to TG is a major function of adipocytes. Genes involved in lipogenesis, such as a lipoprotein lipase (LPL), ACC, fatty acid synthase (FAS), and stearoyl-CoA desaturase (SCD), were decreased following supplementation with mixed isomers of CLA or 10,12 CLA alone (6, 53, 69, 7). PPARγ is a major activator of many lipogenic genes including glycerol-3-phosphate dehydrogenase (GPDH), LPL, and lipin as well as many genes encoding lipid droplet associated proteins such as perilipin, adipocyte differentiation-related protein (ADRP), and cell death-inducing DFFA-like effector c (CIDEC) (82). Thus, the anti-lipogenic action of 10,12 CLA may be explained by inhibition of PPARγ activity. In addition, CLA repression of expression of SREBP-1 and its target genes may play an important role in delipidation. Finally, CLA suppression of insulin signaling may also impair insulin's ability to activate or increase the abundance of a number of lipogenic proteins including LPL, ACC, FAS, SCD-1, and the insulin-dependent glucose transporter (GLUT4).

CLA Increases Markers of Inflammation

Adverse side effects have been reported for CLA supplementation such as elevated levels of inflammatory markers, lipodystrophy, steatosis, and insulin resistance. Most adverse side effects are due to the 10,12 CLA isomer. Treatment with 10,12 CLA increases the expression or secretion of inflammatory markers such as TNFα, interleukin (IL)-1β, IL-6, and IL-8 from adipocyte cultures (53, 9, 7, 79, 81). Secretion of these inflammatory factors further exacerbates inflammatory signaling in WAT via paracrine and autocrine signaling (7). For example, TNFα stimulates lipolysis, inhibits lipogenesis, and thus promotes delipidation in adipoyctes (83, 84). Our lab has linked 10,12 CLA-mediated inflammation to an increase in intracellular calcium accumulation (85), and MEK/ERK and NFκB activation (7, 9). Activated NFκB can transrepress PPARγ via direct binding to PPARγ or its co-activators, thereby preventing binding of PPARγ to target genes (86, reviewed in 87). Moreover, CLA increases the phosphorylation of phospholipase (PLA)2 (85) and the expression of cyclooxygenase (COX)-2, an enzyme involved in the synthesis of PGs, and the secretion of $PGF_{2\alpha}$ (78, 88). These inflammatory pathways are known to antagonize PPARγ activity and insulin sensitivity (75, 88, 89, 90).

Consistent with these *in vitro* data, 10,12 CLA supplementation increases the levels of inflammatory cytokines and PGs in humans (91, 92). For example, women supplemented with 5.5 g/day of a CLA mixture for 16 weeks had higher levels of C-reactive protein in serum and 8-iso-$PGF_{2\alpha}$ in urine (41). 10,12 CLA supplementation in mice resulted in macrophage recruitment in WAT (79). In contrast, 9,11 CLA appears to have anti-inflammatory actions (17).

CLA Causes Insulin Resistance

Insulin resistance has been reported *in vivo* (53, 93, 94, 95) and *in vitro* (6, 9, 78, 88) following supplementation with a CLA mixture or 10,12 CLA alone. For example, 10,12 CLA supplementation of 3.4 g/day for 12 weeks in obese men with metabolic syndrome increased serum glucose and insulin levels and decreased insulin sensitivity (93). Supplementation with a CLA mixture in type 2 diabetics increased fasting plasma glucose levels and reduced insulin sensitivity (94). Mice fed 1% (w/w) 10,12 CLA displayed elevated fasted and feeding plasma insulin levels and had reduced insulin sensitivity (71). Consistent with these data, the mRNA levels of adiponectin, a key adipokine associated with insulin sensitivity, decreases following supplementation with 10,12 CLA *in vivo* (33, 79, 90) and *in vitro* (78, 80, 96, 97). Additionally, our lab group has shown that 10,12 CLA decreases fatty acid uptake and insulin-stimulated glucose uptake in primary cultures of newly differentiated human adipocytes (7). The reduction of fatty acid and glucose uptake is likely due to decreases in PPARγ activity and protein levels, and thus the suppression of genes involved in glucose and fatty acid uptake and storage, such as aP2, and GLUT4.

CLA Causes Lipodystrophy

The combination of inflammation and insulin resistance results in reduced FA and glucose uptake in WAT, leading to lipid accumulation in the blood (hyperlipidemia), liver (steatosis), or muscle. CLA-mediated hyperlipidemia and steatosis has been reported in several animal studies (33, 71, 98). For example, 1% (w/w) CLA time-dependently

increased insulin levels and led to increased liver weight and liver lipid accumulation in C57BL/6J mice (33). Aging C57BL/6J mice fed 0.5% 10,12 CLA displayed increased insulin resistance and liver hypertrophy (98).

Cell Types Responsible for CLA-Mediated Inflammation in WAT

The primary cell types in adipose tissue responsible for mediating inflammation and insulin resistance are under debate. Adult human WAT has been reported to contain ~ 50-70% adipocytes, ~ 20-40% preadipocytes, and ~ 1-30% infiltrated macrophages (14, 99). These percentages also depend on body composition. For example, Tchoukalova et al. (100) found that WAT of lean women contain approximately 30% preadipocytes, whereas WAT of obese women contain only 17% preadipocytes. However, the localization and secretory pattern of cytokines in WAT are unknown. Some researchers suggest that non-fat cells are the key players in cytokine release from adipose tissue (101, 102). Fain et al. (101) has shown that non-fat cells are responsible for up to 90% of adipokine release from adipose tissue compared to mature adipocytes. These samples, obtained from obese women directly after undergoing bariatric surgery, were analyzed for basal levels of inflammatory markers. Our lab group has shown that lipopolysaccharide (LPS)-stimulated preadipocytes display a greater extent of inflammatory signaling than adipocytes (103). On the other hand, LaRosa et al. (53) showed that 10,12 CLA-induced inflammatory genes were higher in murine adipocytes compared to preadipocytes. In addition, Ajuwon et al. (104) demonstrated that a saturated fatty acid, palmitate, induces IL-6 and TNFα expression in 3T3-L1 mature adipocytes.

Skurk et al. (105) proposes that adipocyte size is an important determinant of adipokine secretion, where proinflammatory cytokine secretion (i.e., IL-6, IL-8, MCP-1, and TNFα) was significantly higher from large adipocytes compared to smaller adipocytes. Interestingly, Suganami et al. (106, 107) reported that activated hypertrophied adipocytes release saturated free fatty acids (FFA) that subsequently promote TNFα secretion from macrophages, leading to an inflammatory cycle in adipose tissue. Taken together, these conflicting results suggest that differences in the inflammatory capacity of preadipocytes vs. adipocytes, may be due to 1) inflammatory stimuli, 2) adipocyte size, and 3) crosstalk with other cell types.

Conclusions

There is an abundance of evidence in animals suggesting that CLA consumption may reduce the incidence or risk of developing obesity depending on the type and abundance of CLA isomer consumed and the physiological status of the animal model. Data on the anti-obesity properties of 10,12 CLA in animals, especially mice, are the most reproducible. However, these potential benefits are not without risks, as the 10,12 isomer is associated with increased levels of inflammatory markers, lypodystrophy, and insulin resistance. More clinical studies are needed to determine the efficacy of CLA isomers in humans, and more mechanistic animal and cell studies are needed to determine the precise, isomer-specific mechanisms of action of CLA, potential side effects, and the cell types responsible for these adverse effects.

Chapter III will address the hypothesis that 10,12 CLA initiates inflammatory signaling from adipocytes vs. preadipocytes. Chapter IV will address the hypothesis that 10,12 CLA mediates inflammatory signaling via activation of the MAPK, JNK, using the chemical JNK inhibitor SP00125. Chapter V will address the hypothesis that diacylglycerol kinases play a role in 10,12 CLA-mediated inflammation, insulin resistance, and delipidation using the DGK inhibitor R59022.

Table 2.1 CLA content of various foods.

Food	mg/g fat	Food	mg/g fat
Meats/Fish		**Dairy**	
Corned beef	6.6	Condensed milk	7
Lamb	5.8	Colby cheese	6.1
Fresh ground beef	4.3	Butter fat	6.1
Salami	4.2	Ricotta	5.6
Beef smoked sausage	3.8	Homogenized milk	5.5
Knackwurst	3.7	Cultured buttermilk	5.4
Smoked ham	2.9	American processed cheese	5
Veal	2.7	Mozarella	4.9
Smoked turkey	2.4	Plain yogurt	4.8
Fresh ground turkey	2.6	Butter	4.7
Chicken	0.9	Sour cream	4.6
Pork	0.6	Cottage cheese	4.5
Egg yolk	0.6	Low fat yogurt	4.4
Salmon	0.3	2% milk	4.1
Vegetable oils		Medium cheddar	4.1
Safflower oil	0.7	Ice cream	3.6
Sunflower oil	0.4	Parmesan	3
Peanut	0.2	Frozen yogurt	2.8

Sources: Based on values reported in Lin, H. et al. J. Dairy Sci **1995,** *78* (11), 2358-2365; Chin, S. et al. J. Food Comp. Anal. **1992**, *5*, 185-197; Fritsche, J.; Steinhardt, H. Food Res. Tech. **1998**, *206*, 77-82; and the University of Wisconsin Food Research Institute (Dr. Pariza, Director)

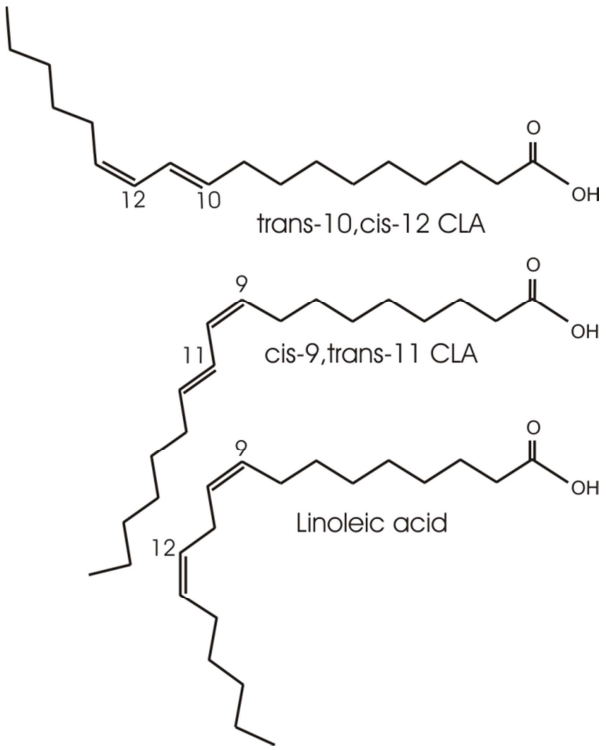

Figure 2.1 Stuctures of linoleic acid, cis-9, trans11 CLA, and trans-10, cis-12 CLA.

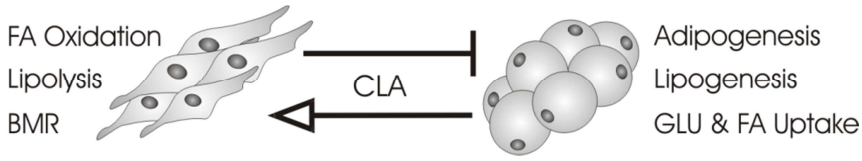

Figure 2.2 Reported mechanisms by which 10,12 CLA decreases adipose tissue mass and obesity.

References

1. World Health Organization. 2011. Media Centre Fact Sheet.

2. Flegal KM, Carroll MD, Ogden CL, Curtin LR. 2010. Prevalence and Trends in Obesity Among US Adults, 1999-2008. JAMA. 303:235-241.

3. Behan DF and Cox SH. 2010. Obesity and its Relation to Mortality and Morbidity Costs. Society of Actuaries.

4. Flegal KM, Carroll MD, Kuezmarki RJ, Johnson CL. 1998. Overweight and obesity in the United States: prevalence and trends, 1960-1994. Int J Obes Relat Metab Disord. 22:39-47.

5. Brown JM, Halverson YD, Lea-Currie R, Geigerman C, and McIntosh M. 2001. Trans-10, cis-12, but not cis-9, trans-11, conjugated linoleic acid attenuates lipogenesis in primary cultures of stromal vascular cells from human adipose tissue. J Nutr. 131:2316-2321.

6. Brown JM, Sandberg-Boysen M, Skov S, Morrison R, Storkson J, Lea-Currie R, Pariza M, Mandrup S, and McIntosh M. 2003. Isomer-specific regulation of metabolism and PPARγ by conjugated linoleic acid (CLA) in human preadipocytes. J Lipid Res. 44:1287-300.

7. Brown J, Boysen M, Chung S, Fabiyi O, Morrison R, Mandrup S, and McIntosh M. 2004. Conjugated linoleic acid (CLA) induces human adipocyte delipidation: autocrine/paracrine regulation of MEK/ERK signaling by adipocytokines. J Biol Chem. 279:26735-26747.

8. Kennedy, A., K. Martinez, S. Chung, K. Lapoint, R. Hopkins, S. F. Schmidt, K. Andersen, S. Mandrup, and M. McIntosh. 2010. Inflammation And Insulin Resistance Induced By Trans-10, Cis-12 Conjugated Linoleic Acid Depend On Intracellular Calcium Levels In Primary Cultures Of Human Adipocytes. *J Lipid Res* 51: 1906-1917. PMC2882722.

9. Chung S, Brown JM, and McIntosh M. 2005. Trans-10, cis-12 CLA increases adipocytes lipolysis and alters lipid droplet-associated proteins: role of mTOR and ERK signaling. J Lipid Res. 46:885-895.

10. Center for Disease Control. 2008. Physical Activity and Good Nutrition: Essential Elements to Prevent Chronic Diseases and Obesity- At a Glance 2008. http://www.cdc.gov/nccdphp/publications/aag/dnpa.htm (accessed October 15, 2008).

11. Hotamisligil GS., Shargill NS., & Spiegelman, BM. 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 259: 87-91.

12. Kern PA., Saghizaheh M., Ong JM., Bosch RJ., Deem R., & Simsolo RB. 1995. The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. J Clin Invest. 95: 2111-2119.

13. Uysal KT., Wiesbrock SM., Marino MW., & Hotamisligil GS. 1997. Protection from obesity induced insulin resistance in mice lacking TNF-α function. Nature. 389: 610-614.

14. Trayhurn P., & Wood SI. 2004. Adipokines: Inflammation and the pleiotropic role of white adipose tissue. Brit J Nutr. 92: 347-355.

15. Griinari, J.; Cori, B.; Lacy, S.; Chouinard, P.; Nurmela, K.; Bauman, D. Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by delta-9 desaturase. J. Nutr. 2000, *130* (9), 2285-2291.

16. Wallace, R.J.; McKain, N.; Shingfield, K.J.; Devillard, E. Isomers of conjugated linoleic acids are synthesized via different mechanisms in ruminal digesta and bacteria. J. Lipid Res. 2007, *48* (10), 2247-2254.

17. Reynolds, C.M.; Draper, E.; Keogh, B.; Rahman, A.; Moloney, A.; Mills, K.H.; Loscher, C.E.; Roche, H.M. A conjugated linoleic acid-enriched beef diet attenuates lipopolysaccharide-induced inflammation in mice in part through PPARg-mediated suppression of toll-like receptor 4. J. Nutr. 2009, *139* (12), 2351-2357.

18. Bell, J.; Griinari, J; Kennelly, J. 2006. Effect of safflower oil, flaxseed oil, monensin, and vitamin E on concentration of conjugated linoleic acid in bovine milk fat. J. Dairy Sci. 2006, *89* (2), 733-748.

19. Pariza, M.W.; Park, Y.; Cook, M.E. The biologically active isomers of conjugated linoleic acid. Prog. Lipid Res. 2001, *40* (4), 283-298.

20. Ritzenthaler, K.L.; McGuire, M.K.; Falen, R.; Schultz, T.D.; Dasgupta, N.; and McGuire, M.A. Estimation of conjugated linoleic acid intake by written dietary assessment methodologies underestimates actual intake evaluated by food duplicate methodology. J. Nutr. 2001, *131* (5), 1548–1554.

21. Mougios, V.; Matsakas, A.; Petridou, A.; Ring, S.; Sagredos, A.; Melissopoulou, A.; Tsigilis, N.; Nikolaidis, M. Effect of supplementation with conjugated linoleic acid on human serum lipids and body fat. J. Nutr. Biochem. 2001, *12* (10), 585-594.

22. Petridou, A.; Mougios, V.; Sagredos A. Supplementation with CLA: isomer incorporation into serum lipids and effect on body fat of women. Lipids 2003, *38* (8), 805-811.

23. Whigham, L.D.; Watras, A.C.; Schoeller, D.A. Efficacy of conjugated linoleic acid for reducing fat mass: a meta-analysis in humans. Am. J. Clin. Nutr. 2007, *85* (5), 1203-1211.

24. Wang, Y.W.; Jones, P.J. Conjugated linoleic acid and obesity control: efficacy and mechanisms. Int. J. Obes. Relat. Metab. Disord. 2004, *28* (8), 941-955.

25. Park, Y.; Storkson, J.; Albright, K.; Liu, W.; Pariza, M. Evidence that trans-10, cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. Lipids 1999, 34 (3), 235-241.

26. House, R.L.; Cassady, J.P.; Eisen, E.J.; Eling, T.E.; Collins, J.B.; Grissom, S.F.; Odle, J. Functional genomic characterization of delipidation elicited by trans-10, cis-12-conjugated linoleic acid (t10c12-CLA) in a polygenic obese line of mice. Physiol. Genomics. 2005, *21* (3), 351-361.

27. Brandebourg, T.D.; Hu, C.Y. Isomer-specific regulation of differentiating pig preadipocytes by conjugated linoleic acids. J. Anim. Sci. 2005, *83* (9), 2096-2105.

28. Raff, M.; Tholstrup, T.; Toiubro, S.; Bruun, J.; Lund, P.; Straarup, E.; Christensen, R.; Sandberg, K.; Mandrup, S. Conjugated linoleic acids reduce body fat in healthy postmenopausal women. J. Nutr. 2009, *139* (7), 1347-1352.

29. Park, Y.; Albright, K.J.; Liu, W.; Storkson, J.M.; Cook, M.E.; Pariza, M.W. Effect of conjugated linoleic acid on body composition in mice. Lipids 1997, *32* (8), 853-858.

30. Sisk, M.B.; Hausman, D.B.; Martin, R.J.; Azain, M.J. Dietary conjugated linoleic acid reduces adiposity in lean but not obese Zucker rats. J. Nutr. 2001, *131* (6), 1668–1674.

31. Clément, L.; Poirier, H.; Niot, I.; Bocher, V.; Guerre-Millo, M.; Krief, S.; Staels, B.; Besnard, P. Dietary trans-10,cis-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse. J. Lipid Res. 2002, *43* (9), 1400-1409.

32. Meadus, W.J.; MacInnis, R.; Dugan, M. Prolonged dietary treatment with conjugated linoleic acid stimulates porcine muscle peroxisome proliferator activated receptor gamma and glutamine–fructose aminotransferase gene expression in vivo. J. Mol. Endocrinol. 2002, *28* (2), 79–86.

33. Poirier, H.; Rouault, C.; Clément, L.; Niot, I.; Monnot, M.; Guerre-Millo, M.; Besnard P. Hyperinsulinemia triggered by dietary conjugated linoleic acid is associated with a decrease in leptin and adiponectin plasma levels and pancreatic beta cell hyperplasia in the mouse. Diabetologia 2005, *48* (6), 1059-1065.

34. Gaullier, J.M.; Halse, J.; Høivik, H.O.; Høye, K.; Syvertsen, C.; Nurminiemi, M.; Hassfeld, C.; Einerhand, A.; O'Shea, M.; Gudmundsen O. Six months supplementation with conjugated linoleic acid induces regional-specific fat mass decreases in overweight and obese. Br. J. Nutr. 2007, *97* (3), 550-560.

35. Nazare, J.A.; de la Perrière, A.B.; Bonnet, F.; Desage, M.; Peyrat, J.; Maitrepierre, C.; Louche-Pelissier, C.; Bruzeau, J.; Goudable, J.; Lassel, T.; Vidal, H.; Laville, M. Daily intake of conjugated linoleic acid-enriched yoghurts: effects on energy metabolism and adipose tissue gene expression in healthy subjects. Br. J. Nutr. 2007, *97* (2), 273-280.

36. Laso, N.; Brugué, E.; Vidal, J.; Ros, E.; Arnaiz, J.A.; Carné, X.; Vidal, S.; Mas, S.; Deulofeu, R.; Lafuente, A. Effects of milk supplementation with conjugated linoleic acid (isomers cis-9, trans-11 and trans-10, cis-12) on body composition and metabolic syndrome components. Br. J. Nutr. 2007, *98*, 860-867.

37. Azain, M.J.; Hausman, D.B.; Sisk, M.B.; Flatt, W.P.; Jewell, D.E. Dietary conjugated linoleic acid reduces rat adipose tissue cell size rather than cell number. J. Nutr. 2000, *130*, 1548-1554.

38. West, D.B.; Blohm, F.Y.; Truett, A.A.; DeLany, J.P. Conjugated linoleic acid persistently increases total energy expenditure in AKR/J mice without increasing uncoupling protein gene expression. J. Nutr. 2000, *130*, 2471-2477.

39. Terpstra, A.H.; Javadi, M.; Beynen, A.C.; Kocsis, S.; Lankhorst, A.E.; Lemmens, A.G.; Mohede, I.C. Dietary conjugated linoleic acids as free fatty acids and triacylglycerols similarly affect body composition and energy balance in mice. J. Nutr. 2003, *133*, 3181-3186.

40. Lambert, E.V.; Goedecke, J.H.; Bluett, K.; Heggie, K.; Claassen, A.; Rae, D.E.; West, S.; Dugas, J.; Dugas, L.; Meltzeri, S.; Charlton, K.; Mohede, I. Conjugated linoleic acid versus high-oleic acid sunflower oil: effects on energy metabolism, glucose tolerance, blood lipids, appetite and body composition in regularly exercising individuals. Br. J. Nutr. 2007, *97*, 1001-1011.

41. Tholstrup, T.; Raff, M.; Straarup, E.M.; Lund, P.; Basu, S.; Bruun, J.M. An oil mixture with trans-10, cis-12 conjugated linoleic acid increases markers of inflammation and in vivo lipid peroxidation compared with cis-9, trans-11 conjugated linoleic acid in postmenopausal women. J. Nutr. 2008, *138*, 1445-1451.

42. Schoeller, D. A.; Watras, A.C.; Whigham, L.D. A meta-analysis of the effects of conjugated linoleic acid on fat-free mass in humans. Appl. Physiol. Nutr. Metab. 2009, *34*, 975-978.

43. Rahman, M.M.; Bhattacharya, A.; Banu, J.; Fernandes, G. Conjugated linoleic acid protects against age-associated bone loss in C57BL/6 female mice. J. Nutr. Biochem. 2007, *18*, 467-474.

44. Banu, J.; Bhattacharya, A.; Rahman, M.; O'Shea, M.; Fernandes, G. Effects of conjugated linoleic acid and exercise on bone mass in young male Balb/C mice. Lipids Health Dis. 2006, *5*, 7.

45. Banu, J.; Bhattacharya, A.; Rahman, M.; Fernandes, G. Beneficial effects of conjugated linoleic acid and exercise on bone of middle-aged female mice. J. Bone. Miner. Metab. 2008, *26*, 436-445.

46. Platt, I.D.; El-Sohemy, A. Regulation of osteoblast and adipocyte differentiation from human mesenchymal stem cells by conjugated linoleic acid. J. Nutr. Biochem. 2009, *20*, 956-964.

47. Roy, B.D.; Bourgeois, J.; Rodriguez, C.; Payne, E.; Young, K.; Shaughnessy, S.G.; Tarnopolosky, M.A. Conjugated linoleic acid prevents growth attenuation induced by corticosteroid administration and increases bone mineral content in young rats. Appl. Physiol. Nutr. Metab. 2008, *33*, 1096-1104.

48. Mizunova, W.; Haramizu, S.; Shibakusa, T.; Okabe, Y.; Fushiki, T. Dietary conjugated linoleic acid increases endurance capacity and fat oxidation in mice during exercise. Lipids 2005, *40* (3), 265-271.

49. Rahman, M.M.; Halade, G.; Jamali, A.E.; Fernandes, G. Conjugated linoleic acid (CLA) prevents age-associated skeletal muscle loss. BBRC 2005, *383*, 513-518.

50. Pinkoski, C.; Chilibeck, P.D.; Candow, D.G.; Esliger, D.; Ewaschuk, J.B.; Facci, M.; Farthing, J.P.; Zello, G.A. The effects of conjugated linoleic acid supplementation during resistance training. Med. Sci. Sports. Exerc. 2006, *38* (2), 339-348.

51. Cornish, S.M.; Candow, D.G.; Jantz, N.T.; Chilibeck, P.D.; Little, J.P.; Forbes, S.; Abeysekara, S.; Zello, G.A. Conjugated linoleic acid combined with creatine monohydrate and whey protein supplementation during strength training. Int. J. Sport. Nutr. Exerc. Metab. 2009, *19* (1), 79-96.

52. Nagao, K.; Inoue, N.; Wang, Y.M.; Hirata, J.; Shimada, Y.; Nagao, T.; Matsui, T.; Yanagita, T. The 10trans,12cis isomer of conjugated linoleic acid suppresses the development of hypertension in Otsuka Long-Evans Tokushima Fatty rats. Biochem Biophys Res Commun. 2003, *306* (1), 134-138.

53. LaRosa, P.C.; Miner, J.; Xia, Y.; Zhou, Y.; Kachman, S.; Fromm, M.E. Trans-10, cis-12 conjugated linoleic acid causes inflammation and delipidation of white adipose tissue in mice: a microarray and histological analysis. Physiol. Genomics 2006, *27*, 282-294.

54. Wendel, A.A.; Purushotham, A.; Liu, L.F.; Belury, M.A. Conjugated linoleic acid induces upcoupling protein 1 in white adipose tissue of ob/ob mice. Lipids 2009, *in press*.

55. Roche, H.M.; Noone, E.; Sewter, C.; Mc Bennett, S.; Savage, D.; Gibney, M.J.; O'Rahilly, S.; Vidal-Puig, A.J. Isomer-dependent metabolic effects of conjugated linoleic acid: insights from molecular markers sterol regulatory element-binding protein-1c and LXRalpha. Diabetes 2002, *51*, 2037-2044.

56. Choi, J.S.; Koh, I.U.; Jung, M.H.; Song, J. Effects of three different conjugated linoleic acid preparations on insulin signaling, fat oxidation and mitochondrial function in rats fed a high-fat diet. Br. J. Nutr. 2007, *98*, 264-275.

57. Ribot, J.; Portillo, M.P.; Picó, C.; Macarulla, M.T.; Palou, A. Effects of trans-10, cis-12 conjugated linoleic acid on the expression of uncoupling proteins in hamsters fed an atherogenic diet. Br. J. Nutr. 2007, *97*, 1074-1082.

58. Priore, P., Giudetti, A.M.; Natali, F.; Gnoni, G.V.; Geelen, M.J. Metabolism and short-term metabolic effects of conjugated linoleic acids in rat hepatocytes. Biochim. Biophys. Acta. 2007, *1771*, 1299-1307.

59. Ferramosca, A.; Savy, V.; Conte, L.; Zara, V. Dietary combination of conjugated linoleic acid (CLA) and pine nut oil prevents CLA-induced fatty liver in mice. J. Agric. Food. Chem. 2008, *56*, 8148-8158.

60. Gudbrandsen, O.A.; Rodríguez, E.; Wergedahl, H.; Mørk, S.; Reseland, J.E.; Skorve, J.; Palou, A.; Berge, R.K. Trans-10, cis-12-conjugated linoleic acid reduces the haptic triacylglycerol content and the leptin mRNA level in adipose tissue in obese Zucker fa/fa rats. Br. J. Nutr. 2009, *102*, 803-812.

61. Evans, M.; Geigerman, C., Cook, J.; Curtis, L.; Kuebler, B.; McIntosh, M. Conjugated linoleic acid suppresses triglyceride accumulation and induces apoptosis in 3T3-L1 preadipocytes. Lipids 2000, *35*, 899-910.

62. Jiang, S.; Wang, Z.; Riethoven, J.J.; Xia, Y.; Miner, J.; Fromm, M. Conjugated linoleic acid activates AMP-activated protein kinase and reduces adiposity more effectively when used with metformin in mice. J. Nutr. 2009. *in press*.

63. Zabala, A.; Fernandez-Quintela, A.; Macarulla, M.T.; Simon, E.; Rodriguez, V.M.; Navarro, V.; Portillo, M.P. Effects of conjugated linoleic acid on skeletal muscle triacylglycerol metabolism in hamsters. Nutrition 2006, *22*, 528-533.

64. Inque, N.; Nagao, K.; Wang, Y.; Noguchi, H.; Shirouchi, B.; Yanagita, T. Dietary conjugated linoleic acid lowered tumor necrosis factor-α content and altered expression of genes related to lipid metabolism and insulin sensitivity in the skeletal muscle of zucker rats. J. Agric. Food. Chem. 2006, *54*, 7935-7939.

65. Macarulla, M.T.; Fernández-Quintela, A.; Zabala, A.; Navarro, V.; Echevarría, E.; Churruca, I.; Rodríguez, V.M.; Portillo, M.P. Effects of conjugated linoleic acid on liver composition and fatty acid oxidation are isomer-dependent in hamster. Nutrition 2005, *21*, 512-519.

66. Close, R.N.; Schoeller, D.A.; Watras, A.C.; Nora, E.H. Conjugated linoleic acid supplementation alters the 6-mo change in fat oxidation during sleep. Am. J. Clin. Nutr. 2007, *86*, 797-804.

67. Zambell, K.L.; Keim, N.L.; Van Loan, M.D.; Gale, B.; Benito, P,; Kelley, D.S.; Nelson, G.J. Conjugated linoleic acid supplementation in humans: effects on body composition and energy expenditure. Lipids 2000, *35* (7), 777-782.

68. Kamphius, M.M.; Lejeune, M.P.; Saris, W.H.; Westerterp-Plantenga, M.S. The effect of conjugated linoleic acid supplementation after weight loss on body weight regain, body composition, and resting metabolic rate in overweight subjects. Int. J. Obes. Relat. Metab. Disord. 2003, *27*, 840-847.

69. Evans, M.; Lin, X.; Odle, J.; McIntosh, M. Trans-10, cis-12 conjugated linoleic acid increases fatty acid oxidation in 3T3-L1 preadipocytes. J. Nutr. 2002, *132*, 450-455.

70. Brown, M.; Evans, M.; McIntosh, M. Linoleic acid partially restores the triglyceride content of conjugated linoleic acid-treated cultures of 3T3-L1 preadipocytes. J. Nutr. Biochem. 2001, *12*, 381-387.

71. Tsuboyama-Kasaoka, N.; Takahashi, M.; Tanemura, K.; Kim, H.J.; Tange, T.; Okuvama, H.; Kasai, M.; Ikemoto, S.; Ezaki, O. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. Diabetes 2000, *49*, 1534-1542.

72. Noto, A.; Zahradka, P.; Yurkova, N.; Xie, X.; Truong, H.; Nitschmann, E.; Ogborn, M.R.; Taylor, C.G. Dietary conjugated linoleic acid decreases adipocyte size and favorably modifies adipokine status and insulin sensitivity in obese, insulin-resistant rats. Metabolism 2007; *56*: 1601-1611.

73. Miner, J.L.; Cederberg, C.A.; Nielsen, M.K.; Chen, X.; Baile, C.A. Conjugated linoleic acid (CLA), body fat, and apoptosis. Obes. Res. 2001, *9*, 129-134.

74. Hargrave, K.M.; Li, C.; Meyer, B.J.; Kachman, S.D.; Hartzell, D.L.; Della-Fera, M.A.; Miner, J.L.; Baile, C.A. Adipose depletion and apoptosis induced by trans-10, cis-12 conjugated linoleic Acid in mice. Obes. Res. 2002, *10*, 1284-1290.

75. Liu, L.F.; Purushotham, A.; Wendel, A.A.; Belury, M.A. Combined effects of rosiglitazone and conjugated linoleic acid on adiposity, insulin sensitivity, and hepatic steatosis in high-fat-fed mice. Am. J. Physiol. Gastrointest. Liver Physiol. 2007, *292*, G1671-G1682.

76. Brodie, A.E.; Manning, V.A.; Ferguson, K.R.; Jewell, D.E.; Hu, C.Y. Conjugated linoleic acid inhibits differentiation of pre- and post- confluent 3T3-L1 preadipocytes but inhibits cell proliferation only in preconfluent cells. J. Nutr. 1999, *129*, 602-606.

77. Choi, Y.; Kim, Y.C.; Han, Y.B.; Park, Y.; Pariza, M.W.; Ntambi, J.M. The trans-10,cis-12 isomer of conjugated linoleic acid downregulates stearoyl-CoA desaturase 1 gene expression in 3T3-L1 adipocytes. J. Nutr. 2000, *130*, 1920-1924.

78. Kang, K.; Liu, W.; Albright, K.J.; Park, Y.; Pariza, M.W. trans-10,cis-12 CLA inhibits differentiation of 3T3-L1 adipocytes and decreases PPAR gamma expression. Biochem. Biophys. Res. Commun. 2003, *303*, 795-799.

79. Poirier, H.; Shapiro, J.S.; Kim, R.J.; Lazar, M.A. Nutritional supplementation with trans-10,cis-12 conjugated linoleic acid induces inflammation of white adipose tissue. Diabetes 2006, *55*, 1634-1640.

80. Miller, J.R.; Siripurkpong, P.; Hawes, J.; Majdalawieh, A.; Ro, H.S.; McLeod, R.S. The trans-10, cis-12 isomer of conjugated linoleic acid decreases adiponectin assembly by PPARgamma-dependent and PPARgamma-independent mechanisms. J. Lipid Res. 2008, *49*, 550-562.

81. Kennedy, A.; Chung, S.; LaPoint, K.; Fabiyi, O.; McIntosh, M.K. Trans-10, cis-12 conjugated linoleic acid antagonizes ligand-dependent PPARgamma activity in primary cultures of human adipocytes. J. Nutr. 2008, *138*, 455-461.

82. Neilson, R.; Pedersen, T.; Hagenbeek, D.; Moulos, P.; Sierbaek, R.; Megens, E.; Denissov, S.; Borgesen, M.; Francoijs, K.; Mandrup, S.; Stunnenberg, H. Genome-wide profiling of PPARgamma:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. Genes Dev. 2008, *22*, 2953-2967.

83. Kawakami M, Murase T, Ogawa H, Isibashi S, Mori N, Takaku F, Shibata S. 1987. Human recombinant TNF suppresses lipoprotein lipase activity and stimulates lipolysis in3T3-L1 cells. J Biochem. 101:331-338.

84. Petruschke T, Hauner H. 1993. Tumor necrosis factor-alpha prevents the differentiation of huan adipocyte precursor cells and causes delipidation of newly developed fat cells. J Clin Endocrinol Metab. 76:742-747.

85. Kennedy A, Martinez K, Chung S, LaPoint K, West T, Hopkins R, Schmidt S, Andersen K, Mandrup S, McIntosh M. (2010) Inflammation and insulin resistance induced by trans-10, cis-12 conjugated linoleic acid are dependent on intracellular calcium levels in primary cultures of human adipocytes. J Lipid Res 51:1906-1917.

86. Takada I, Suzawa M, Kato S. 2005. Nuclear receptors as targets for drug development: crosstalk between peroxisome proliferator-activated receptor gamma and cytokines in bone marrow-derived mesenchymal stem cells. J Pharmacol Sci. 97:184-189.

87. Ye J. 2008. Regulation of PPARgamma function by TNF-alpha. Biochem Biophys Res Commun. 374:405-408.

88. Kennedy, A.; Overman, A.; LaPoint, K.; Hopkins, R.; West, T.; Chuang, C.C.; Martinez, K.; Bell, D.; McIntosh, M. Conjugated linoleic acid-mediated inflammation and insulin

resistance in human adipocytes are attenuated by resveratrol. J. Lipid Res. 2009, *50*, 225-232.

89. Purushotham, A.; Wendel, A.A.; Liu, L.; Belury, M.A. Maintenance of adiponectin attenuates insulin resistance induced by dietary conjugated linoleic acid in mice. J. Lipid Res. 2007, *48*, 444-452.

90. Liu, L.F.; Clipstone, N. Prostaglandin F2alpha inhibits adipocyte differentiation via a Gαq-calcium-calcineurin-dependent signaling pathway. J. Cell Biochem. 2007, *100*, 161-173.

91. Steck, S.E.; Chalecki, A.M.; Miller, P.; Conway, J.; Austin, G.L.; Hardin, J.W.; Albright, C.D.; Thuillier, P. Conjugated linoleic acid supplementation for twelve weeks increases lean body mass in obese humans. J. Nutr. 2007, *137*, 1188–1193.

92. Moloney, F.; Toomey, S.; Loscher, C.; Noone, E.; Nugent, A.; Allan, B.; Roche, H. Anti-diabetic effect of cis-9, trans-11 conjugated linoleic acid may be mediated via anti-inflammatory effects in white adipose tissue. Diabetes 2007, *56* (3), 574-582.

93. Risérus, U.; Arner, P.; Brismar, K.; Vessby, B. Treatment with dietary trans10cis12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. Diabetes Care 2002, *25*, 1516-1521.

94. Moloney, F.; Yeow, T.P.; Mullen, A.; Nolan, J.J.; Roche, H.M. Conjugated linoleic acid supplementation, insulin sensitivity, and lipoprotein metabolism in patients with type 2 diabetes mellitus. Am. J. Clin. Nutr. 2004, *80*, 887-895.

95. Thrush, A.B.; Chabowski, A.; Heigenhauser, G.J.; McBride, B.W.; Or-Rashid, M.; Dyck, D.J. Conjugated linoleic acid increases skeletal muscle ceramide content and decreases insulin sensitivity in overweight, non-diabetic humans. Appl. Physiol. Nutr. Metab. 2007, *32*, 372-382.

96. Ahn, I.S.; Choi, B.H.; Ha, J.H.; Byun, J.M.; Shin, H.G.; Park, K.Y.; Do, M.S. Isomer-specific effect of conjugated linoleic acid on inflammatory adipokines associated with fat accumulation in 3T3-L1 adipocytes. J. Med. Food 2006, *9*, 307-312.

97. Pérez-Matute, P.; Marti, A.; Martínez, J.A.; Fernández-Otero, M.P.; Stanhope, K.L.; Havel, P.J.; Moreno-Aliaga, M.J. Conjugated linoleic acid inhibits glucose metabolism, leptin and adiponectin secretion in primary cultured rat adipocytes. Mol. Cell. Endocrinol. 2007, *268*, 50-58.

98. Halade, G.V.; Rahman, M.M.; Fernandes, G. Effect of CLA isomers and their mixture on aging C57BL/6J mice. Eur. J. Nutr. 2009, *48*, 409-418.

99. Hauner H. 2005. Secretory factors from human adipose tissue and their functional role. *Proc Nutr Soc.* 64: 163-169.

100. Tchoukalova Y., Koutsari C., & Jensen M. 2007.Committed subcutaneous preadipocytes are reduced in human obesity. *Diabetologia.* 50: 151-157.

101. Fain JN., Madan AK., Hiler ML., Cheema P., Bahouth SW. 2004. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology.* 145: 2273-2282.

102. Gil A., Aguilera CM., Gil-Campos M., & Cañete R. 2007. Altered signalling and gene expression associated with the immune system and the inflammatory response in obesity. *Br J Nutr.* 98; Suppl 1: S121-S126.

103. Chung S., LaPoint K., Martinez K., Kennedy A., Sandberg MB., & McIntosh MK. 2006. Preadipocytes mediate LPS-induced inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes. *Endocrinology.* 147: 5340-5351.

104. Ajuwon KM., & Spurlock ME. 2005. Palmitate activates the NF-kappaB transcription factor and induces IL-6 and TNFalpha expression in 3T3-L1 adipocytes. *J Nutr.* 135: 1841-1846.

105. Skurk T., Alberti-Huber C., Herder C., & Hauner H. 2007. Relationship between adipocyte size and adipokine expression and secretion. J Clin Endocrinol Metab. 92: 1023-1033.

106. Suganami T., Nishida J., & Ogawa Y. 2005. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. *Ateriosclr Thromb Vasc Biol.* 25: 2062-2068.

107. Suganami T., Tanimoto-Koyama K., Nishida J., Itoh M., Yuan X., Mizuarai S., Kotani H., Yamaoka S., Miyake K., Aoe S., Kamei Y., & Ogawa Y. 2007. Role of Toll-like receptor-4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. necrosis factor alpha. *Aterioscler Thromb Vasc Biol.* 27:84-91.

CHAPTER III

TRANS-10, *CIS*-12 CONJUGATED LINOLEIC ACID INSTIGATES INFLAMMATION IN HUMAN ADIPOCYTES COMPARED TO PREADIPOCYTES

Abstract

We previously showed in primary human (pre)adipocytes that lipopolysaccharide (LPS) and *trans*-10, *cis*-12 (10,12) conjugated linoleic acid (CLA) activate inflammatory signaling that promotes insulin resistance. Because our published data demonstrated that preadipocytes were the primary instigators of inflammatory signaling in LPS-treated cultures, we hypothesized that they played the same role in 10,12 CLA-mediated inflammation. To test this hypothesis, we employed four distinct models. In Model 1, a Differentiation Model, CLA activation of mitogen activated protein kinases (MAPK) and induction of interleukin (IL)-8, IL-6, IL-1β, and cyclo-oxygenase (COX)-2 were greatest in differentiated compared to undifferentiated cultures. In Model 2, a Cell Separation Model, the mRNA levels of these inflammatory proteins were increased by 10,12 CLA compared to BSA vehicle in the adipocyte fraction (ADF) and the preadipocyte fraction (PDF). In Model 3, a Co-Culture Insert Model, inserts containing ~50% adipocytes (AD50) or ~100% preadipocytes (AD0) were suspended over wells containing AD50 or AD0 cultures. 10,12 CLA-induced IL-8, IL-6, IL-1β, and COX-2 mRNA levels were highest in AD50 cultures when co-cultured with AD0 inserts. In Model 4, a Conditioned Media (CM) Model, CM collected from CLA-treated AD50, but not AD0 cultures, induced IL-8 and IL-6 mRNA levels and activated phosphorylation of MAPK in naïve

AD0 and AD50 cultures. Consistent with these data, 10,12 CLA-mediated secretions of IL-8 and IL-6 from AD50 cultures were higher than from AD0 cultures. Notably, blocking adipocytokine secretion prevented the inflammatory capacity of CM from 10,12 CLA-treated cultures. These data suggest that CLA instigates release of inflammatory signals from adipocytes that subsequently activate adjacent preadipocytes.

Introduction

Obesity is the most prevalent nutrition-related disease in the U.S., where 66% of the adult population is classified as overweight or obese (1). Obesity is linked to chronic diseases, such as type 2 diabetes, hypertension, and cardiovascular disease. Annual health care costs for treating the overweight and obese population are ~ $100 billion. Thus, maintaining ideal body weight will decrease the incidence of and health care costs associated with obesity. However, current strategies that promote effective and safe weight loss or maintenance are lacking.

Notably, a commercially-available weight loss supplement containing two equal levels of conjugated linoleic acid (CLA) isomers [i.e., cis-9, trans-11 (9,11) and trans-10, cis-12 (10,12)] decreases adiposity or increases lean body mass in animals (2-6) and some humans (7-10). It appears that 10,12 CLA, rather than 9,11 CLA, is the anti-obesity isomer in this supplement (reviewed in 11). Potential mechanisms responsible for these anti-obesity properties of 10,12 CLA include 1) decreasing energy intake or increasing energy expenditure (12-16), 2) decreasing lipogenesis or increasing lipolysis

(17-20), 3) decreasing adipogenesis or increasing delipidation (21-25), or 4) increasing adipocyte apoptosis (26-28).

There is, however, concern about potential adverse side effects of CLA supplementation, including lipodystrophy (28), steatosis (29), macrophage infiltration to white adipose tissue (WAT; 5), and insulin resistance (9, 30-32). For example, the 10,12 isomer of CLA caused increased insulin resistance (33) and increased markers of oxidative stress [e.g., iso-prostaglandin (PG) $F_{2\alpha}$] and atherosclerosis [e.g., C-reactive protein (CRP); 34] in obese men with Metabolic Syndrome. Consistent with these data, CLA supplementation (i.e., equal levels of 9,11 and 10,12 isomers) adversely affected insulin and glucose metabolism in patients with type 2 diabetes (35). More recently, it was reported that supplementing postmenopausal women with an equal mixture of 10,12 and 9,11 CLA increased serum triglyceride (TG) levels, CRP, and plasminogen activator inhibitor (PAI)-1, and urinary levels of iso-$PGF_{2\alpha}$ compared to the 9,11 isomer alone (32). Furthermore, insulin and tumor necrosis factor (TNF)α levels were elevated in the serum and WAT, respectively, in women consuming the CLA mixture compared to the olive oil controls (9).

Similarly, we have demonstrated that 10,12 CLA, but not 9,11 CLA, decreased the TG content of primary cultures of newly differentiated human adipocytes (18, 19, 23, 25). However, 10,12 CLA increased the expression levels of genes and proteins linked to chronic inflammation in these cultures, resulting in adverse metabolic consequences, such as insulin resistance (23, 25, 36, 37). Indeed, chronic inflammation driven by nuclear factor kappa B (NF-κB) and specific mitogen activated protein kinases (MAPK)

antagonize peroxisome proliferator activated receptor (PPAR)γ activity, thereby suppressing the transcriptional activation of genes needed for glucose and fatty acid uptake and conversion to lipids. Consistent with these data, we have demonstrated that 10,12 CLA-mediated suppression of PPARγ activity and target gene expression is linked to the activation of extracellular signal-related kinase (ERK)1/2 (23, 37) and NF-κB (36). Therefore, 1) the effective and safe use of CLA for weight loss or maintenance remains unclear, 2) the precise mechanism by which 10,12 CLA promotes inflammation and delipidation is unknown, and 3) the cell type in our primary cultures of human adipocytes responsible for mediating the inflammatory effects of CLA is unknown. To better understand the connection between inflammation and delipidation induced by CLA in our cultures, it is important to first identify the cell type in our cultures that is responsible for initiating the inflammatory effects of CLA. Thus, the objective of this research was to identify the role that preadipocytes vs. adipocytes play in mediating 10,12 CLA-mediated inflammation in primary cultures of newly differentiated human adipocytes.

Experimental Procedures

Materials and Models

Materials- All cell culture-ware and Hyclone fetal bovine serum (FBS) were purchased from Fisher Scientific (Norcross, GA). Adipocyte media (AM-1) was purchased from Zen-Bio. Isomers of CLA (+98% pure) were purchased from Matreya

(Pleasant Gap, PA). Lightning Chemiluminescence Substrate was purchased from Perkin Elmer Life Science (Boston, MA). Immunoblotting buffers and precast gels were purchased from Invitrogen (Carlsbad, CA). Primary antibodies for rabbit polyclonal phospho-p44/42 (Thr202/Tyr204, Cat # 9101), p44/p42 (Cat # 9102), phospho-SAPK/JNK (c-Jun-NH_2-terminal kinase; Thr183/Tyr185, Cat # 9251), SAPK/JNK (Cat # 9252), phospho-c-Jun (Ser63 Cat # 9261S), phospho-STAT3 (Tyr705, 3E2, Cat # 9138), STAT3 (Cat # 9139), c-Jun (60AB, Cat # 9165), and p38 (Cat # 9217) were purchased from Cell Signaling Technologies (Beverly, MA). Mouse monoclonal phospho-p38 (pT180/pY182, Cat # 612280) was purchased from BD Transduction Laboratories (San Jose, CA). The primary antibodies for activating transcription factor (ATF) 3 (C-19, Cat # sc-188) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc20357) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Gene expression assays for IL-1β, interleukin (IL)-8, IL-6, cyclooxygenase (COX)-2, adiponectin (apm1), adipocyte-specific fatty acid binding protein (aP2), adipocyte enhancer binding protein (AEBP)-1, and monocyte chemoattractant protein (MCP)-1 were purchased from Applied Biosystems Inc. (Foster City, CA). PicoGreen reagent was purchased from Molecular Probes (Eugene, OR). BioPlex singleplex assays for IL-6 and IL-1β were purchased from Bio-Rad (Hercules, CA). Recombinant human IL-6 was purchased from Fitzgerald Industries International (Concord, MA). Monoclonal anti-human IL-6 antibody (ab) was purchased from R&D Systems (Minneapolis, MN). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and AL-8810 were purchased from Cayman Chemical (Ann Arbor, MI). All other chemicals and

reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Cell Culture Models- Abdominal WAT was obtained from non-diabetic Caucasian and African American females with a body mass index ≤ 32.0 and between the ages of 20 and 50 years old who had undergone elective surgery as previously described (23). These selection criteria allow for reduced variation in gender, age, and obesity status. Institutional Review Board approval was granted through the University of North Carolina at Greensboro and the Moses H. Cone Memorial Hospital. Stromal vascular (SV) cells from human WAT were isolated via collagenase digestion and subsequently grown as previously described (23). CLA was administered at a physiological level of 50 µM. Each experiment was repeated at least twice using a mixture of cells from at least three different subjects unless otherwise indicated.

Because our previous CLA studies had been conducted in primary cultures of human adipocytes containing ~50% preadipocytes and 50% adipocytes (see 36 for marker analyses of these cells), we did not know which cells were instigating inflammation or insulin resistance in response to CLA treatment. To better understand the extent to which preadipocytes and adipocytes initiated inflammation in these cultures, we developed four distinct cell models described below.

Model 1- Differentiation Model. Four human (pre)adipocyte cell models containing ~ 0, 10, 30, and 50% adipocytes (i.e., AD0, AD10, AD30, and AD50) were established by modulating exposure to differentiation media (DM-1) containing 250 µM isobuthylmethylxanthine (IBMX) and 1 µM Rosiglitazone (BRL, a PPARγ agonist

generously provided by Dr. Per Sauerberg at Novo Nordisk A/S, Copenhagen, Denmark). SV cells were seeded into 35 mm dishes at 4 x 10^4 cells/cm^2. AD0 cultures received no DM-1 for 8 d (NT). AD10, AD30, and AD50 cultures were supplemented with DM-1 for 5 h, 3 d, or 6 d, respectively (**Fig. 3.1A**). Cells were treated with 50 µM 10,12 CLA for 12 h and harvested on d 8. For MAPK and AP-1 activation shown in **Fig. 3.3**, cells were treated for 6, 12, and 24 h.

Model 2- Cell Separation Model. SV cells were grown in 100 mm dishes containing ~ 3 million cells and were differentiated to ~ 30% adipocytes (AD30). On day 8, cells were treated with 50 µM 10,12 CLA or BSA vehicle for 12 h (**Fig. 3.4**). Cells were washed with Hanks Balanced Salt Solution (HBSS) containing 0.5 mM EDTA and trypsinized with trypsin-like enzyme at 37ºC for 10 min. Cells were layered on 6% iodixanol (Optiprep;Axis-Shield, Oslo, Norway; ~1.03 g/ml) in HBSS containing 0.5% BSA in a 15 ml centrifuge tube and centrifuged at 650 x g for 20 min at 4ºC. The floating adipocytes were collected from the top of the tube and placed in a 1.5 ml microcentrifuge tube and resuspended in HBSS and recentrifuged for 5 min at 5000 x g to remove any cell debris. The remaining supernatant was removed and the SV cells were collected from the pellet and transferred to a microcentrifuge tube. TriReagent was added to each tube for RNA extraction.

Model 3- Co-Culture Insert Model. SV cells were seeded in cell culture inserts seeded with 0.2 million cells per insert or in 6-well Multiwell™ plates seeded at 4 x 10^4 cells/cm^2. Inserts and wells received either AM-1 until d 8 to achieve AD0 cultures or DM-1 for 6 d to achieve AD50 cultures. At d 8 of differentiation, inserts were transferred

over underlying wells and a recovery period of ~15 h was allotted to allow for a decrease in potential stress due to the transfer (**Fig. 3.5**). Next, both wells and inserts were supplemented with 50 μM 10,12 CLA or BSA vehicle control for 12 h and inflammatory gene expression was measured via qPCR in cultures harvested from the underlying wells.

Model 4- Conditioned Media (CM) Model. SV cells were seeded in 60 mm dishes at 4 x 10^4 cells/cm^2. Cultures received either AM-1 for 8 d or DM-1 for 6 d to achieve AD0 and AD50 cultures, respectively (**Fig. 3.6**). On d 8, cells were treated with 50 μM 10,12 CLA or BSA control for 24 h after which CM was collected and stored at -80°C for subsequent experiments. Naïve AD0 and AD50 cultures were treated with a 1:1 ratio of thawed CM and fresh AM-1 at d 8 of differentiation.

Methods

Lipid and Nuclear Staining and TG Content- Intracellular lipid and nuclei were visualized by staining cultures with ORO and Mayer's Hematoxylin, respectively, as previously described (18). For establishing the Differentiation Model, photomicrographs were taken using an Olympus Microscope Digital Camera Model DP71 to provide micrographs of the degree of lipid accumulation in relation to nuclei in the cultures. In **Fig. 3.1B**, three fields were captured per well with three replicates per treatment group (i.e., AD0, AD10, AD30, and AD50). Therefore, a total of nine pictures were taken per treatment group. Data were expressed as the percentage of ORO-stained adipocytes to total cell number. TG levels were determined with a modified commercially-available

TG assay as previously described (19). To normalize the TG content, DNA was isolated using a DNeasy® Blood & Tissue Kit (QIAGEN, Valencia, CA) and then quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen; Molecular Probes, Carlsbad, CA).

RNA Isolation and qPCR- RNA was isolated from cell cultures using TriReagent (Molecular Research Center; Cincinnati, OH) following the manufacturer's protocol for RT-PCR. For real-time quantitative (q) PCR, 2 µg total RNA was used to generate first-strand cDNA using Applied Biosystems High Capacity cDNA Archive Kit (Foster City, CA). qPCR was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems) using TaqMan® Universal PCR Master Mix and Taqman Gene Expression Assays. To account for possible variation related to cDNA input amounts or the presence of PCR inhibitors, the endogenous reference gene GAPDH was simultaneously quantified for each sample in separate wells of the same 96-well plate.

Immunobloting- Immunoblotting was conducted as previously described using NuPage precast gels from Invitrogen (23).

Secretion of IL-6 and IL-8 via Multiplex Cytokine Assay- The concentrations of IL-6 and IL-8 were determined using the BioPlex® Suspension Array System from Bio-Rad (Hercules, CA) following the manufacturer's protocol.

Quantification of PGE_2 and PGF_2- PGE_2 and PGF_2 were measured with a stable isotope dilution gas chromatographic/negative ion chemical ionization-mass spectrometric (GC/NICI/MS) assay (38). Briefly, PGE_2-d_4 (2.12 ng) and PGF_2-d_4 (0.84

ng) internal standards were added to the media samples. The sample was then acidified to pH 3.0 with 1 N HCl and extracted on a C18 Sep-Pak. PGE_2 and PGF_2 were eluted with ethyl acetate:heptane and evaporated under a stream of nitrogen (N_2). PGE_2 and PGF_2 in methoxylamine solution were extracted with ethyl acetate and evaporated with N_2. The pentafluorobenzyl esters were purified by thin layer chromatography (TLC; PGF_2 and PGE_2 methyl esters were used as TLC standards), converted to O-methyloxime pentafluorobenzyl ester trimethylsilyl derivatives, and PGE_2 and PGF_2 were dissolved in undecane that was dried over a bed of calcium hydride. GC/NICI/MS analysis was performed as described previously with the ions for PGE_2 (m/z 526) and the [$^4H_2$]-PGE_2 as internal standard (m/z 528) and the ions for PGF_2 (m/z 569) and the [$^4H_2$]-PGF_2 as internal standard (m/z 573).

Quantification of Isoprostanes (IsoP)s- Total F_2-IsoPs were measured by GC/MS with selective ion monitoring (39, 40). Briefly, cells were resuspended in 0.5 ml of methanol containing 0.005% butylated hydroxytoluene, sonicated, and then subjected to chemical saponification with 15% KOH to hydrolyze bound F_2-IsoPs. The cell lysates were adjusted to a pH of 3.0, followed by the addition of 0.1 ng of $^4H_2$-labeled 15-F_{2t}-IsoP internal standard. F_2-IsoPs were subsequently purified by C_{18} and silica Sep-Pak extraction and by TLC. They were subsequently analyzed with pentafluorobenzyl ester, a trimethylsilyl ether derivative, via GC/NICI/MS assay.

Lipolysis Assay- [^{14}C]-oleic acid release into CM was measured after 3, 9, or 24 h treatment of (pre)adipocyte cultures with 10,12 CLA or BSA vehicle as previously described (20).

Statistical Analysis- Statistical analyses were performed for data in **Figs. 3.1B- C, and 9B** using one-way ANOVA. Analyses were performed for data in **Figs. 3.2, 3.4B-C, 3.5B, 3.6B, 3.7, 3.8A,** and **3.9A** using two-way ANOVA with interaction (JMP version 6.03, SAS Institute). Data in **Figs. 3.8B** were analyzed by a multifactor ANOVA with interaction. Student's t tests were used to compute individual pairwise comparisons of least square means ($P < 0.05$). Data are expressed as the means ± SE. Number of replicates and independent experiments varies with each outcome.

Results

Differentiation Model

Cell Types in Our Cultures- We previously identified the predominate type of cells in our primary cultures of newly differentiated human adipocytes based on marker analysis (41). Undifferentiated cultures lacking DM-1 supplementation consisted of preadipocytes (i.e., + for Pref-1 and - for aP2 and PPARγ). Differentiated cultures supplemented with DM-1 media consisted of preadipocytes and adipocytes (i.e., + for Pref-1 or aP2 and PPARγ, respectively). None of the cells were positive for markers of myocytes (e.g., MyoD) or macrophages (e.g., CD-68, MAC-1). Based on these findings, undifferentiated cells (i.e., lacking aP2 or PPARγ expression, TG content, or visible lipid droplets) discussed in this article will be referred to as preadipocytes.

The Percentage of Lipid-filled Adipocytes Increases as the Duration of Rosiglitazone (BRL) Treatment Increases- We previously demonstrated in primary cultures of newly

differentiated human adipocytes that 10,12 CLA promotes inflammatory signaling, insulin resistance, and delipidation (23, 25, 36, 37). We have also shown that preadipocytes are the primary instigators of inflammation and insulin resistance induced by lipopolysaccharide (LPS) in our cultures (41). Based on these data, we originally hypothesized that preadipocytes would be the instigators of the inflammatory response to 10,12 CLA. In order to determine whether the preadipocytes or the adipocytes were responsible for 10,12 CLA-mediated inflammatory signaling, we developed a Differentiation Model in which various levels of differentiation were achieved as described in the Materials and Models section. As shown in **Fig. 3.1A**, the percentage of adipocytes stained with ORO relative to total cell number increased as the duration of 1 μM BRL treatment increased from no treatment (NT) to 6 d of treatment. BRL untreated cultures showed no ORO-stained adipocytes, whereas cultures that received 1 μM BRL for 5 h, 3 d, or 6 d yielded ~ 10, 30, or 50% ORO-stained adipocytes, respectively (**Fig. 3.1B**). Furthermore, TG content in AD30 and AD50 cultures was significantly greater than in AD0 or AD10 cultures (**Fig. 3.1C**). Therefore, by manipulating the duration of treatment with the PPARγ agonist BRL, a Differentiation Model was established that had four distinct levels of lipid-filled adipocytes in the cultures.

Adipocytes Play an Essential Role in 10,12 CLA-induced Inflammatory Gene Expression and Activation of MAPK and Activating Protein (AP)-1 Subunits- Gene expression of preadipocyte (i.e., AEBP-1) and adipocyte [aP2 and adiponectin (apm1)] markers were analyzed to verify levels of preadipocytes and adipocytes in each group of the Differentiation Model (**Fig. 3.2A**). The expression AEBP-1 was highest in the AD0

cultures and decreased as the level of differentiation increased. In contrast, the mRNA levels of aP2 and apm1 were lowest in the AD0 cultures, and increased as the level of differentiation increased. Consistent with previous findings (23), 10,12 CLA attenuated aP2 and apm1 gene expression compared to BSA control in AD30 and AD50 cultures.

To determine which experimental group (i.e., AD0, AD10, AD30, or AD50) displayed the greatest level of inflammatory signaling in response to 10,12 CLA treatment, gene expression of inflammatory cytokines and COX-2 were analyzed. Cultures were treated with 50 μM 10,12 CLA, 9,11 CLA, or BSA vehicle for 12 h. These dose and time points were selected based on previous time course and dose response studies conducted in our lab (25, 37). In contrast to previous findings with LPS as an inflammatory stimulant, 10,12 CLA treatment caused the greatest increase in the expression of IL-8, IL-6, IL-1β, and COX-2 in AD50 cultures compared to all other groups (**Fig. 3.2B**). The induction of these genes by 10,12 CLA was greater in AD50 compared to AD30 cultures. Furthermore, 10,12 CLA did not induce inflammatory genes in AD0 or AD10 cultures compared to BSA. Consistent with our previous findings (19, 23, 37), the inflammatory effect of CLA was specific to the 10,12 isomer, where 9,11 CLA did not induce inflammatory gene expression compared to BSA vehicle.

Given the role of MAPK and AP-1 in activating inflammatory gene expression during cellular stress, their activation by 10,12 CLA in AD50 vs. AD0 cultures was examined. 10,12 CLA robustly increased the phosphorylation of ERK, JNK, and p38 at 12 and 24 h in AD50 vs. AD0 cultures compared to BSA controls (**Fig. 3.3A**). Similarly, 10,12 CLA increased the levels of ATF3 and P-c-Jun at 12 and 24 h in the AD50 cultures, but not in

the AD0 cultures, compared to BSA controls (**Fig. 3.3B**). Collectively, these data suggest that adipocytes are essential for 10,12 CLA-mediated activation of MAPK and transcription factors and induction of genes associated with inflammation.

Cell Separation Model

10,12 CLA Induces Inflammatory Gene Expression in Adipocytes and Preadipocytes from Mixed Cultures- The Cell Separation Model, as described in the Methods section and shown in **Fig. 3.4A**, was used to demonstrate that adipocytes are responsible for the inflammatory response induced by 10,12 CLA in mixed cultures of preadipocytes and adipocytes. On d 8, cells were treated with 50 μM 10,12 CLA or BSA vehicle for 12 h and fractionated to yield a floating ADF and a pelleted PDF. The preadipocyte marker AEBP-1 was expressed at higher levels in the PDF compared to the ADF (**Fig. 3.4B**). Adipocyte-specific genes, aP2 and apm1, were expressed at higher levels in the ADF compared to the PDF (**Fig. 3.4B**). As expected, 10,12 CLA decreased aP2 and apm1 gene expression in the ADF.

Surprisingly, the expression of IL-8, IL-6, and COX-2 induced by 10,12 CLA was greater than BSA vehicle controls in the PDF and the ADF (**Fig. 3.4C**). Thus, we hypothesized that the inflammatory response to 10,12 CLA in the PDF may be due to cross-talk between adipocytes and preadipocytes prior to fractionation, as there is cell-to-cell contact in these mixed cultures (AD30) during the 12 h treatment with 10,12 CLA. Alternatively, it is possible that immature adipocytes contaminated the PDF. However,

adipogenic gene expression was much greater in the ADF compared to the PDF, so the possibility that a significant number of adipocytes contaminated the PDF is unlikely. Thus, a third model was utilized to better understand 10,12 CLA-induced inflammatory gene expression and potential cross-talk between adipocytes and preadipocytes.

Co-Culture Insert Model

Preadipocytes Enhance the Inflammatory Response to 10,12 CLA in Adipocytes- To determine the extent to which adipocytes communicate with preadipocytes during 10,12 CLA treatment, AD0 and AD50 cultures were grown in inserts and suspended over underlying wells containing AD0 or AD50 cultures during treatment with 50 µM 10,12 CLA or BSA vehicle for 12 h (**Fig. 3.5A**). Gene expression was measured only in cells grown in underlying wells.

Consistent with the Differentiation Model, 10,12 CLA did not induce inflammatory gene expression in preadipocytes (AD0), regardless of whether preadipocyte (AD0 + AD0 insert) or adipocyte (AD0 + AD50 insert) inserts were suspended above them (**Fig 3.5B**). Also consistent with the Differentiation Model, 10,12 CLA-induced inflammatory gene expression was greater in AD50 vs. AD0 cultures. However, the mRNA levels of genes induced by 10,12 CLA were greatest in AD50 cultures when AD0-containing inserts were suspended above them (AD50 + AD0 insert) compared to when AD50 cultures were suspended above them (AD50 + AD50 inserts). These data suggest that the presence of preadipocytes enhanced the increase of inflammatory gene expression in

AD50 cultures by 10,12 CLA treatment, once again suggesting cross-talk between preadipocytes and adipocytes. Therefore, a fourth model, the Conditioned Media (CM) Model, was implemented to clarify which cultures, AD50 or AD0, are responsible for 10,12 CLA-mediated inflammation.

Conditioned Media (CM) Model

Adipocytes Secrete Inflammatory Signals that Activate Preadipocytes- This fourth model, as described in the Methods section and shown in **Fig. 3.6A**, was developed to better understand the inflammatory capacity of adipocytes *vs.* preadipocytes as well as crosstalk between both cell-types. It can be inferred from the Co-Culture Insert Model that cross-talk occurs between adipocytes and preadipocytes, as evidenced by the increase in inflammatory gene expression in AD50 cultures when AD0-containing inserts are suspended above them. Data from the Cell Separation Model also suggest that cross-talk occurs between adipocytes and preadipocytes. Therefore, we hypothesized that CM from 10,12 CLA-treated AD50, but not AD0 cultures, would promote inflammatory protein activation and gene expression in naïve AD0 and AD50 cultures.

CM was collected from AD50 and AD0 cultures treated with 50 μM 10,12 CLA or BSA vehicle for 24 h. A 24 h time point was chosen based on previous dose data showing that the secretion of IL-8, IL-6, and $PGF_{2\alpha}$ by 24 h after 10,12 CLA treatment was greater than earlier time points in mixed cultures (36, 37). As hypothesized, CM from 10,12 CLA-treated AD50 cultures increased IL-8 and IL-6 gene expression in naïve

AD50 and AD0 cultures (**Fig. 3.6B**). MCP-1 and ATF3 gene expression were also increased in naïve AD0 and AD50 cultures by CM from 10,12 CLA-treated AD50 (data not shown). In contrast, CM from CLA-treated AD0 cultures did not increase inflammatory gene expression in naïve AD50 or AD0 cultures. Notably, CM from 10,12 CLA-treated AD50 cultures caused a greater level of inflammatory gene expression in naïve AD0 *vs.* AD50 cultures.

Consistent with data in **Fig. 3.6B**, CM from AD50 cultures treated with 10,12 CLA *vs.* BSA vehicle increased P-ERK, P-JNK, and P-38 in naïve AD0 and AD50 cultures (**Fig. 3.6C)**. Next, we wanted to determine the extent to which CM from AD0 compared to AD50 cultures treated with 10,12 CLA for 15 min impacted MAPK phosphorylation. As hypothesized, CM from AD50 cultures increased the P-ERK, P-JNK, and P-p38, particularly in naive AD0 cultures compared to naïve AD50 cultures (**Fig. 3.6D)**. In contrast, CM from AD0 cultures did not increase the levels of P-ERK, P-JNK, or P-p38 in either set of cultures. Collectively, these data suggest that 10,12 CLA selectively activates adipocytes that secrete inflammatory adipocytokines, PGs, or free fatty acids (FFA) that signal to preadipocytes, resulting in increased inflammatory protein activation and gene expression in preadipocytes.

Therefore, we wanted to identify inflammatory candidates in the CM from cultures treated with 10,12 CLA. IL-6 and IL-8 levels (**Fig. 3.7A**) as well as PGF_2 and PGE_2 (**Fig. 3.7B**) were higher in CM from 10,12 CLA-treated AD50 cultures, but not in AD0 cultures compared to BSA controls. However, the increase in PGE_2 in AD50 cultures was not higher than basal levels found in AD0 cultures. Consistent with the PGF_2 data, iso-

prostane F2 levels were higher in AD50 cultures treated with 10,12 CLA compared to BSA controls (**Fig. 3.7C**).

Due to the role that elevated FFA play in inducing inflammation and insulin resistance, we suspected that 10,12 CLA-mediated lipolysis may also be contributing inflammatory FFAs to the CM. Surprisingly, 10,12 CLA treatment increased [^{14}C]-oleic acid release from AD0 cultures at 9 h (**Fig. 3.7D**) and 24 h (data not shown), but had no effect on AD50 cultures. These data suggest that 10,12 CLA-driven lipolytic activity does not contribute to the inflammatory capacity of AD50 CM.

In order to determine if adipocytokines are necessary for the inflammatory capacity of AD50 CM, AD50 cultures were pretreated with 1 µg/ml brefeldin A (BA) for 1 h to prevent cytokine secretion by inhibiting protein transport from the ER to the golgi apparatus. Subsequently, cultures were treated with 50 µM CLA or BSA for 24 h, after which CM was collected and added to naïve AD0 or AD50 cultures (at a 1:1 ratio) for 3 h and cells were harvested to measure inflammatory gene expression. As hypothesized, BA pretreatment prevented CLA-mediated adipocytokine secretion (**Fig. 3.8A**). Furthermore, pretreatment with BA prevented AD50 CM-mediated increases of IL-8 and IL-6 mRNA levels in AD0 and AD50 cultures (**Fig. 3.8B**). Similar effects were found for MCP-1 and ATF-3 gene expression (data not shown).

In order to more specifically identify inflammatory adipocytokines or PGs responsible for mediating inflammatory signaling in AD0 cultures, IL-8, IL-6, and $PGF_{2\alpha}$ were targeted for further investigation. IL-8 α and β receptors, IL-6 receptor and signal transducer (ST), and $PGF_{2\alpha}$ receptor expression were measured in our primary cultures of

human preadipocytes. Consistent with a previous report (42), IL-8 α and β receptors were undetectable in human preadipocytes (data not shown). Interestingly, IL-6 receptor and ST and $PGF_{2\alpha}$ receptor expression were higher in AD0 vs. AD50 cultures (**Fig. 3.9A**). Consequently, AD50 cultures were pretreated with an IL-6 neutralizing ab or the FP prostanoid receptor antagonist AL-8810 for 30 min and subsequently treated with 10,12 CLA or BSA vehicle for 24 h. First, AD50 cultures were analyzed for inflammatory mRNA levels. Whereas there was no effect of 0.01 μg/ml IL-6 ab on IL-8 mRNA levels, 50 μM AL-8810 prevented CLA induction of IL-8 in AD50 cultures (**Fig. 3.9B**). Next, CM from these AD50 treated cultures was collected and added to AD0 cultures at a 1:1 ratio, after which IL-8 gene expression was measured at 3 h and P-STAT3, P-ERK, and P-JNK levels were measured at 1 h. IL-6 neutralizing ab and AL-8810 attenuated IL-8 gene expression (**Fig. 3.9B**), but not other inflammatory genes induced by CLA CM including IL-6 and MCP-1 (data not shown). Neutralizing IL-6 attenuated P-STAT3 levels in a dose-dependent manner (**Fig. 3.9C**). However, only 0.1 μg/ml IL-6 neutralizing ab modestly attenuated P-ERK and P-JNK levels increased by CLA CM. As expected, AL-8810 had no effect on P-STAT3 levels, but 10 μM AL-8810 reduced P-JNK levels, and modestly reduced P-ERK levels (**Fig. 3.9C**). The specificity of the IL-6 neutralizing ab and AL-8810 was confirmed (**Fig. 3.9D**). The IL-6 ab dose-dependently reduced IL-6-mediated increases in P-STAT3 and P-ERK levels, and AL-8810 dose-dependently reduced $PGF_{2\alpha}$-mediated increases in P-ERK levels. These data suggest that IL-6 and $PGF_{2\alpha}$ contribute, in part, to the inflammatory capacity of CLA CM from AD50 cultures. Taken together, these data imply that adipocytes instigate the inflammatory

response to 10,12 CLA treatment in newly differentiated cultures of primary human adipocytes, which in turn activate inflammatory pathways in neighboring preadipocytes (**Fig. 3.10**).

Discussion

Interpretation of Data from the Four Cell Models- We demonstrated using four experimental models, that adipocytes are the instigators of 10,12 CLA-induced inflammation in primary cultures of newly differentiated human adipocytes. Data from the Differentiation Model showed that 10,12 CLA-induced inflammatory signaling was greater in differentiated cultures (AD10-50) compared to undifferentiated cultures (AD0) as evidenced by increased expression of inflammatory genes and phosphorylation of MAPK and AP-1. Results from the Cell Separation Model suggest that adipocytes signal to preadipocytes during treatment with 10,12 CLA in mixed cultures (AD30), as there was an increase in inflammatory gene expression in the PDF, yet no induction of inflammatory genes in cultures containing only preadipocytes (AD0) as shown in the Differentiation and Co-Culture Insert Models. Next, it was demonstrated in the Co-Culture Insert Model that not only are adipocytes required for an inflammatory response to CLA, but that preadipocytes also enhance the inflammatory response in differentiated cultures to 10,12 CLA treatment. Finally, using the Conditioned Media Model, we demonstrated that CM from differentiated cultures (AD50), but not undifferentiated cultures (AD0), treated with 10,12 CLA yielded bioactive CM containing inflammatory

adipocytokines and PGs that promote increased inflammatory signaling in naïve (pre)adipocytes. Notably, CM from the differentiated cultures caused greater increases in inflammatory gene expression in preadipocytes (AD0) *vs.* adipocytes (AD50). Blocking adipocytokine release with brefeldin A prevented CLA CM induction of IL-6, IL-8, and MCP-1 Moreover, neutralizing IL-6 and blocking the $PGF_{2\alpha}$ receptor attenuated CLA CM-induced IL-8 gene expression or STAT3 phosphorylation in preadipocytes. However, other genes induced by CLA CM including IL-6 and MCP-1 were not affected. Therefore, IL-6 and $PGF_{2\alpha}$ may be key factors in CM contributing to the inflammatory response in preadipocytes, but likely are not solely responsible. Further investigation is needed to determine other important inflammatory mediators in CLA CM and potential synergism of these bioactive factors. Taken together, these studies show that adipocytes are the main initiators of 10,12 CLA-mediated inflammatory signaling in primary cultures of newly differentiated human adipocytes.

Cell Types in WAT and Their Inflammatory Capacity- Adult human WAT has been reported to contain ~50-70% adipocytes, ~ 20-40% preadipocytes, and ~1-30% infiltrated macrophages (43, 44). These percentages depend on body composition and age. For example, it has been reported that WAT of lean women contain ~30% preadipocytes, whereas WAT of obese women contain only 17% preadipocytes (45). However, the localization and secretory pattern of cytokines in WAT are unknown. Some researchers suggest that non-fat cells such as macrophages are the key players in cytokine release from adipose tissue (46, 47). According to Fain et al. 2004, non-fat cells are responsible for up to 90% of adipokine release from WAT compared to mature adipocytes. These

samples, obtained from obese women directly after undergoing bariatric surgery, were analyzed for basal levels of inflammatory markers. Similarly, results from our lab suggested that 10,12 CLA-mediated cytokine release was predominately from SV cells freshly isolated from human subcutaneous WAT as opposed to cultured newly differentiated adipocytes (23, 36). However, this heterogeneous mixture of SV cells had never been exposed to adipocyte media (AM-1) for 6-12 d as in the current study. Thus, we do not know if these SV cultures contained cells other than preadipocytes (i.e., macrophages) that may respond differently to CLA.

In contrast to these studies, our current results show that cultured preadipocytes grown in adipocyte media do not secrete adipocytokines in response to 10,12 CLA treatment, suggesting that the microenvironment of cultured (pre)adipocytes may affect experimental outcomes. For example, it has been proposed that adipocyte size is an important determinant of adipokine secretion, where proinflammatory cytokine secretion (i.e., IL-6, IL-8, MCP-1, TNFα) was higher from large adipocytes compared to smaller adipocytes (48). Interestingly, Suganami et al. (49, 50) reported that activated hypertrophied adipocytes release saturated free fatty acids that subsequently promote TNFα secretion from macrophages, leading to an inflammatory cycle in adipose tissue. Taken together, these results suggest that differences in the inflammatory capacity of preadipocytes, adipocytes, or other inflammatory cells like macrophages in WAT may be due to 1) the type of inflammatory stimuli employed, 2) the microenvironment of cultures, 3) adipocyte size, 4) degree of adiposity, or 5) crosstalk with other cell types.

Shortcomings of Cell Models- Due to our use of primary SV cells, it is difficult to achieve fully-differentiated cultures containing 100% adipocytes (AD100). Our greatest level of differentiation was approximately 50% adipocytes (AD50) on days 8-12. Therefore, it is difficult to clearly ascertain the function of adipocytes alone, being that preadipocytes are always present in our cultures. The Cell Separation Model provides us the ability to separately analyze inflammatory gene expression from each fraction. However, these cells have direct cell-to-cell contact during treatment. The Insert Model provides an environment where these cells are not in direct contact. Yet, the most differentiated cultures still contain ~50% adipocytes. Therefore, future studies are needed to determine the effect of CLA on pure cultures of mature adipocytes. These could be achieved by 1) developing a more effective differentiation cocktail, 2) using freshly isolated floating adipocytes during the isolation of SV cells, or 3) using fully differentiated cultures of murine 3T3-L1 adipocytes. Another challenge with using primary human adipocytes is that the degree of differentiation varies between experiments.

Although there are some limitations in using primary cultures, our model of primary cultures of newly differentiated human adipocytes is a particular strength due to its direct application to (pre)adipocytes in human WAT compared to using animal (pre)adipocytes or cell lines of adipocytes. This model also allows us to examine the direct effects of CLA on cross-talk between preadipocytes and adipocytes as occurs *in vivo*, and their impact on cell signaling, gene expression, and metabolism.

Implications and Future Experiments- Results presented in this manuscript clearly show that adipocytes are essential for the inflammatory response to CLA. On the basis of these studies, we propose that treating primary cultures of newly differentiated human adipocytes with 10,12 CLA increases the phosphorylation of MAPK (i.e., ERK 1/2, JNK, and p38) and the activation of the transcription factor AP-1 (i.e., c-Jun and ATF3), which leads to the production of inflammatory adipocytokines and PGs through upregulating inflammatory genes (i.e., COX-2, IL-6, IL-1β, IL-8, MCP-1, and ATF3). These inflammatory signals subsequently activate preadipocytes, leading to inflammatory cytokine secretion from preadipocytes, thus continuing the inflammatory cycle. These studies are expected to lead to discovering the earliest mechanism by which 10,12 CLA causes adipocyte delipidation. As a consequence, it will enable researchers to determine the potentially-safe and effective use of CLA as a dietary strategy to promote weight loss.

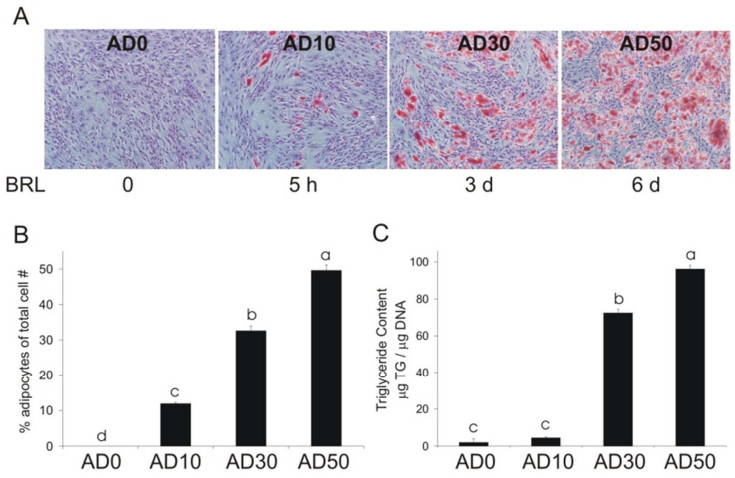

FIGURE 3.1. The percentage of lipid-filled adipocytes increases with the duration of BRL supplementation. Four human (pre)adipocyte cell models containing ~ 0, 10, 30, and 50% adipocytes were established by modulating exposure to DM-1. AD0 cultures received no DM-1 for 8 d (NT). AD10, AD30, and AD50 cultures were supplemented with DM-1 containing 1 µM BRL and 250 µM IBMX for 5 h, 3 d, or 6 d, respectively. **A)** On d 9-10, cells were fixed with baker's formalin, stained with ORO to detect adipocytes, and counter-stained with Mayer's Hematoxylin to detect non-differentiated cells. Three pictures per well were taken, each of a different field. **B)** The total number of cells stained with ORO were counted and expressed as a percentage of total cell number. **C)** TG content was determined using a colorimetric assay. Data are expressed as µg TG/ µg DNA. Data in panels **A-C** are representative of three independent experiments. Means ± SEM not sharing a common superscript differ significantly ($p < 0.05$).

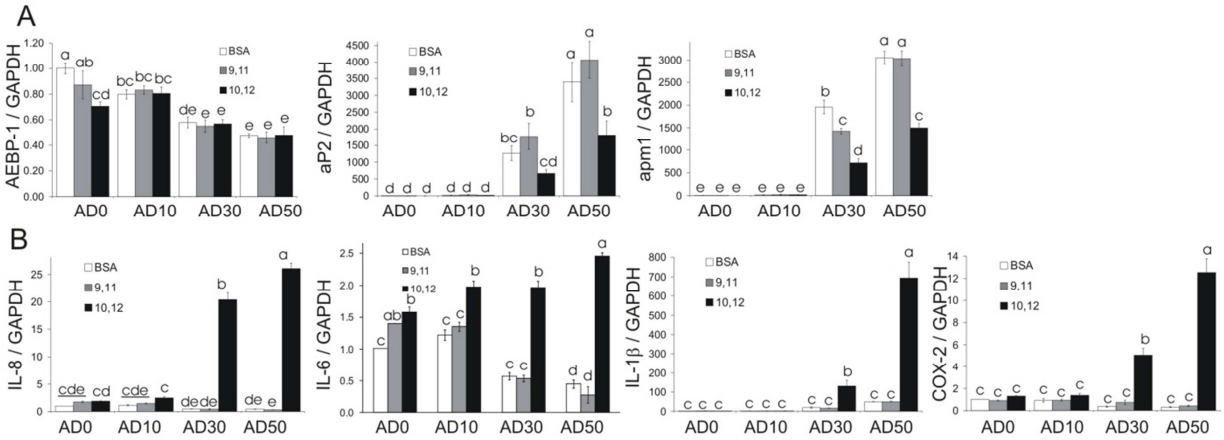

FIGURE 3.2. 10,12 CLA-induction of inflammatory gene expression increases as the degree of differentiation increases. Using the Differentiation Model, each experimental group (i.e., AD0, AD10, AD30, and AD50) was treated with BSA vehicle, 50 μM 10,12 CLA or 50 μM 9,11 CLA as a positive control for 12 h. Cells were harvested for RNA extraction and mRNA analyses via qPCR on d 9 for subsequent analysis of **A)** marker gene analyses to verify increases in adipocyte number with increasing duration of DM-1 treatment and **B)** inflammatory genes increased by 10,12 CLA compared to 9,11 CLA and BSA controls. Data were normalized to BSA vehicle in AD0 cultures. Means ± SEM (n = 3) not sharing a common superscript are significantly different ($p < 0.05$). Data in panels **A** and **B** are representative of three independent experiments.

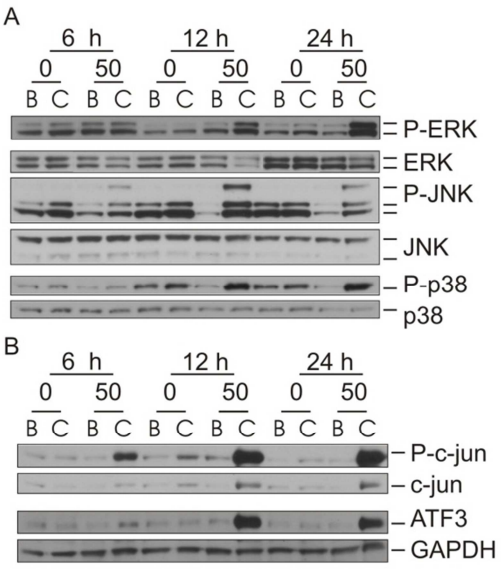

FIGURE 3.3. Time-dependent increase in the activation of MAPK and transcription factors in AD50 vs. AD0 cultures treated with 10,12 CLA. AD0 and AD50 cultures were treated with 50 μM 10,12 CLA (C) or BSA (B) control for 6, 12, or 24 h. Cells were harvested and analyzed for protein expression via immunoblot. Membranes were probed with antibodies targeting **A)** phospho (P)- and total ERK, JNK, and p38, and **B)** P-c-Jun, c-Jun, ATF3, and GAPDH (n = 2). Data in panels **A** and **B** are representative of three independent experiments.

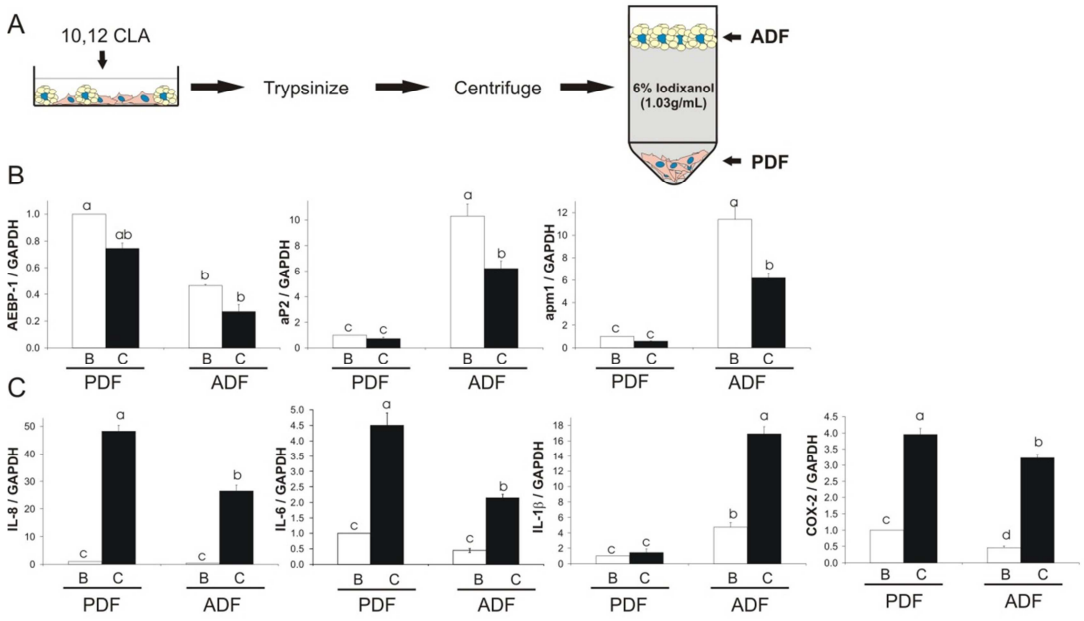

FIGURE 3.4. 10,12 CLA induces inflammatory gene expression in the PDF and ADF fractions of newly differentiated primary human adipocytes. **A)** Human SV cells were supplemented with 1 μM BRL for 3 d yielding cultures containing ~ 30% adipocytes (AD30). Cultures were treated with 50 μM 10,12 CLA or BSA vehicle for 12 h and next fractionated using 6% iodixanol (1.03 g/ml). The lipid-laden ADF was floated, and the PDF was pelleted. **B)** Fractionations were verified by measuring gene expression of AEBP-1, aP2, and apm1 using qPCR. **C)** Relative mRNA expression of IL-8, IL-1β, COX-2, and ATF3 were investigated using qPCR. Data in panels **B-C** were normalized to BSA vehicle in the PDF. Means ± SEM (n = 3) not sharing a common superscript differ significantly ($p < 0.05$). Data in panels **B** and **C** are representative of three independent experiments.

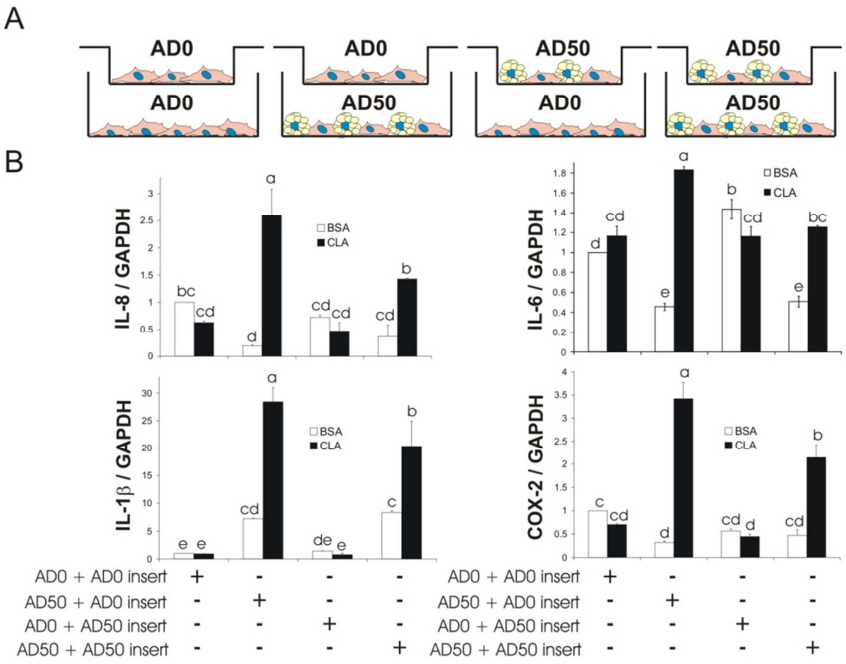

FIGURE 3.5. 10,12 CLA-induced inflammatory gene expression is greatest in AD50 cultures in the presence of AD0-containing inserts. A) Inserts containing either preadipocytes (AD0) or preadipocytes and adipocytes (AD50) were suspended over 6-well plates containing either AD0 or AD50 cultures. **B)** Next, both wells and inserts were supplemented with 50 μM 10,12 CLA or BSA vehicle control for 12 h and inflammatory gene expression in the cells in the underlying wells were subsequently analyzed via qPCR. Means ± SEM (n = 3) not sharing a common superscript differ significantly ($p < 0.05$). Data are representative of two independent experiments.

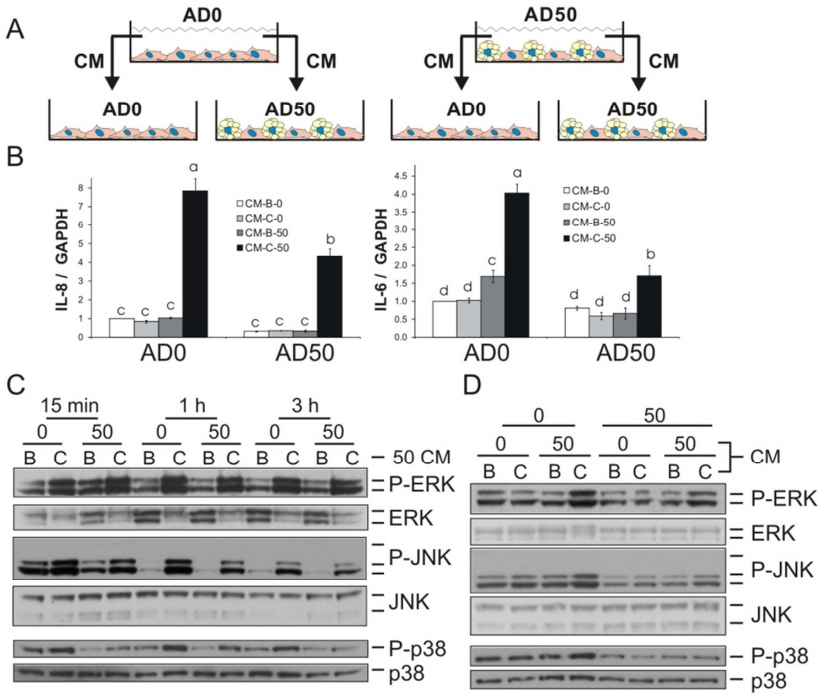

FIGURE 3.6. CM from 10,12 CLA-treated AD50 cultures induces inflammatory genes and activates MAPK in naïve AD0 and AD50 cultures. A) CM was collected from AD0 and AD50 cultures that were treated with 50 µM 10,12 CLA or BSA control for 24 h. A 1:1 ratio of CM and AM-1 was added to AD0 and AD50 cultures for 3 h. **B)** Next, inflammatory genes were analyzed via qPCR. Means ± SEM (n = 4) not sharing a common superscript differ significantly ($p < 0.05$). **C)** AD0 and AD50 cultures were treated with AD50 CM for 15 min, 1 h, and 3h. **D)** AD0 cultures were treated with AD50 and AD0 CM for 15 min. **C-D**) Cells were harvested and analyzed for protein expression via immunobloting. Membranes were probed with antibodies targeting P- and total ERK, JNK, and p38 (n = 2). Data in panels **B-D** are representative of three independent experiments.

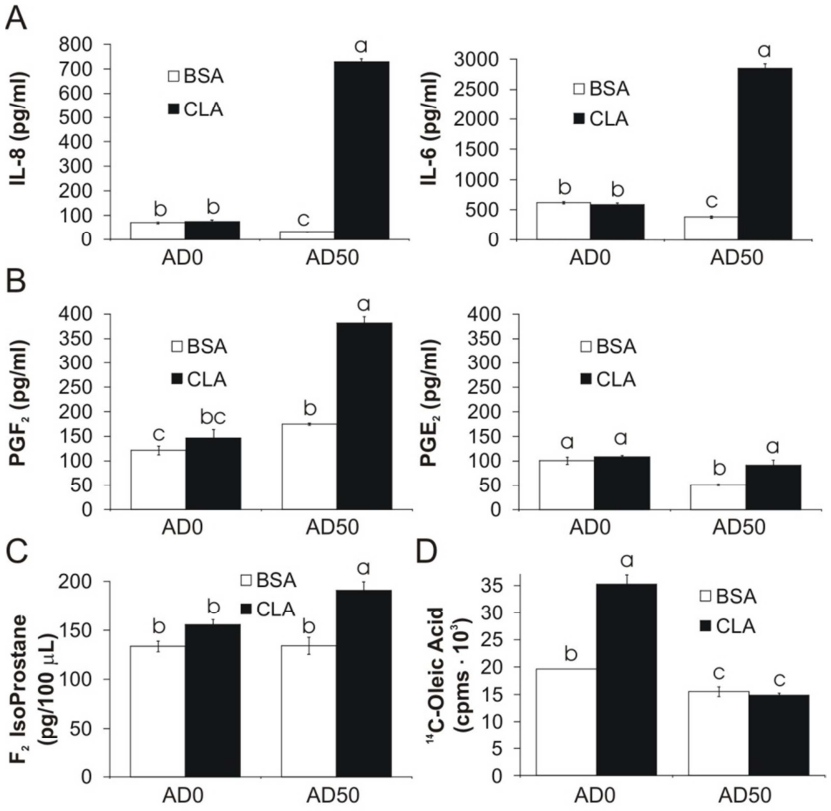

FIGURE 3.7. 10,12 CLA-treated AD50 cultures secrete adipocytokines and PGF_2. Cells were treated with 50 µM 10,12 CLA or BSA control for 24 h. **A)** CM was collected and IL-8 and IL-6 were measured in CM using BioRad's BioPlex suspension array system. **B)** CM was collected and PGF_2 and PGE_2 were measured using a stable isotope dilution GC/NICI/MS assay. **C)** Cells were harvested in PBS and total F_2-IsoProstanes were measured using GC/MS with selective ion monitoring. **D)** AD0 and AD50 cultures were treated with 20 µl HBSS containing 12.5 nM [^{14}C]-oleic acid (0.2 µCi; specific activity = 40-60 mCi/mmol) for 12 h. Media was removed and cells were washed three times with HBSS containing 2% BSA. Subsequently, each well was treated with 250 µl DMEM containing 50 µM 10,12 CLA or BSA + phloretin, a fatty acid uptake inhibitor, for 9 h. 200 µl media was collected from each well and measured for [^{14}C]-oleic acid by scintillation counting. Means ± SEM (n = 4 **A-C**; n = 3 **D**) not sharing a common superscript differ significantly ($p < 0.05$). Data in panels **A-C** and in panel **D** are representative of two or three independent experiments, respectively.

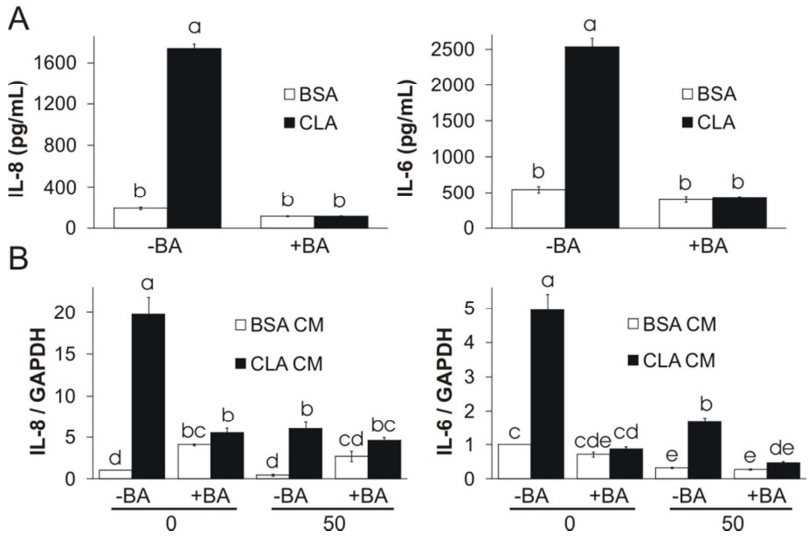

FIGURE 3.8. Brefeldin A (BA) prevents CM from 10,12 CLA AD50 cultures from inducing inflammatory gene expression. AD50 cultures were pretreated with 1 µg/ml BA for 1 h to prevent cytokine secretion, and treated with 50 µM CLA or BSA for 24 h. **A)** IL-8 and IL-6 were measured in CM using BioRad's BioPlex suspension array system. **B)** CM was collected and 1 mL was added to naïve AD0 or AD50 cultures containing 1 mL of AM-1 (1:1 ratio) for 3 h and cells were harvested to measure inflammatory gene expression via qPCR. Means ± SEM (n = 2 **A**; n = 3 **B**) not sharing a common superscript differ significantly ($p < 0.05$). Data in panel **A** and **B** are representative of two or three independent experiments, respectively.

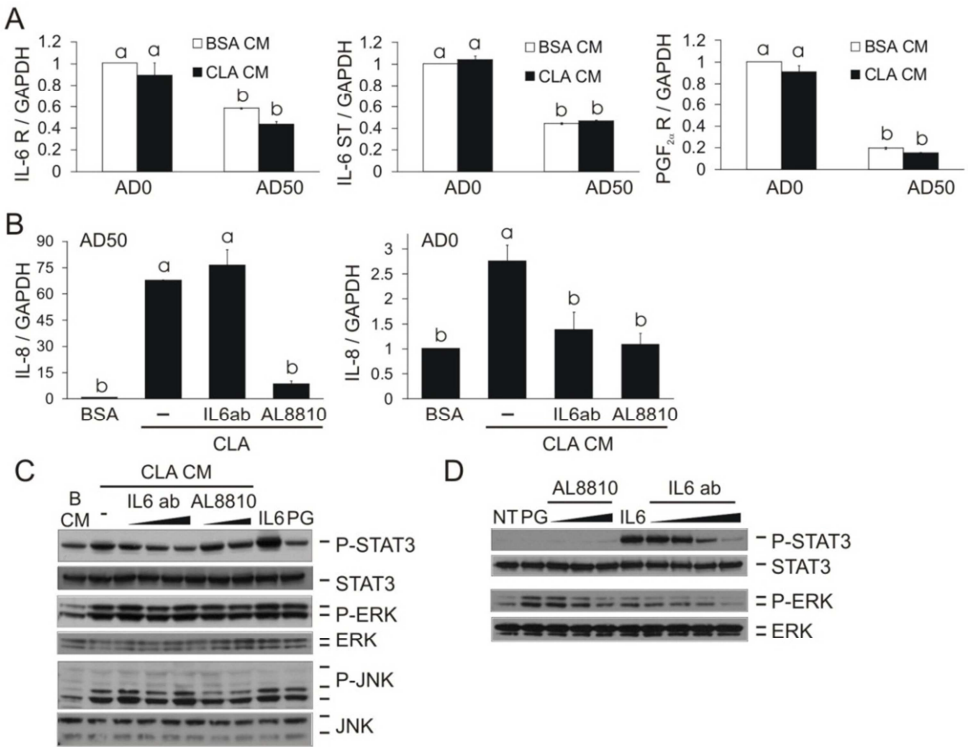

FIGURE 3.9. IL-6 neutralizing antibody (IL-6 ab) and $PGF_{2\alpha}$ analog and FP prostanoid receptor antagonist, AL-8810, attenuate CLA CM-induced IL-8 gene expression and P-STAT3 levels. A) AD0 and AD50 cultures treated with BSA or CLA CM at a 1:1 ratio of CM to AM-1 for 3 h were analyzed for IL-6 receptor (R), IL-6 signal transducer (ST), and $PGF_{2\alpha}$ R expression via qPCR. **B**) AD50 cultures were pretreated with 0.01 ug/ml IL-6 ab or 50 µM AL-8810 for 30 min, and subsequently treated with 50 µM 10,12 CLA or BSA vehicle for 24 h, after which IL-8 mRNA levels were measured. Next, AD0 cultures were treated with AD50 CM for 3 h and IL-8 mRNA levels were measured. **C**) AD50 cultures were pretreated with 0.01, 0.1, and 1 µg/ml IL-6 ab or 1 and 10 µM AL-8810 for 30 min. Then cultures were treated with 50 µM 10,12 CLA or BSA vehicle for 24 h, after which CM was collected and added to AD0 cultures for 1 h. Cultures were treated with 0.1 µg/ml recombinant human (rh) IL-6 and 10 µM $PGF_{2\alpha}$ (PG) for 30 min as positive controls. **D**) AD0 cultures were pretreated with 0.01, 0.1, 1, or 10 µg/ml IL-6 ab or 0.5, 5, or 50 µM AL-8810 for 30 min, and subsequently treated with 0.1 µg/ml rh IL-6 or 10 µM PG for 30 min, respectively. **C-D**) Cells were harvested and analyzed for levels of P- and total STAT3, ERK, or JNK via immunoblotting. Means ± SEM (n = 3 **A**; n = 2-3 **B-D**) not sharing a common superscript differ significantly ($p < 0.05$). Data in panels **A-D** are representative of three independent experiments.

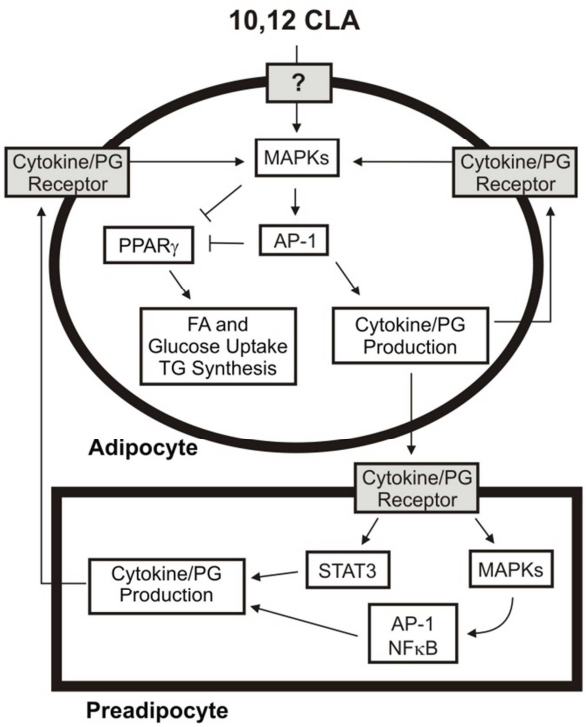

FIGURE 3.10. Working Model. Treating primary cultures of newly differentiated human adipocytes with 10,12 CLA increases the phosphorylation of MAPK (i.e., ERK 1/2, JNK, and p38) and the activation of the transcription factor AP-1 (i.e., c-Jun, c-Fos, ATF2, and ATF3), which leads to the production of inflammatory cytokines and PGs (i.e., PGE_2, PGF_2) through upregulating inflammatory genes (i.e., COX-2, IL-1β, IL-8, and ATF3). These inflammatory signals subsequently activate preadipocytes, leading to inflammatory cytokine secretion from preadipocytes, thus continuing the inflammatory cycle. Furthermore, activation of STAT3, MAPK, and AP-1 by 10,12 CLA antagonizes PPARγ and associated target genes, ultimately leading to delipidation through decreased glucose and fatty acid uptake and TG content in adipocytes.

References

1. Center for Disease Control. (2008) Physical Activity and Good Nutrition: Essential Elements to Prevent Chronic Diseases and Obesity- At a Glance 2008. http://www.cdc.gov/nccdphp/publications/aag/dnpa/.htm (accessed October 15, 2008)

2. Park Y, Storkson J, Albright K, Liu W, and Pariza M. 1999. Evidence that trans-10, cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. Lipids. 34:235-241.

3. Terpstra AH, Beynen AC, Everts H, Kocsis S, Katan MB, and Zock PL. 2002. The decrease in body fat in mice fed conjugated linoleic acid is due to increases in energy expenditure and energy loss in the excreta. J Nutr. 132:940-945

4. Bhattacharya A, Rahman MM, Sun D, Lawrence R, Mejia W, McCarter R, O'Shea M, and Fernandes G. 2005. The combination of dietary conjugated linoleic acid and treamill exercise lowers gain in body fat mass andenhances lean bdoy mass in high fat-fed male Balb/C mice. J Nutr. 135:1124-1130.

5. Poirier H, Shapiro JS, Kim RJ, and Lazar MA. 2006. Nutritional supplementation with rans-10,cis-12 conjugated linoleic acid induces inflammation of white adipose tissue. Diabetes. 55:1634-1640.

6. Banu J, Bhattacharya A, Rahman M, and Fernandes G. 2008. Beneficial effects of conjugated linoleic acid and exercise on bone of middle-aged female mice. J Bone Miner Metab. 26:436-45.

7. Steck SE, Chalecki AM, Miller P, Conway J, Austin GL, Hardin JW, Albright CD, and Thuillier P. 2007. Conjugated linoleic acid supplementation for twelve weeks increases lean body mass in obese humans. J Nutr. 137:1188–1193.

8. Gaullier JM, Halse J, Høivik HO, Høye K, Syvertsen C, Nurminiemi M, Hassfeld C, Einerhand A, O'Shea M, and Gudmundsen O. 2007. Six months supplementation with conjugated linoleic acid induces regional-specific fat mass decreases in overweight and obese. Br J Nutr. 97:550-60.

9. Raff M, Tholstrup T, Toubro S, Bruun J, Lund P, Straarup L, Christensen R, Sandberg M, and Mandrup S. 2009. Conjugated linoleic acids reduce body fat in healthy postmenopausal women. J Nutr. 139:1347-52.

10. Norris L, Collene A, Asp M, Hsu J, Liu L, Richardson J, Li D, Bell D, Osei K, Jackson R, and Belury M. 2009. Comparison of dietary conjugated linoleic acid with safflower oil on body composition in obese postmenopausal women with type 2 diabetes mellitus. Am J Clin Nutr. 90:1-9.

11. Kennedy A, Martinez K, Schmidt S, Mandrup S, LaPoint K, and McIntosh M. 2009. Antiobesity mechanisms of action of conjugated linoleic acid. J Nutr Biochem. 21:179-9.

12. West DB, Blohm FY, Truett AA, and DeLany JP. 2000. Conjugated linoleic acid persistently increases total energy expenditure in AKR/J mice without increasing uncoupling protein gene expression. J Nutr. 130:2471-2477.

13. Nagao K, Wang YM, Inoue N, Han SY, Buang Y, Noda T, Kouda N, Okamatsu H, and Yanagita T. 2003. The 10trans, 12cis isomer of conjugated linoleic acid promotes energy metabolism in OLETF rats. Nutrition. 19:652-656.

14. House RL, Cassady JP, Eisen EJ, Eling TE, Collins JB, Grissom SF, and Odle J. 2005. Functional genomic characterization of delipidation elicited by trans-10, cis-12-conjugated linoleic acid (t10c12-CLA) in a polygenic obese line of mice. Physiol Genomics. 21:351-361.

15. Close RN, Schoeller DA, Watras AC, and Nora EH. 2007. Conjugated linoleic acid supplementation alters the 6-mo change in fat oxidation during sleep. Am J Clin Nutr 86:797-804.

16. So MH, Tse IM, and Li ET. 2009. Dietary fat concentration inluences the effects of trans-10, cis-12 conjugated linoleic acid on temporal patterns of energy intake and hypothalamic expression of appetite-controlling genes in mice. J Nutr. 139:145-151.

17. Brodie AE, Manning VA, Ferguson KR, Jewell DE, and Hu CY. 1999. Conjugated linoleic acid inhibits differentiation of pre- and post- confluent 3T3-L1 preadipocytes but inhibits cell proliferation only in preconfluent cells. J Nutr. 129:602-606.

18. Brown JM, Halverson YD, Lea-Currie R, Geigerman C, and McIntosh M. 2001. Trans-10, cis-12, but not cis-9, trans-11, conjugated linoleic acid attenuates lipogenesis in primary cultures of stromal vascular cells from human adipose tissue. J Nutr. 131:2316-2321.

19. Brown JM, Sandberg-Boysen M, Skov S, Morrison R, Storkson J, Lea-Currie R, Pariza M, Mandrup S, and McIntosh M. 2003. Isomer-specific regulation of metabolism and PPARγ by conjugated linoleic acid (CLA) in human preadipocytes. J Lipid Res. 44:1287-300.

20. Chung S, Brown JM, and McIntosh M. 2005. Trans-10, cis-12 CLA increases adipocytes lipolysis and alters lipid droplet-associated proteins: role of mTOR and ERK signaling. J Lipid Res. 46:885-895.

21. Azain MJ, Hausman DB, Sisk MB, Flatt WP, and Jewell DE. 2000. Dietary conjugated linoleic acid reduces rat adipose tissue cell size rather than cell number. J Nutr. 130:1548-1554.

22. Kang K, Miyazaki M, Ntambi JM, and Pariza MW. 2004. Evidence that the anti-obesity effect of conjugated linoleic acid is independent of effects on stearoyl-CoA desaturase1 expression and enzyme activity. Biochem Biophys Res Commun. 315:532-537.

23. Brown J, Boysen M, Chung S, Fabiyi O, Morrision R, Mandrup S, and McIntosh M. 2004. Conjugated linoleic acid (CLA) induces human adipocyte delipidation: autocrine/paracrine regulation of MEK/ERK signaling by adipocytokines. J Biol Chem. 279:26735-26747.

24. LaRosa PC, Miner J, Xia Y, Zhou Y, Kachman S, and Fromm ME. 2006. Trans-10, cis-12 conjugated linoleic acid causes inflammation and delipidation of white adipose tissue in mice: a microarray and histological analysis. Physiol Genomics. 27:282-294.

25. Kennedy A, Chung S, LaPoint K, Fabiyi O, and McIntosh M. 2008. Trans-10, cis-12 conjugated linoleic acid antagonizes ligand-dependent PPARgamma activity in primary cultures of human adipocytes J. Nutr. 138:455-461.

26. Evans M, Geigerman C, Cook J, Curtis L, Kuebler B, and McIntosh M. 2000. Conjugated linoleic acid suppresses triglyceride accumulation and induces apoptosis in 3T3-L1 preadipocytes. Lipids. 35:899-910.

27. Miner JL, Cederberg CA, Nielsen MK, Chen X, and Baile CA. 2001. Conjugated linoleic acid (CLA), body fat, and apoptosis. Obes Res. 9:129-134.

28. Tsuboyama-Kasaoka N, Takahashi M, Tanemura K, Kim HJ, Tange T, Okuyama H, Kasai M, Ikemoto S, and Ezaki O. 2000. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. Diabetes. 49:1534-1542.

29. Wendel AA, Purushotham A, Liu LF, and Belury MA. 2008. Conjugated linoleic acid fails to worsen insulin resistance but induces hepatic steatosis in the presence of leptin in ob/ob mice. J Lipid Res. 49:98-110.

30. Houseknecht K, Vanden Heuvel J, Moya-Camerena S, Protocarrero C, Peck L, Nickel K, and Belury M. 1998. Dietary conjugated linoleic acid normalizes impaired glucose

tolerance in the Zucker diabetic fatty fa/fa rat. Biochem Biophys Res Comm. 244:678-682.

31. Clément L, Poirier H, Niot I, Bocher V, Guerre-Millo M, Krief S, Staels B, and Besnard, P. 2002. Dietary trans-10,cis-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse. J Lipid Res. 43:1400-1409.

32. Tholstrup T, Raff M, Straarup EM, Lund P, Basu S, and Bruun JM. 2008. An oil mixture with trans-10, cis-12 conjugated linoleic acid increases markers of inflammation and in vivo lipid peroxidation compared with cis-9, trans-11 conjugated linoleic acid in postmenopausal women. J Nutr. 138:1445-1451.

33. Risérus U, Arner P, Brismar K, and Vessby B. 2002. Treatment with dietary trans10cis12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. Diabetes Care. 25:1516-1521.

34. Risérus U, Basa S, Jovinge S, Fredrickson G, Arnlov J, and Vessby B. 2002. Supplementation with conjugated linoleic acid causes isomer-dependent oxidative stress and elevated C-reactive protein: a potential link to fatty acid-induced insulin resistance. Circulation. 106:1925–1929.

35. Moloney F, Yeow TP, Mullen A, Nolan JJ, and Roche HM. 2004. Conjugated linoleic acid supplementation, insulin sensitivity, and lipoprotein metabolism in patients with type 2 diabetes mellitus. Am J Clin Nutr. 80:887-895.

36. Chung S, Brown JM, Provo JN, Hopkins R, and McIntosh MK. 2005. Conjugated linoleic acid promotes human adipocyte insulin resistance through NFkappaB-dependent cytokine production. J Biol Chem. 280:38445-38456.

37. Kennedy A, Overman A, Lapoint K, Hopkins R, West T, Chuang C-C, Martinez K, Bell D, and McIntosh M. 2009. Conjugated linoleic acid-mediated inflammation and insulin resistance in human adipocytes are attenuated by resveratrol. J Lipid Res. 50:225-232.

38. Awas J, Soteriou M, Drougas J, Stokes K, Roberts L, and Pinson C. 1996. Plasma prostaglandin E1 concentrations and hemodynamics during intravenous infusions of prostaglandin E1 in humans and swine. Transplantation. 61:1624-1629.

39. Morrow JD, and Roberts LJ. 1999. Mass spectrometric quantification of F2-isoprostanes in biological fluids and tissues as measure of oxidant stress. Methods Enzymol. 300:3-12.

40. Milatovic D, Yin Z, Gupta RC, Sydoryk M, Albrecht J, Aschner JL, and Aschner M. 2007. Manganese induces oxidative impairment in cultured rat astrocytes. Toxicol Sci. 98:198-205.

41. Chung S, LaPoint K, Martinez K, Kennedy A, Sandberg MB, and McIntosh MK. 2006. Preadipocytes mediate lipopolysaccharide-induced inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes. Endocrinology. 147:5340-5351.

42. Gerhardt C, Romero Cancello L, Camoin R, Camoin L, and Strosberg A. 2001. Chemokines control fat accumulation and leptin secretion by cultured human adipocytes. Mol Cell Endocrinol. 175:81–92.

43. Trayhurn P, and Wood SI. 2004. Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr. 92:347-355.

44. Hauner H. 2005. Secretory factors from human adipose tissue and their functional role. Proc Nutr Soc. 64:163-169.

45. Tchoukalova Y, Koutsari C, and Jensen M. 2007. Committed subcutaneous preadipocytes are reduced in human obesity. Diabetologia. 50:151-157.

46. Fain JN, Madan AK, Hiler ML, Cheema P, and Bahouth SW. 2004. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology. 145:2273-2282.

47. Gil A, Aguilera CM, Gil-Campos M, and Cañete R. 2007. Altered signalling and gene expression associated with the immune system and the inflammatory response in obesity. Br J Nutr. 98 Suppl 1: S121-S126.

48. Skurk T, Alberti-Huber C, Herder C, and Hauner H. 2007. Relationship between adipocyte size and adipokine expression and secretion. J Clin Endocrinol Metab. 92:1023-1033.

49. Suganami T, Nishida J, and Ogawa Y. 2005. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. Aterioscler Thromb Vasc Biol. 25:2062-2068.

50. Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, Kotani H, Yamaoka S, Miyake K, Aoe S, Kamei Y, and Ogawa Y. 2007. Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. Aterioscler Thromb Vasc Biol. 27:84-91.

CHAPTER IV

SP600125 ATTENUATES *TRANS*-10, *CIS*-12 CONJUGATED LINOLEIC ACID-MEDIATED REGULATION OF INFLAMMATORY AND LIPOGENIC GENE EXPRESSION

Abstract

Supplementation with a mixture of *trans*-10, *cis*-12 (t10,c12) and *cis*-9, *trans*-11 (c9,t11) isomers of conjugated linoleic acid (CLA), or t10,c12 CLA alone, reduces body weight and fat deposition in animals and some humans. However, these anti-obesity actions of t10,c12 CLA are routinely accompanied by increased markers of inflammation and insulin resistance. Thus, we examined the extent to which blocking c-Jun NH2-terminal kinase (JNK) signaling using the JNK inhibitor SP600125 attenuated markers of inflammation and insulin resistance in primary human adipocytes treated with t10,c12 CLA. SP600125 attenuated t10,c12 CLA-mediated phosphorylation of cJun and increased protein levels of activating transcription factor (ATF) 3, two downstream targets of JNK. SP600125 attenuated t10,c12 CLA-mediated induction of inflammatory genes, including interleukin (IL)-6, IL-8, IL-1β, ATF3, monocyte chemoattractant protein (MCP)-1, and cyclooxygenase-2. Consistent with these data, SP600125 prevented t10,c12 CLA-mediated secretion of IL-8, IL-6, and MCP-1. SP600125 prevented t10,c12

CLA suppression of lipogenic genes including peroxisome proliferator activated receptor gamma, liver x receptor, sterol regulatory element binding protein, acetyl-CoA carboxylase, and stearoyl-CoA desaturase. Additionally, SP600125 blocked t10,c12 CLA-mediated induction of suppresser of cytokine synthesis-3 and suppression of adiponectin and insulin-dependent glucose transporter 4 mRNA levels. Collectively, these data suggest that JNK signaling plays an important role in t10,c12 CLA-mediated regulation of inflammatory and lipogenic gene expression in primary cultures of human adipocytes.

Introduction

Obesity is a global health issue with ~ 500 million people classified as obese and 1.5 billion overweight in 2008, including 43 million children under the age of five reported in 2010 [1]. One potential strategy for reducing adiposity is consumption of conjugated linoleic acid (CLA), unsaturated fatty acids found in ruminant meats and dairy products, or in dietary supplements and fortified foods. Conjugated linoleic acid refers to a group of conjugated octadecadienoic acid isomers derived from linoleic acid, a fatty acid that contains 18 carbons and 2 double bonds in cis configuration at the 9th and 12th carbons (i.e., *cis*-9, *cis*-12 octadecadienoic acid). Microbes in the gastrointestinal tract of ruminant animals convert linoleic acid into different isoforms of CLA through biohydrogenation. This process changes the position and configuration of the double bonds, resulting in a single bond between one or both of the two double bonds (i.e., *cis*-9, *trans*-11 (c9,t11) or

trans-10, *cis*-12 (t10,c12) octadecadienoic acid). Consuming a mixture of c9,t11 and t10,c12 CLA isomers, or t10,c12 CLA alone, reduces body fat mass in rodents, particularly mice, and some humans [reviewed in 2]. However, the isomer-specific mechanism by which CLA reduces adiposity is unclear. Furthermore, a number of clinical studies report potential side effects of CLA supplementation including increased levels of markers of inflammation (e.g., inflammatory cytokines, chemokines, or prostaglandins), insulin resistance, hyperlipidemia, and lipodystrophy [3-7]. These anti-obesity and adverse side effects of CLA appear to be due primarily to the t10,c12 isomer. In contrast, the c9,t11 isomer appears to have anti-inflammatory and anti-diabetic properties without reducing body weight [8].

We have demonstrated that t10,c12 CLA reduces glucose and fatty acid uptake and triglyceride content in cultures of human adipocytes, in part, by activating extracellular signal-related kinase (ERK) [9] and nuclear factor kappa B (NFκB) [10]. These *in vitro* data have been confirmed *in vivo* [11]. Activated NFκB [12-14] and ERK [15-17] induce markers of inflammation and antagonize peroxisome proliferator activated receptor (PPAR)γ activity, thereby causing insulin resistance. However, the extent to which t10,c12 CLA activates other kinases or transcription factors that impact inflammatory signaling, insulin sensitivity, and triglyceride content in human adipocytes, and their mechanism of action, are unclear.

We recently demonstrated that t10,c12 CLA increased the phosphorylation levels of c-Jun NH2-terminal kinase (JNK) and downstream targets cJun and activating

transcription factor (ATF3), members of the redox-sensitive transcription factor activating protein-1 (AP-1), that induce inflammatory gene transcription [18-19]. c-Jun NH2-terminal kinase activation is known to enhance inflammation and insulin resistance associated with obesity, and lack of JNK1 or JNK2 reduces body fat and improves insulin sensitivity *in vivo* [20-21] and *in vitro* [22]. However, the role of JNK in activating cJun or ATF3 in CLA-treated cultures and the extent to which this activation regulates inflammatory and lipogenic gene expression has not been investigated.

Based on these data, we hypothesized that JNK plays an important role in t10,c12 CLA-mediated activation of AP-1 and induction of inflammatory genes and suppression of lipogenic genes. To test this hypothesis, we employed the chemical JNK1-3 inhibitor, SP600125. By using this inhibitor, we demonstrate that JNK is involved in the regulation of t10,c12 CLA-induced inflammatory signaling, and suppression of gene markers for adipogenesis, lipogenesis, and insulin signaling in cultures of newly-differentiated human adipocytes. Therefore, JNK may be an important target for preventing 10,12 CLA-mediated inflammation.

Materials and Methods

Materials

All cell culture ware were purchased from Fisher Scientific (Norcross, GA). Lightning Chemiluminescence Substrate was purchased from Perkin Elmer Life Science (Boston,

MA). Immunoblotting buffers and precast gels were purchased from Invitrogen (Carlsbad, CA). Adipocyte media was purchased from Zen Bio (Research Triangle Park, NC). The Nuclear Extract Kit was purchased from Active Motif (Carlsbad, CA). Polyclonal antibodies for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ATF3 and monoclonal antibody for anti-PPARγ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-total and anti-phospho (P) JNK (Thr183/Try185) and P-cJun (Ser63) antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Anti-nucleoporin was purchased from BD transduction laboratories (San Jose, CA). Hyclone fetal bovine serum was purchased from Fisher Scientific. Isomers of CLA (+98% pure) were purchased from Matreya (Pleasant Gap, PA). The cell permeable selective JNK1-3 inhibitor SP600125 (#420119; Anthra[1,9-*cd*]pyrazol-6(2*H*)-one, 1,9-pyrazoloanthrone; JNKII) was purchased from EMD Chemicals (Gibbstown, NJ). This inhibitor of JNK1-3 is competitive with respect to ATP, and has over a 300-fold greater selectivity for JNK compared to other mitogen-activated protein kinase (MAPK) including ERK and p38 [23], and specifically inhibits the phosphorylation of cJun serine residues 63 and 67 [24]. All other reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Culturing of human primary adipocytes

Abdominal white adipose tissue was obtained with consent from the Institutional Review Boards at the University of North Carolina at Greensboro and the Moses Cone Memorial

Hospital, during elective abdominoplasty of non-diabetic Caucasian and African American females between the ages of 20-50 years old with a body mass index ≤ 32.0. These selection criteria allow for reduced variation in gender, age, and obesity status. Tissue was digested using collagenase; stromal vascular cells were isolated as previously described [9]. Stromal vascular cells were differentiated with adipocyte media (AM-1) containing 1 μM rosiglitazone and 250 μM 1-methyl-3-isobutylxanthine for 3 d, which yielded cultures containing ~30-50% adipocytes. On days 6-12, cells were pretreated with 5, 20, or 80 μM SP600125 JNK inhibitor dissolved in DMSO for 30 min, and subsequently treated with 50 μM t10,c12 CLA or bovine serum albumin (BSA) vehicle control for 12-24 h depending on the experimental outcome measured. All cultures were normalized to contain the same amount of BSA and DMSO vehicles. Each independent experiment was repeated at least twice using a mixture of cells from three subjects, unless otherwise indicated.

Fatty acid preparation

t10,c12 CLA was delivered as a free acid complexed to 7.5% fatty acid-free BSA at a 4:1 molar ratio as previously described [9].

RNA isolation and PCR

Total RNA was isolated from the cultures using Tri Reagent purchased from Molecular Research Center (Cincinnati, OH), according to manufacturer's protocol. For quantitative real time PCR, 2.0 ug total RNA was converted into first strand cDNA using Applied Biosystems High-Capacity cDNA Archive Kit (Foster City, CA). Real time PCR was performed in an Applied Biosystems 7500 FAST Real Time PCR System using Taqman Gene Expression Assays. To account for possible variation in cDNA input or the presence of PCR inhibitors, the endogenous reference gene GAPDH was simultaneously quantified for each sample, and these data normalized accordingly. The Relative Standard Curve Method using seven, two-fold dilutions ranging from 100 – 1.56 ng RNA was used to check primer efficiency and linearity of each transcript according to Applied Biosystem's Guide to Performing Relative Quantification of Gene Expression Using Real-Time Quantitative PCR.

Nuclear and cytosolic separation

Nuclear and cytosolic cellular fractions were prepared using a commercially available kit from Active Motif as previously described [10].

Immunoblotting

Immunoblotting using 20 ug of protein per lane was conducted using 4-12% NuPage precasted gels (Invitrogen) as previously described [10]. Briefly, PVDF membranes were blocked with 5% milk in TBST for 1 h and washed 3 x in TBST for 5 min. Blots were incubated overnight at 4°C with primary antibodies targeting P-JNK, P-cJun, total cJun, and ATF3 at a dilution of 1:1000, and subsequently incubated in the respective horseradish peroxidase-conjugated secondary antibody at a dilution of 1:5000 at room temperature for 1 h. Primary and secondary antibodies targeting GAPDH were used at a 1:5000 dilution. Primary and secondary antibodies targeting PPARγ were used at dilutions 1:200 and 1:2000, respectively. After washing, blots were treated with chemiluminescence reagent for 1 min and film was exposed using a SRX-101A Konica Minolta flim developer. Densitometry was performed using a Kodak Image Station 440 CF by Perkin Elmer and Kodak Molecular Imaging Software Version 4.0.

Secretion of IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1

The concentrations of IL-6, IL-8, and MCP-1 were determined using the BioPlex® Suspension Array System from Bio-Rad (Hercules, CA) following the manufacturer's protocol. Briefly, media was collected from cultures that were pretreated with 5, 20, or 80 μM SP600125 for 30 min, and subsequently treated with 50 μM t10,c12 CLA or BSA for 24 h. The media was centrifuged at 13,200 x g for 10 min at 4°C to clear the samples

of any precipitate. Samples and standards were run in duplicate. Based on the manufacturer's report 'Bio-Plex Pro Human Cytokine, Chemokine, and Growth Factor Assays - Bulletin 5828,' the intra-assay % CVs for IL-6, IL-8, and MCP-1 are 7, 9, and 9%, respectively. The inter-assay % CVs are 11, 4, and 7%, respectively.

Statistical analyses

Data are expressed as the means ± S.E. Data were analyzed using one-way analysis of variance followed by Tukey's-HSD tests for each pair for multiple comparisons. Differences were considered significant if $p < 0.05$. All analyses were performed using JMP IN, Version 9 Software (SAS Institute, Cary, NC).

Results

The JNK inhibitor SP600125 attenuates t10,c12 CLA-mediated activation of cJun and ATF3

We previously demonstrated that treatment of newly-differentiated human adipocytes with 50 μM t10,c12 CLA, but not c9,t11 CLA, for 6 h increased phosphorylation levels of JNK and cJun, which was sustained for 24 h when compared to vehicle (BSA)-treated cultures in total cell extract [18-19]. However, a direct role for JNK in the activation of cJun and upregulation of inflammatory genes in response to t10,c12 CLA treatment has not been determined. In order to implicate a role for JNK in the activation of cJun, we

examined JNK and cJun phosphorylation in cytosol and nuclear fractions after 6, 12, and 24 h of treatment with t10,c12 CLA. Phosphorylation of JNK was increased at 6 h and was sustained at 24 h in the cytosolic fraction, and increased in the nuclear fraction at 12 and 24 h with t10,c12 CLA. Interestingly, t10,c12 CLA increased total cJun levels compared to the BSA control in the nuclear fraction at all time points. Consistently, cJun phosphorylation by t10,c12 CLA was detected almost exclusively in the nuclear fraction at all time points (Fig. 1a). Based on the robust t10,c12 CLA-mediated activation of JNK and cJun, we investigated the extent to which pretreatment with the JNK inhibitor SP600125 blocked t10,c12 CLA--mediated phosphorylation of cJun after 12 h of treatment. Concentrations of SP600125 ranging between 5-80 uM were chosen based on studies using Jurkat T cells in which the IC_{50} for blocking cJun phosphorylation was 10 uM and using CD4+ cells isolated from human peripheral blood mononuclear cells in which the IC_{50} for blocking cyclooxygenase (COX)-2 and tumor necrosis factor alpha expression was 5 and 10 uM, respectively [23]. SP600125 suppressed t10,c12 CLA-mediated phosphorylation of cJun and increase in the protein levels of ATF3, an AP-1 family member, in total cell lysates (Fig. 1b). These data suggest that JNK is involved in t10,c12 CLA-mediated cJun activation.

SP600125 attenuates t10,c12 CLA induction of inflammatory genes

Next, we determined the extent to which SP600125 blocked t10,c12 CLA-induced inflammatory gene expression. Pretreatment of cultures with SP600125 attenuated

t10,c12 CLA-induction of IL-8, IL-6, IL-1β, MCP-1, COX2, and ATF3 (Fig. 2a). Consistent with these data, SP600125 attenuated t10,c12 CLA-mediated secretion of IL-8, IL-6, and MCP-1 (Fig. 2b). Collectively, these data demonstrate that SP600125 suppresses t10,c12 CLA-mediated induction of inflammatory gene expression and protein secretion.

SP600125 blocks t10,c12 CLA suppression of lipogenic genes

Inflammatory transcription factors such as NFκB and AP-1 in concert with MAPKs like ERK have been shown to inhibit lipogenic gene expression, in part, by suppressing PPARγ activity [12-17]. Due to the hypothesized role of JNK in regulating t10,c12 CLA-induced inflammation, we investigated the impact of SP600125 on t10,c12 CLA-mediated suppression of lipogenic gene expression and PPARγ protein levels. Consistent with our hypothesis, SP600125 blocked t10,c12 CLA-mediated suppression of the expression of transcription factors that regulate lipid metabolism (i.e., PPARγ/α, sterol regulatory element binding protein (SREBP)-1c, liver X receptor (LXR)α) and several of their downstream targets (i.e., acetyl-CoA carboxylase (ACC)-1, stearoyl-CoA desaturase (SCD)-1) in a concentration-dependent manner (Fig. 3a). Additionally, 20 uM SP modestly prevented a CLA-mediated decrease in PPARγ protein levels (Fig. 3b). Taken together, these data suggest that JNK activity regulates t10,c12 CLA-mediated suppression of lipogenic genes, which may contribute to the ability of t10,c12 CLA to decrease the triglyceride levels in adipocytes.

SP600125 prevents t10,c12 CLA-mediated regulation of genes involved in insulin signaling

We previously demonstrated that t10,c12 CLA-induced inflammation leads to a suppression of insulin signaling and sensitivity [9,10,18,19]. Therefore, we determined the extent to which JNK impacted the expression of genes associated with insulin signaling. SP600125 attenuated t10,c12 CLA-mediated induction of suppressor of cytokine synthesis (SOCS)-3, a protein reported to cause insulin resistance (Fig. 4). Consistent with these data, t10,c12 CLA-mediated suppression of insulin-dependent glucose transporter (GLUT) 4 and adiponectin mRNA levels, which are positively associated with insulin sensitivity, was attenuated by SP600125 in a concentration-dependent manner (Fig. 4). Further studies are needed to confirm a role for JNK in CLA-mediated suppression of insulin-stimulated glucose uptake.

Discussion

Consistent with our hypothesis, t10,c12 CLA-mediated activation of cJun and ATF3 were attenuated by the chemical JNK inhibitor, SP600125. SP600125 also blocked t10,c12 CLA-induced inflammatory gene expression and cytokine secretion, and suppression of lipogenic genes and markers of insulin signaling. Taken together, these data suggest that JNK plays a role in t10,c12 CLA-mediated induction of markers of inflammation and insulin resistance in cultures of human adipocytes. However, due to the potential lack of specificity of chemical inhibitors (i.e., they can inhibit other kinases including ERK and

p38, albeit at much lower selectivities [23]), JNK gene silencing experiments are needed to confirm these results. Knockdown experiments are also need to determine the extent to which JNK signaling impairs insulin-stimulated glucose uptake in t10,c12 CLA-treated cultures, because such longer-term studies were not possible with SP600125 (data not shown).

Treatment times differed based on the experimental outcome measured. Historically, we have shown in our primary cultures of newly differentiated primary human adipocytes that t10,c12 CLA increases phosphorylation of MAPKs between 6-24 h, induces inflammatory gene expression and protein secretion between 12-48 h, decreases adipogenic gene expression from 18-72 h, and PPARγ protein from 24-48 h [9,10, 18, 19, 32, 33]. Therefore, we chose a 12 h time point to examine JNK phosphorylation, 18 h for inflammatory gene expression, 24 h for cytokine/chemokine secretion, and 24 h for adipogenic, lipogenic, and insulin-sensitizing gene expression. The timing of these events fits our hypothesis that JNK phosphorylation occurs prior to t10,c12 CLA-mediated induction and secretion of inflammatory proteins, leading to the suppression of adipogenic/lipogenic gene and protein levels.

c-Jun NH2-terminal kinase-mediated activation of cJun results in induction of several inflammatory genes including IL-8, IL-6, and COX2, and also genes involved in cell death or apoptosis. For example, SP600125 or RNA interference of JNK in 3T3-L1 adipocytes prevented free fatty acid-induced MCP-1 expression [26]. Consistent with these data, we showed that inhibiting JNK with the chemical inhibitor SP600125

prevented t10,c12 CLA-mediated inflammatory gene expression and protein secretion (Fig. 2). This prevention was accompanied by an increase in the expression lipogenic and insulin sensitizing genes and PPARγ protein levels that promote lipogenesis or insulin signaling (Fig. 3).

One possible explanation for these data is that by inhibiting JNK activity, t10,c12 CLA was unable to decrease PPARγ activity, which drives the expression of genes that promote glucose and fatty acid uptake and triglyceride synthesis and deposition in adipocytes. Indeed, PPARγ2 activity is regulated by phosphorylation [27]. Notably, phosphorylation at serine residue 112 by ERK or JNK has been reported to decrease PPARγ activity by ubiquination and proteosome degradation [28], and by decreasing its ligand-dependent and ligand-independent transactivating functions [15, 29-31]. Consistent with this hypothesis, we have shown that t10,c12 CLA increases PPARγ phosphorylation prior to reducing PPARγ protein levels. Furthermore, supplementation with Rosiglitazone, a PPARγ agonist [32], or resveratrol, an anti-inflammatory polyphenol [33], attenuates delipidation by t10,c12 CLA. Thus, inhibiting t10,c12 CLA-mediated JNK signaling may increase PPARγ activity, thereby increasing the expression of lipogenic and insulin-signaling genes. Consistent with this hypothesis, SP600125 supplementation of t10,c12 CLA-treated cultures increased the expression of lipogenic and insulin-signaling genes compared to cultures treated with t10,c12 CLA alone (Fig. 3).

We previously showed using the ERK inhibitor U0126 that ERK activation by t10,c12 CLA is one mechanism by which t10,c12 CLA suppresses lipogenic gene expression and insulin-stimulated glucose uptake [9]. Therefore, it is tempting to speculate that t10,c12 CLA antagonizes PPARγ activity via activation of MAPK like ERK and JNK, thereby inhibiting PPARγ target genes. In support of this hypothesis, knockdown of JNK1, but not JNK2, increased basal and troglitazone-stimulated PPARγ reporter activity [34].

c-Jun NH2-terminal kinase deficiency in animals on a high-fat diet protects them from developing insulin resistance [20]. Similarly, mitochondrial dysfunction-induced insulin resistance in 3T3-L1 adipocytes is prevented by knockdown of JNK1 [35]. Several reports show that JNK directly phosphorylates insulin receptor substrate (IRS)-1 at serine 307, thus inactivating insulin receptor signaling. However, we did not observe any effects of t10,c12 CLA or SP600125 on IRS-1 ser 307 phosphorylation (data not shown), in spite of SP600125 blocking t10,c12 CLA induction of SOCS-3, a protein reported to phosphorylate ser 307 on IRS-1. Nevertheless, t10,c12 CLA-mediated suppression of the mRNA levels adiponectin and GLUT4, proteins associated with insulin sensitivity, was completely prevented by SP600125 (Fig. 4).

Taken together, these data suggest that JNK may be an important target for preventing t10,c12-CLA mediated inflammation. Further studies are needed to confirm a role for JNK in t10,c12 CLA-mediated insulin resistance. RNA interference studies targeting JNK are also needed to confirm these data. Future research will also focus on

identifying upstream activators of JNK and ERK and potential mechanisms by which these MAPK pathways are linked to insulin resistance and suppression of lipogenesis in adipocytes.

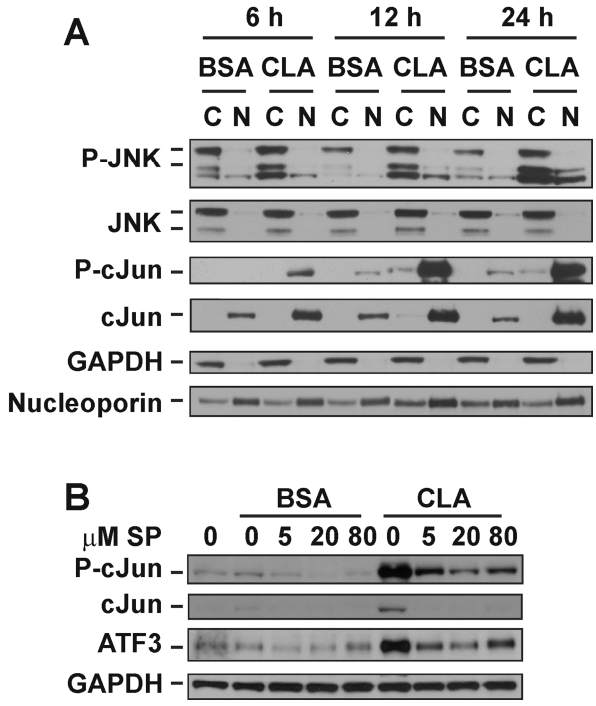

FIGURE 4.1. The JNK inhibitor SP600125 attenuates t10,c12 CLA-mediated activation of cJun and ATF3. (a) Cultures of newly-differentiated human adipocytes were treated with BSA vehicle or 50 μM t10,c12 CLA for 6, 12, or 24 h. Nuclear and cytosolic fractions were prepared using the Nuclear Extract Kit from Active Motif (Carlsbad, CA) and analyzed for the determination of the protein levels of P-JNK, JNK, P-cJun, cJun, GAPDH, and nucleoporin. **(b)** Cultures were pretreated for 30 min with 5, 20, or 80 μM SP600125 (SP) followed by a 12 h treatment with BSA vehicle or 50 μM t10,c12 CLA. Subsequently, total cell lysates were harvested for the determination of the protein levels of P-cJun, cJun, ATF3, and GAPDH. Data are representative of two **(a)** or three **(b)** independent experiments.

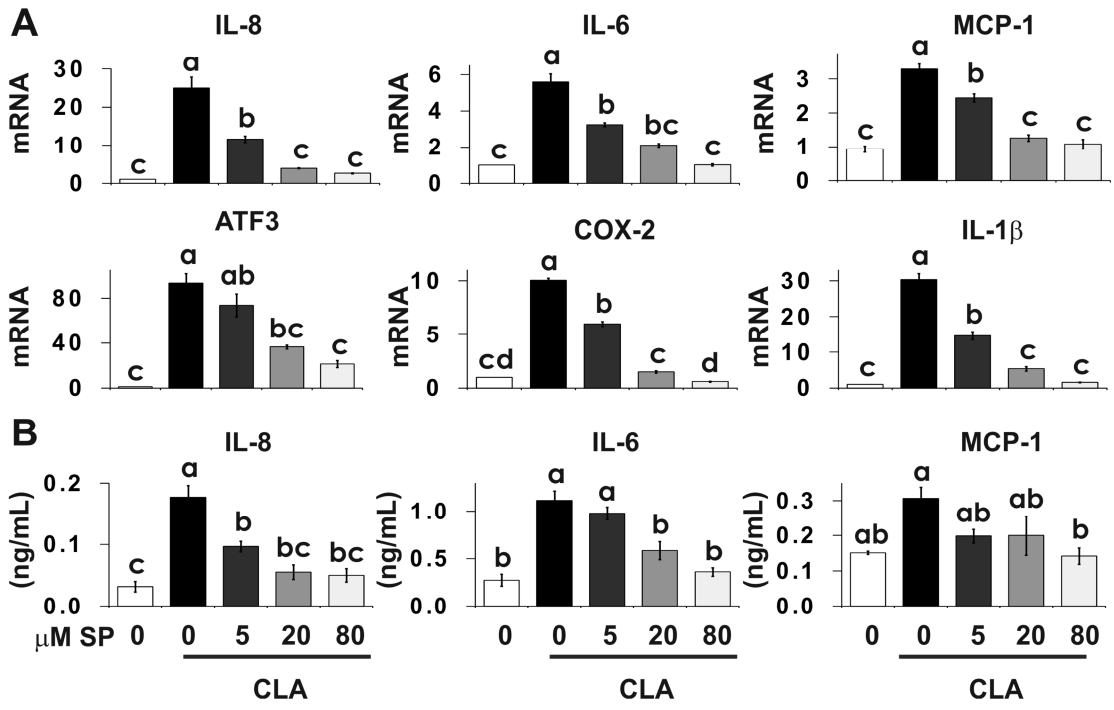

FIGURE 4.2. SP600125 attenuates t10,c12 CLA induction of inflammatory genes. (a) Cultures of newly-differentiated human adipocytes were pretreated with 5, 20, or 80 μM SP600125 (SP) for 30 min, followed by treatment with BSA vehicle or 50 μM t10,c12 CLA for 18 h (**a**) or 24 h (**b**). Subsequently, RNA was harvested and the mRNA levels of IL-8, IL-6, IL-1β, MCP-1, ATF3, and COX-2 were measured by real time qPCR and normalized to GAPDH endogenous control. Means (+SE; n=2-3 (**a**) or n=4 (**b**)) not sharing a lower case letter differ significantly ($p<0.05$). **(b)**. Media were collected and analyzed for IL-8, IL-6, and MCP-1 levels using the BioRad Multiplex System. Data are representative of two (**b**) or three (**a**) independent experiments.

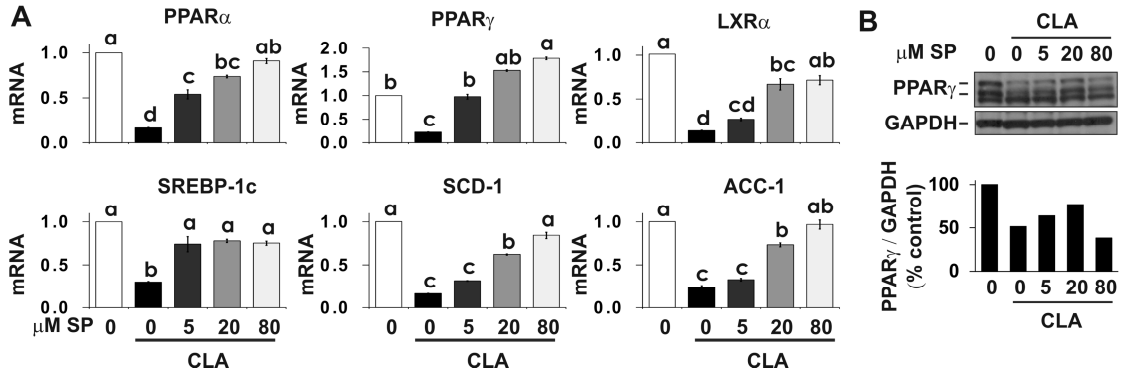

FIGURE 4.3. SP600125 blocks t10,c12 CLA suppression of lipogenic genes. Cultures of newly-differentiated human adipocytes were pretreated with 5, 20, or 80 μM SP600125 (SP) for 30 min, followed by treatment with BSA vehicle or 50 μM t10,c12 CLA for 24 h. **(a)** RNA was harvested and the mRNA levels of PPARγ, PPARα, LXRα, SREBP-1c, ACC-1, and SCD-1 were measured using real time qPCR and normalized to GAPDH endogenous control. Means (+SE; n=2-3) not sharing a lower case letter differ significantly ($p<0.05$). **(b)** Cultures were harvested for the determination of the protein levels of PPARγ and GAPDH. PPARγ levels were quantified by densitometry and normalized to the loading control, GAPDH. Densitometry values are expressed as % of BSA vehicle control. Data are representative of two **(b)** or three **(a)** independent experiments.

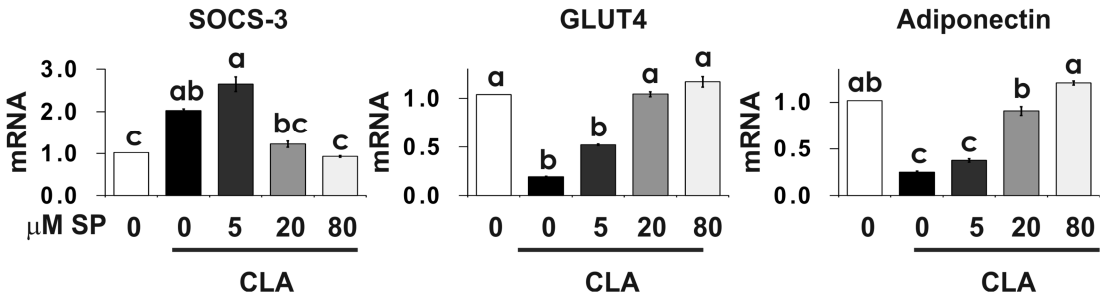

FIGURE 4.4. SP600125 prevents t10,c12 CLA-mediated regulation of genes involved in insulin signaling. Cultures of newly-differentiated human adipocytes were pretreated with 5, 20, or 80 μM SP600125 (SP) for 30 min, followed by treatment with BSA vehicle or 50 μM t10,c12 CLA for 24 h. Subsequently, RNA was harvested and the mRNA levels of SOCS-3, GLUT4, and apm1 were measured by real time qPCR and normalized to GAPDH endogenous control. Means (+SE; n=2-3) not sharing a lower case letter differ significantly ($p<0.05$).

References

1. World Health Organization. (Accessed March 7, 2011). Obesity and Overweight. www.who.int/mediacentre/factsheets/fs311/en/index.html.

2. Kennedy A, Martinez K, Schmidt S, Mandrup S, Lapoint K, McIntosh M (2010) Anti-obesity Mechanisms of Action of Conjugated Linoleic Acid. J Nutr Biochem 21:171-179.

3. Risérus U, Arner P, Brismar K, Vessby B (2002) Treatment with dietary trans10cis12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. Diabetes Care 25:1516-1521.

4. Moloney F, Yeow TP, Mullen A, Nolan JJ, Roche HM (2004) Conjugated linoleic acid supplementation, insulin sensitivity, and lipoprotein metabolism in patients with type 2 diabetes mellitus. Am J. Clin Nutr 80:887-895.

5. Steck SE, Chalecki AM, Miller P, Conway J, Austin GL, Hardin JW, Albright CD, Thuillier P (2007) Conjugated linoleic acid supplementation for twelve weeks increases lean body mass in obese humans. J Nutr 137:1188–1193.

6. Thrush AB, Chabowski A, Heigenhauser GJ, McBride BW, Or-Rashid M, Dyck DJ (2007) Conjugated linoleic acid increases skeletal muscle ceramide content and decreases insulin sensitivity in overweight, non-diabetic humans. Appl Physiol Nutr Metab 32:372-382.

7. Tholstrup T, Raff M, Straarup EM, Lund P, Basu S, Bruun JM (2008) An oil mixture with trans-10, cis-12 conjugated linoleic acid increases markers of inflammation and in vivo lipid peroxidation compared with cis-9, trans-11 conjugated linoleic acid in postmenopausal women. J Nutr 138:1445-51.

8. Moloney F, Toomey S, Noone E, Nugent A, Allan B, Loscher C, Roche H (2007) Antidiabetic effects of cis-9, trans, 11 conjugated linoleic acid may be mediated via anti-inflammatory effects in white adipose tissue. Diabetes 56:574-582.

9. Brown JM, Boysen M, Chung S, Fabiyi O, Morrision R, Mandrup S, McIntosh M (2004) Conjugated linoleic acid (CLA) induces human adipocyte delipidation: autocrine/ paracrine regulation of MEK/ERK signaling by adipocytokines. J Biol Chem 279:26735-47.

10. Chung S, Brown JM, Provo JN, Hopkins R, McIntosh M (2005) Conjugated linoleic acid promotes human adipocyte insulin resistance through NFkappaB-dependent cytokine production, J Biol Chem 280:38445-56.

11. Poirier H, Shapiro H, Kim R, Lazar M (2006) Nutritional supplementation with trans-10, cis-12 conjugated linoleic acid induces inflammation of white adipose tissue. Diabetes 55:1634-1641.

12. Ruan H, Hacohen N, Golub T, Van Parijs L, Lodish H (2002) Profiling gene transcription in vivo reveals adipose tissue as an intermediate target for tumor necrosis alpha: implications for insulin resistance. Diabetes 51:1319-1336.

13. Ruan H, Pownall H, Lodish H (2003) Troglitazone antagonizes tumor necrosis factor-a induced reprogramming of adipocyte gene expression by inhibiting the transcriptional regulatory functions of NFkB. J Biol Chem 278:28181-28192.

14. Suzawa M, Takada I, Yanagisawa J, Ohtake F, Ogawa S, Yamauchi T, Kadowaki T, Takeuchi Y, Shibuya H, Gotoh Y, Matsumoto K, Kato S (2003) Cytokines suppress adipogenesis and PPAR-gamma function through the TAK1/TAB1/NIK cascade. Nat Cell Biol 5:224-230.

15. Adams M, Reginato M, Shao D, Lazar M, Chatterjee V (1997) Transcriptional activation by peroxisome proliferator activated receptor gamma is inhibited by phosphoryaltion at a consensus mitogen-activated protein kinase site. J Biol Chem 272:5128-5132.

16. Camp H, Tafuri S (1997) Regulation of peroxisome proliferators-activated receptor γ activity by mitogen activated protein kinase. J Biol Chem 272:10811-10816.

17. de Mora J, Porras A, Ahn N, Santos E (1997) Mitogen-activated protein kinase activation is not necessary for, but antagonizes, 3T3-L1 adipocytic differentiation. Mol Cell Biol 17:6068-6075.

18. Kennedy A, Martinez K, Chung S, LaPoint K, West T, Hopkins R, Schmidt S, Andersen K, Mandrup S, McIntosh M (2010) Inflammation and Insulin Resistance Induced by Trans-10, Cis-12 Conjugated Linoleic Acid are Dependent on Intracellular Calcium Levels in Primary Cultures of Human Adipocytes. J Lipid Res 51:1906-1917.

19. Martinez K, Kennedy A, West T, Milatovic D, Aschner M, (2010) McIntosh M Trans-10, cis-12 Conjugated Linoleic Acid Promotes Inflammation to a Greater

Extent in Human Adipocytes Compared to Preadipocytes. J Biol Chem 285:17701-17712.

20. Hirosumi J, Tuncman G, Chang G, Gorgun C, Uysal K, Maeda K, Karin M, Hotamisligil G (2002) A central role of JNK in obesity and insulin resistance. Nature 420:333-336.

21. Tuncman G, Hirosumi H, Solinas G, Chang L, Karin M, Hotamisligil G (2006) Functional in vivo interactions between JNK1 and JNK2 isoforms in obesity and insulin resistance. Proc Natl Acad Sci USA 103:10741-10746.

22. Davis J, Gabler N, Walker-Daniels J, Spurlock M (2009)The c-Jun N terminal kinase mediates the induction of oxidative stress and insulin resistance by palmitate and toll-like receptor 2 and 4 ligands in adipocytes. Horm Metab Res 41:523-530.

23. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA 98:13681-13686.

24. Gupta S, Barrett T, Whitmarsh A, Cavanagh J, Sluss H, Derijard B, Davis R (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO J 15:2760-2770.

25. Holzberg D, Knight G, Dittrich-Breiholz O, Schneider H, Dorrie A, Hoffman E, Resch K, Krach M (2003) Disruption of c-JUN-JNK complex by a cell permeable peptide containing the c-JUN g domain induces apoptosis and affects a distinct set of interleukin-1-induced inflammatory genes. J Biol Chem 41:40213-40223.

26. Jiao P, Chen Q, Shah S, Du J, Tao J, Tzamelim I, Wan W, Xu G (2009) Obesity-related upregulation of monocyte chemotatic factors in adipocytes. Diabetes 58:104-115.

27. Hu E, Kim JB, Sarraf P, Spiegelman BM (1996) Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. Science 274:2100-03.

28. Floyd ZE, Stephens JM (2002) Interferon-gamma-mediated activation and ubiquitin-proteasome-dependent degradation of PPARgamma in adipocytes. J Biol Chem 277:4062-68.

29. Shao DL, Rangwala SM, Bailey ST, Krakow SL, Reginato MJ, Lazar MA (1998) Interdomain communication regulating ligand binding by PPARgamma. Nature 396:377-80.

30. Diradourian C, Girard J, Pégorier JP (2005) Phosphorylation of PPARs: from molecular characterization to physiological relevance. Biochimie 87:33-38.

31. Burns KA, Vanden Heuvel JP (2007) Modulation of PPAR activity via phosphorylation. Biochim Biophys Acta 1771:952-60.

32. Kennedy A, Chung S, LaPoint K, Fabiyi O, McIntosh MK (2008) Trans-10, cis-12 conjugated linoleic acid antagonizes ligand-dependent PPARgamma activity in primary cultures of human adipocytes. J Nutr 138:455-61.

33. Kennedy A, Overman A, Lapoint K, Hopkins R, West T, Chuang CC, Martinez K, Bell D, McIntosh M (2009) Conjugated linoleic acid-mediated inflammation and insulin resistance in human adipocytes are attenuated by resveratrol. J Lipid Res 50:225-32.

34. Rozo A, Vijayvargia R, Weiss H, Ruan H (2008) Silencing JNK1 and JNK2 accelerate basal lipolysis and promote fatty acid re-esterification in mouse adipocytes. Diabetologia 51:1493-1504.

35. Kim T, Leitner J, Adochio R, Draznin B (2009) Knockdown of JNK rescues 3T3-L1 adipocytes from insulin resistance induced by mitochondrial dysfunction. Biochem Biophys Res Commun 378:772-776.

CHAPTER V

R59022 ATTENUATES *TRANS*-10, *CIS*-12 CONJUGATED LINOLEIC ACID-MEDIATED INFLAMMATION AND INSULIN RESISTANCE IN PRIMARY HUMAN ADIPOCYTES

Abstract

Diacylglycerol kinases (DGKs) convert diacylglycerol into phosphatidic acid, which has been reported to stimulate calcium release from the ER. Based on our published data showing that *trans*-10, *cis*-12 conjugated linoleic acid (t10,c12 CLA)-mediated intracellular calcium accumulation is linked to inflammation and insulin resistance, we hypothesized that chemically inhibiting DGKs with R59022 would prevent t10,c12 CLA-mediated inflammatory signaling and insulin resistance in human adipocytes. Consistent with our hypothesis, R59022 attenuated t10,c12 CLA-induced interleukin (IL)-8, IL-6, monocyte chemoattractant protein-1 (MCP-1), and protein secretion, and attenuated t10,c12 CLA-mediated suppression of peroxisome proliferator activated receptor γ gene and protein levels, delipidation, insulin-stimulated glucose uptake, and glucose transporter 4 and adiponectin mRNA levels. DGKη was targeted for investigation based on our findings that 1) DGKη was highly expressed in our primary cultures of newly-differentiated human adipocytes and induced by t10,c12 CLA-treatment, 2) t10,c12 CLA-induced DGKη expression was dose-dependently decreased with R59022 pretreatment, and 3) t10,c12 CLA increased DGKη translocation to the plasma membrane compared to

the vehicle control. Taken together, these data suggest that DGKs mediate t10,c12 CLA-induced inflammatory signaling and insulin resistance in primary human adipocytes.

Introduction

Overweight and obesity is a global health issue affecting 1.6 billion individuals worldwide [1]. This high prevalence of overweight and obesity has resulted in an annual economic cost of 300 billion in the U.S. and Canada in 2009 [2]. One potential strategy for reducing adiposity is consumption of conjugated linoleic acid (CLA), a group of conjugated octadecadienoic acid isomers derived from linoleic acid, a fatty acid (FA) that contains 18 carbons and 2 double bonds in the *cis* configuration at the 9^{th} and 12^{th} carbons (i.e., *cis*-9, *cis*-12 octadecadienoic acid). CLA is found in ruminant meats and dairy products, as microbes in the gastrointestinal tract of ruminant animals convert linoleic acid into different isoforms of CLA through biohydrogenation. This process changes the position and configuration of the double bonds, resulting in a single bond between the two double bonds. The major isomers produced include *cis*-9, *trans*-11 (c9,t11) and *trans*-10, *cis*-12 (t10,c12) octadecadienoic acid. Food sources of CLA contain ~80% c9,t11 CLA and 10% t10,c12 CLA, and the remaining 10% is composed of other isomers. CLA is also produced chemically from linoleic acid for inclusion in supplements and fortified foods, yielding a composition containing ~40% c9,t11 CLA, ~40% t10,c12 CLA isomers, and the remaining 20% of other isomers.

Consuming a mixture of c9,t11 and t10,c12 CLA isomers, or t10,c12 CLA alone, reduces body fat mass in rodents, particularly mice, and some humans [reviewed in 3]. However, the isomer-specific mechanism by which CLA reduces adiposity is unclear. Furthermore, a number of clinical studies report potential side effects of CLA supplementation including increased levels of markers of inflammation (e.g., inflammatory cytokines, chemokines, or prostaglandins), insulin resistance, hyperlipidemia, and lipodystrophy [4-8]. These anti-obesity and adverse side effects of CLA appear to be due primarily to the t10,c12 CLA isomer. In contrast, the c9,t11 CLA isomer appears to have anti-inflammatory and anti-diabetic properties without reducing body weight [9].

Several mechanisms by which t10,c12 CLA supplementation reduces body weight and body fat mass have been reported [reviewed in 3]. These include increasing energy expenditure via increasing basal metabolic rate, lipid oxidation, and thermogenesis, and lean body mass via increasing bone mineral density and muscle growth. Other mechanisms include adipocyte lipolysis, adipocyte apoptosis, and reducing glucose and FA uptake in adipose tissue. Our lab has extensively studied the mechanisms by which t10,c12 CLA promotes delipidation in adipocytes. We have demonstrated that activation of extracellular signal-regulated kinase (ERK) [10] and nuclear factor kappa B (NFκB) plays a role in t10,c12 CLA-mediated delipidation and insulin resistance [11]. We have also determined that t10,c12 CLA-mediated activation of ERK, cJun N-terminal kinase (JNK), NFκB, and production of reactive oxygen species (ROS) was dependent on accumulation of intracellular calcium levels. For example, BAPTA, a calcium chelator,

TMB-8, which blocks calcium release from the endoplasmic reticulum (ER), and D609, an inhibitor of phospholipase C (PLC), attenuated phosphorylation of ERK and JNK, production of ROS, and induction of inflammatory genes. Additionally, we demonstrated that TMB-8 prevented t10,c12 CLA-mediated NFκB binding to promoters of interleukin (IL)-8 and cyclooxygenase (COX)-2 [12]. Moreover, this type of inflammatory signaling has been directly associated with antagonism of adipogenic transcription factors like peroxisome proliferator activated receptor (PPAR)γ. Activated NFκB [13-15] and ERK [16-18] induce markers of inflammation and antagonize peroxisome proliferator activated receptor (PPAR)γ activity, thereby causing insulin resistance. These data suggest that t10,c12 CLA mediates inflammatory signaling that antagonize adipogenic processes in adipocytes. However, the upstream signals responsible for t10,c12 CLA-mediated increases in intracellular calcium levels, inflammatory signaling, insulin resistance, and reduced triglyceride (TG) content in human adipocytes are unknown.

Diacylglycerol kinases (DGKs) are a family of kinases that phosphorylate diacylglycerol (DAG), resulting in the conversion of DAG into phosphatidic acid (PA). DAG and PA act as second messengers that activate an array of target proteins resulting in significant changes in cellular signaling. For example, DAG activates conventional protein kinase C (cPKC), Unc-13, and protein kinase D, while PA activates atypical PKC, phosphatidylinositol (PI)-4-phosphate 5-kinase (PIP5K), and mammalian target of rapamycin (mTOR), RasGAP, and Raf-1 kinase. Therefore, DGKs are critical in terminating DAG signaling and also initiating PA signaling. In addition to this well-characterized function, DGKs also act as scaffolding proteins and regulate subcellular

signaling via endosomal and nuclear transport. To date, 10 different DGK isozymes have been identified. Each of the DGKs has up to three PKC-like C1 domains and a catalytic region. DGKs are grouped into five different types, based on their structural and functional features. For example, type 1 DGKs that include DGK α, β, and γ, contain recoverin homology domains and EF-hand motifs that serve as calcium-binding domains [reviewed in 19]. Thus, these DGKs are activated in part by calcium binding. Type II DGKs, including DGK δ, η, and κ, contain pleckstrin homology domains, sterile α motif (SAM) domain, and a separated catalytic region. Type III DGKs, including DGK ε, contain no additional functional domains different than other DGK isoforms. Type IV DGKs, including DGK ζ and ι, contain a nuclear localization signal, a myristolated alanine-rich C kinase substrate (MARCKS) phosphorylation domain and four ankyrin repeats. Type V DGKS, including DGK θ, contain three C1 domains, a Gly/Pro-rich domain, and a PH-domain like region [reviewed in 19]. DGKs also display tissue-specific expression. DGKs are highly expressed in the brain, thymus, and muscle. However, DGK expression in adipose tissue or primary human adipocytes is poorly defined. Thus, DGKs are a complex family of kinases.

Several lines of evidence support the involvement of DGKs in t10,c12 CLA-mediated inflammation and insulin resistance. First, DGK-generated PA levels activate mTOR and S6 kinase (S6K) in HEK 293 cells [20]. Interestingly, we reported that t10,c12 CLA robustly increases the phosphorylation of these two proteins in cultures of newly differentiated primary human adipocytes [21]. Additionally, mTOR and S6K activation

have been implicated in the development of insulin resistance, a side effect of CLA supplementation [22, 23]. Furthermore, DGK-mediated PA production has also been shown to increase calcium release from the ER [24]. This finding could provide a mechanism by which t10,c12 CLA increases intracellular calcium levels [12]. Moreover, DGKη has been reported to regultate MEK/ERK activation, which we have found to be necessary, in part, for t10,c12 CLA-mediated insulin resistance [25, 10]. For example, Yasuda et al. (2009) found that DGKη facilitated the transport of c-Raf to the plasma membrane, upstream of MEK/ERK activation in response to epidermal growth factor (EGF) treatment in HeLa cells. Therefore, it is tempting to speculate that t10,c12 CLA-mediated activation of MEK/ERK may involve similar signaling mechanisms. Additionally, DGKα has been shown to regulate tumor necrosis factor (TNF)α-mediated NFκB activation [26], which we have reported to be activated by t10,c12 CLA treatment in adipocytes. Finally, a mixture CLA has been shown to increase expression of DGKζ and increase PA levels in cardiomyocytes [27]. However, this was thought to have been dependent on a PPAR γ-dependent mechanism. We have historically found that t10,c12 CLA decreases PPAR γ activity and protein levels, so whether or not DGKζ plays a role in CLA's effects in adipocytes is unclear. Thus, there are several interesting findings in the literature that suggest a role for DGKs in t10,c12 CLA-mediated signaling in primary human adipocytes.

Based on the close similarity between pathways activated by DGK and t10,c12 CLA, we hypothesized that DGKs play an important role in t10,c12 CLA-mediated

inflammation, insulin resistance, and delipidation. To test this hypothesis, we employed the chemical DGK inhibitor, R59022. By using this inhibitor, we demonstrated that DGKs are involved in the regulation of t10,c12 CLA-induced inflammatory signaling, insulin resistance, and delipidation in newly differentiated primary human adipocytes. Therefore, DGKs may be an important target for preventing 10,12 CLA-mediated inflammation and insulin resistance.

Materials and Methods

Materials

All cell culture ware were purchased from Fisher Scientific (Norcross, GA). Lightning Chemiluminescence Substrate was purchased from Perkin Elmer Life Science (Boston, MA). Immunoblotting buffers and precast gels were purchased from Invitrogen (Carlsbad, CA). Polyclonal antibodies for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and monoclonal antibody for anti-PPARγ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody for DGKη was purchased from Abcam (Cambridge, MA). Anti-total and anti-phospho (P) ERK, JNK, and P-cJun antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Hyclone fetal bovine serum was purchased from Fisher Scientific. Isomers of CLA (+98% pure) were purchased from Matreya (Pleasant Gap, PA). Fluo-3 acetyloxymethyl ester (Fluo-3 AM), pluronic F-127, and probenecid were purchased from Invitrogen by Life Technologies (Carlsbad, CA). Thapsigargin and ionomycin were purchased from

Calbiochem-EMD Biosciences, Inc. (La Jolla, CA). The cell permeable DGK inhibitor R59022 (6-{2-{4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl}-7-methyl-5*H*-thiazolo-(3,2-*a*)pyrimidin-5-one) was purchased from EMD Chemicals (Gibbstown, NJ). This inhibitor functions by inhibiting DAG phosphorylation which is more specific to the Type 1, calcium-sensitive DGKs, but also has less specificity than another DGK inhibitor, R59949 (3-{2-(4-[bis-(4-fluorophenyl)methylene]-1-piperidinyl)ethyl}-2,3-dihydro-2-thioxo 4(1*H*)quinazolinone) [28]. Because we were unaware of the specific DGKs isoforms that may play a role in t10,c12 CLA-mediated signaling, the less specific inhibitor R59022 was used in this study. All other reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Culturing of human primary adipocytes

Abdominal white adipose tissue was obtained with consent from the Institutional Review Boards at the University of North Carolina at Greensboro and the Moses Cone Memorial Hospital, during elective abdominoplasty of non-diabetic Caucasian and African American females between the ages of 20-50 years old with a body mass index ≤ 32.0. These selection criteria allow for reduced variation in gender, age, and obesity status. Tissue was digested using collagenase; stromal vascular cells were isolated as previously described [10]. Stromal vascular cells were differentiated with adipocyte media (AM-1) containing 1 μM rosiglitazone and 250 μM 1-methyl-3-isobutylxanthine for 3 d, which yielded cultures containing ~30-50% adipocytes. On days 7-14, cells were

pretreated with 0.1, 1, 3, 10, or 30 μM R59022 dissolved in DMSO for 30 min, and subsequently treated with 50 -150 μM t10,c12 CLA or bovine serum albumin (BSA) vehicle control for 5 min - 48 h depending on the experimental outcome measured. All cultures were normalized to contain the same amount of BSA and DMSO vehicles. Each independent experiment was repeated at least twice using a mixture of cells from three subjects, unless otherwise indicated.

Fatty acid preparation

t10,c12 and c9,t11 CLA was delivered as a free acid complexed to 7.5% fatty acid-free bovine serum albumin (BSA) at a 4:1 molar ratio as previously described [10]. For measuring $[Ca^{2+}]_i$ levels, t10,c12 CLA dissolved in a 0.1M KOH solution was used.

TG content

TG levels were determined with a modified commercially-available TG assay as previously described [29].

^{3}H-2-deoxyglucose uptake

Cultures of newly-differentiated human adipocytes were supplemented with low-glucose DMEM on day 10. The following day, cultures were pretreated with 0.1, 1, or 10

μM R59022 for 30 minutes and subsequently treated with BSA vehicle or 50 μM t10,c12 CLA for 48 h. Cultures were stimulated for 10 min with 100 nM insulin and treated with 4 nmol ^{3}H-2-deoxyglucose for 90 min and the amount of ^{3}H-2-deoxyglucose was measured via scintillation counting as described previously [29].

^{14}C-oleic acid uptake

Cultures of newly-differentiated human adipocytes were supplemented with low-glucose DMEM on day 10. The following day, cultures were pretreated with 0.1, 1, or 10 μM R59022 for 30 min and subsequently treated with BSA vehicle or 50 μM t10,c12 CLA for 48 h. Cultures were treated with 12.5 nM ^{14}C-oleic acid (0.2 μCi; specific activity = 40-60 mCi/mmol) for 120 min and the amount of radiolabled oleic acid was measured via scintillation counting as described previously [29].

Immunoblotting

Immunoblotting using 20 μg of protein per lane was conducted using 4-12% NuPage precasted gels as previously described [10]. Briefly, PVDF membranes were blocked with 5% milk in TBST for 1 h and washed 3 x in TBST for 5 min. Blots were incubated overnight at 4°C with primary antibodies targeting DGKη, P-ERK, P-JNK, P-cJun, and total cJun at a dilution of 1:1000, and subsequently incubated in the respective horseradish peroxidase-conjugated secondary antibody at a dilution of 1:5000 at room

temperature for 1 h. Primary and secondary antibodies targeting GAPDH were used at a 1:5000 dilution. Primary and secondary antibodies targeting PPARγ were used at dilutions 1:200 and 1:2000, respectively. After washing, blots were treated with chemiluminescence reagent for 1 min and film was exposed using a SRX-101A Konica Minolta film developer. Densitometry was performed using a Kodak Image Station 440 CF by Perkin Elmer and Kodak Molecular Imaging Software Version 4.0.

RNA isolation and PCR

Total RNA was isolated from the cultures using Tri Reagent purchased from Molecular Research Center (Cincinnati, OH), according to manufacturer's protocol. For quantitative real time PCR, 1.0 ug total RNA was converted into first strand cDNA using Applied Biosystems High-Capacity cDNA Archive Kit (Foster City, CA). Real time PCR was performed in an Applied Biosystems 7500 FAST Real Time PCR System using Taqman Gene Expression Assays. To account for possible variation in cDNA input or the presence of PCR inhibitors, the endogenous reference gene GAPDH was simultaneously quantified for each sample, and these data normalized accordingly. The Relative Standard Curve Method using seven, two-fold dilutions ranging from 100 – 1.56 ng RNA was used to check primer efficiency and linearity of each transcript according to Applied Biosystem's Guide to Performing Relative Quantification of Gene Expression Using Real-Time Quantitative PCR.

Secretion of interleukin (IL)-6, IL-8, and monocyte chemoattractant protein (MCP)-1

The concentrations of IL-6, IL-8, and MCP-1 were determined using the BioPlex® Suspension Array System from Bio-Rad (Hercules, CA) following the manufacturer's protocol. Briefly, media was collected from cultures that were pretreated with 30 μM R59022 for 30 min, and subsequently treated with 50 μM t10,c12 CLA or BSA for 24 h. This time point was based on previous time course studies showing that the maximum level of cytokine secretion occurred after 24 h of t10,c12 CLA treatment [11]. The media was centrifuged at 13,200 x g for 10 min at 4°C to clear the samples of cellular debris. Samples and standards were run in duplicate. Based on the manufacturer's report 'Bio-Plex Pro Human Cytokine, Chemokine, and Growth Factor Assays - Bulletin 5828,' the intra-assay % CVs for IL-6, IL-8, and MCP-1 are 7, 9, and 9%, respectively. The inter-assay % CVs are 11, 4, and 7%, respectively.

Measuring $[Ca^{2+}]_i$ levels

$[Ca^{2+}]_i$ levels were determined using the calcium sensitive fluorescent dye Fluo-3 AM. Briefly, cells were preloaded with 5 μM Fluo-3 AM and an anionic detergent, 10% Pluronic F-127, at 37°C for 30 min in the dark. Next, cells were washed with HBSS containing $CaCl_2$ and probenecid, which prevents Fluo-3 AM leakage from cells. Cells were pretreated with R59022 or DMSO vehicle control for 10 min. Subsequently,

baseline fluorescence was measured using a Synergy Multidetection Microplate Reader (BioTek Inc., Winooski, VT) for 1 min at 10 s intervals. Cells were then treated with 5 μM thapsigargin (positive control), ionomycin (positive control), or 150 μM t10,c12 CLA and fluorescence was monitored at 20 s intervals for 7 min. Excitation wavelength was 485 nm, and fluorescence was collected at 528 nm. Changes in the ratio of calcium-dependent fluorescence to prestimulus background fluorescence (F/F_0) were plotted over time. For simplicity, single representative experiments are shown.

Plasma membrane (PM) isolation and DGKη levels

Cultures were treated with 20% serum, 100 ng/ml EGF, 50 uM t10,c12 CLA, or BSA vehicle control for 5 or 15 min. Cells were washed 2 x with 5 ml ice-cold HBSS and harvested in 300 ul TES buffer (20 mM Tris-HCl, 225 mM sucrose, 1 mM EDTA, pH 7.4) containing protease inhibitor at 20°C. Lysate (600 ul) from 2-100 mm culture dishes were homogenized in a 2 ml pre-chilled potter-elvehjem homogenizer and sheared using 15 strokes. The lysate was centrifuged at 16,000 x g for 20 min at 4°C. The supernatant or cytosolic fraction was removed and re-spun to remove excess lipid, and the supernatant was stored at -80°C. The remaining pellet was resuspended in 500 ul TES buffer and centrifuged at 16,000 x g for 20 min at 4°C. The supernatant was removed and discarded. The pellet was resuspended in 500 ul TES buffer and re-homogenized again using 15 strokes with a 2 ml potter-elvehjem homogenizer. The homogenate was layered onto 4 ml of 1.2 M sucrose cushion in a seal-top centrifuge tube. The tube was filled

completely with TES buffer and sealed. The samples were centrifuged at 100,000 x g for 20 min at 4°C. The interphase or PM fraction was removed and re-spun at 100,000 x g for 30 min at 4°C. The pellet was re-suspended in 50 ul RIPA lysis buffer containing protease inhibitor. Immunoblotting was carried out as described previously.

Statistical analyses

Data are expressed as the means ± S.E. Data were analyzed using one-way analysis of variance followed by Student's t tests for each pair for multiple comparisons. Differences were considered significant if $p < 0.05$. All analyses were performed using JMP IN, Version 9 Software (SAS Institute, Cary, NC).

Results

It has been previously reported that DGKs increase $[Ca^{2+}]_i$ levels via PA-mediated secretion from the endoplasmic reticulum (ER) [24]. We have found that 10,12 CLA-mediated inflammatory signaling is dependent on increased $[Ca^{2+}]_i$ [12]. Therefore, we hypothesized that DGKs play a role in 10,12 CLA-mediated inflammation, insulin resistance, and delipidation.

First, we sought to determine the extent to which DGKs played a significant role in the metabolic consequences of t10,c12 CLA treatment such as reduced lipid content and insulin sensitivity. In order to determine if DGKs play a role in t10,c12 CLA-mediated

delipidation, TG levels were measured in cultures pretreated with 3, 10, or 30 μM of the DGK inhibitor R59022 for 30 min and subsequently treated with 10,12 CLA for 24 h. The t10,c12 CLA-mediated reduction in TG was attenuated with 30 μM R59022 (**Fig 5.1A**). Previously, we have demonstrated that t10,c12 CLA reduces ^{14}C-labled oleic acid uptake and insulin-stimulated ^{3}H-deoxy-2-glucose uptake [10]. Therefore, the involvement of DGKs in t10,c12 CLA-mediated suppression of FA uptake and insulin-stimulated glucose uptake was determined. The suppression of FA uptake was dose-dependently attenuated with R59022 (**Fig 5.1B**). t10,c12 CLA-mediated suppression of insulin-stimulated glucose uptake was attenuated with 10 μM R59022 (**Fig 5.1C**).

Due to the role of PPARγ in promoting FA uptake, we hypothesized that R59022 would also prevent CLA's reduction in PPARγ protein levels. Consistent with our hypothesis, 30 μM R59022 attenuated t10,c12 CLA-mediated reduction in PPARγ protein levels (**Fig 5.2A**). Consistently, R59022 pretreatment partially prevented t10,c12 CLA-mediated suppression of adipogenic and lipogenic genes including PPARγ, fatty acid binding protein (aP2), insulin-dependent glucose transporter (GLUT4), and acetyl CoA carboxylase (ACC)-1 (**Fig 5.2B**). These data suggest that DGKs are involved in CLA-mediated delipidation and insulin resistance.

We previously demonstrated that t10,c12 CLA-induced inflammation leads to a suppression of insulin signaling and sensitivity [10-12]. In order to demonstrate the involvement of DGKs in 10,12 CLA-mediated inflammatory signaling, inflammatory gene expression and cytokine/chemokine release were measured in cultures pretreated

with increasing doses of R59022 and subsequently treated with BSA vehicle control or 50 μM 10,12 CLA for 18 h. R59022 significantly attenuated 10,12 CLA-induced IL-8, IL-6, cyclooxygenase (COX)-2, and MCP-1 gene expression (**Fig 5.3A**) and protein secretion (**Fig 5.3B**). These data suggest the DGKs are involved in t10,c12 CLA-mediated inflammatory signaling in primary cultures of newly differentiated human adipocytes.

We have previously shown that t10,c12 CLA-induced inflammation is dependent on MAPK activation [10] and $[Ca^{2+}]_i$ accumulation [12]. Therefore, we examined the role of DGKs in t10,c12 CLA-mediated MAPK (i.e., ERK and JNK) and activator protein (AP)-1 (i.e., cJun) activation, due to their role in upregulating inflammatory gene expression. Indeed, 30 μM R59022 attenuated t10,c12 CLA-mediated ERK, JNK, and c-Jun phosphorylation (**Fig 5.4A**). These data suggest that DGKs are involved in CLA-mediated MAPK and c-Jun phosphorylation. Due to the involvement of intracellular calcium in 10,12 CLA-mediated inflammatory signaling, the role of DGKs in elevating $[Ca^{2+}]_i$ levels by 10,12 CLA was determined. Cultures were pretreated with increasing doses of R59022 for 10 min, and subsequently treated with 10,12 CLA after which $[Ca^{2+}]_i$ were measured using the fluorescent calcium indicator Fluo3-AM. As reported previously, 10,12 CLA increased $[Ca^{2+}]_i$ within 1 min. R59022 decreased $[Ca^{2+}]_i$ elevated by 10,12 CLA (**Fig 5.4B**). In order to better understand how R59022 functions to decrease $[Ca^{2+}]_i$, calcium levels were measured in cultures treated with ionomycin, which causes calcium influx from outside the cell, and thapsigargin, which inhibits calcium-ATPases on the ER causing calcium release from the ER, in the presence or absence of

R59022. Interestingly, R59022 decreased both ionomycin and thapsigargin-mediated calcium accumulation. However, R59022 completely blocked ionomycin-mediated calcium and only partially attenuated thapsigargin-mediated calcium accumulation (**Fig 5.4C**). These data suggest that DGKs may be involved in t10,c12 CLA-mediated increase in $[Ca^{2+}]_i$ from both intra- and extracellular stores.

In order to determine the DGK isoform responsible for these effects, we analyzed basal gene expression of several DGK isoforms including DGKα, δ, γ, η, and ζ. These isoforms were chosen based on microarray analyses of all DGK isoforms (data not shown). DGKα was the most highly expressed isoform (**Fig 5.5A**). DGKδ and DGKη were expressed at similar levels, whereas DGKζ and DGKγ were the least abundant isoforms (**Fig 5.5A**). In order to assess the effect of t10,c12 CLA on DGK expression, cultures were treated with 50 μM t10,c12 CLA from 3 to 24 h. DGKη and DGKδ were modestly induced by t10,c12 CLA treatment after 12-24 h and DGKγ was modestly induced after 24 h of treatment (**Fig 5.5B**). There was no effect of t10,c12 CLA on DGKα or DGKζ expression at any time point (data not shown). The potential self-regulation of DGK expression was determined by analyzing cultures pretreated with R59022 and subsequently treated with t10,c12 CLA for 18 h. R59022 significantly decreased t10,c12 CLA-induced DGKη and DGKδ, but not DGKγ, mRNA levels (**Fig 5.5C**). Based on these results, DGKη and DGK δ were targeted for further study.

The activation of DGKη and DGKδ by t10,c12 CLA was investigated by analyzing their translocation from the cytosol to the PM following t10,c12 CLA treatment using

ultracentrifugation of cell extracts on a sucrose gradient to isolate the PM. Subsequently, PM and cytosolic fractions were immunoblotted for several DGK isoforms. t10,c12 CLA and the positive control epidermal growth factor (EGF) increased DGKη (n=2) within 5 min of treatment, but not DGKδ (data not shown), translocation compared to BSA control (**Fig 5.6**). However, subsequent independent experiments (n=5) did not show any effect of CLA or EGF on DGKη translocation at 5 min, raising doubt about our hypothesis that t10,c12 CLA directly increases DGKη activity. Of the five subsequent reps, EGF and CLA increased DGKη translocation at 15 min (n=1), so there was also inconsistency in the treatment effects across experiments. Based on work by Yasuda and colleagues showing that DGKη facilitated c-Raf and b-Raf translocation to the PM, c-Raf and b-Raf levels were also measured in the PM [25]. EGF, but not t10,c12 CLA, consistently increased c-Raf (n=4) and b-Raf (n=3) translocation to the PM. Taken together, these data suggest that the hypothesized increase in DGKη activity by t10c12 CLA does not occur by a mechanism similar to EGF.

Discussion

Consistent with our hypothesis, R59022 attenuated t10,c12-mediated suppression of TG levels, radiolabeled oleic acid uptake, insulin-stimulated glucose uptake, PPARγ protein levels and target gene expression. Additionally, R59022 suppressed t10,c12 CLA-induced inflammatory gene and protein secretion, and MAPK and cJun phosphorylation, as well as $[Ca^{2+}]_i$. Taken together, these data suggest that DGKs plays role in t10,c12

CLA-mediated induction of inflammation and insulin resistance in cultures of human adipocytes. However, t10,c12 CLA did not consistently increase DGKη translocation to the PM, casting doubt on the extent to which t10,c12 CLA directly increases the activity of this isoform, and the specificity of R59022. DGK silencing experiments are needed to corroborate the DGK chemical inhibitor data.

Further research is needed to confirm a role for DGKs in t10,c12 CLA-mediated inflammation, insulin resistance, and delipidation. As with most chemical inhibitors, there have been issues reported regarding the efficacy and specificity of R59022 [28]. Because we were unsure which DGK isoform might be involved in inflammatory signaling pathways in adipocytes, we decided to use R59022, rather than the more specific Type 1 DGK inhibitor, R59949. In preliminary experiments using R59949, we found it had little effect on reducing inflammatory gene expression and did not significantly attenuate t10,c12 CLA-mediated suppression of insulin-stimulated glucose uptake (data not shown). These findings suggest that Type 1 DGKs may not play a role in t10,c12 CLA signaling. However, DGKα displayed the highest level of expression in our primary cultures of newly differentiated human adipocytes (**Fig 5.5**). Based on data presented here, we expect that DGKη and DGKα may be important targets to investigate in the future. Therefore, gene silencing studies using siRNA targeting DGKη and α will be conducted to determine their role in inflammation and insulin resistance mediated by t10,c12 CLA in primary human adipocytes.

A common mechanism by which FA increase calcium levels is through activation of G-protein coupled receptors (GPCRs), such as GPR40. GPR40, coupled to the G-protein

subunit $G_{\alpha q/11}$, activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into DAG and inositol phosphates (InsPs). InsPs activate receptors on the ER, triggering calcium mobilization [reviewed in 30]. Intriguingly, it was recently reported by Schmidt and colleagues that CLA increases $[Ca^{2+}]_i$ and stimulates insulin release from INS-1E pancreatic cells via activation of the cell surface receptor, FFA1/GPR40 [31]. An alternative mechanism to FA-mediated calcium accumulation has been proposed by Camina and colleagues, whereby PC-specific PLC, activated by a pertussis toxin (PTX)-sensitive g protein, generates choline and DAG, which is converted into PA by DGKs. PA subsequently triggers calcium mobilization from inositiol-3-phosphate–independent calcium pools [24, 32–34]. Consistent with these findings, we reported recently that D609, a phosphatidylcholine (PC)-specific PLC (PC-PLC) inhibitor, prevents t10,c12 CLA-mediated calcium accumulation, ROS production, and inflammatory gene expression (i.e., IL-8, ATF3, and COX-2) [12]. Notably, we have previously shown that PTX attenuates t10,c12 CLA-mediated MEK/ERK phosphorylation and suppression of ^{14}C-oleic acid uptake and insulin-stimulated ^{3}H-2-deoxyglucose uptake, suggesting that t10,c12 may activate inflammatory signaling pathways via activation of a GPCR. Moreover, in the present study, we found that the DGK inhibitor, R59022, decreased t10,c12 CLA-mediated $[Ca^{2+}]_i$ accumulation (**Fig 5.4**). Elevated $[Ca^{2+}]_i$ activates calcium-sensitive proteins like calmodulin (CaM) which activates a number of kinases including CaMKII, which has been shown to inhibit differentiation upon $PGF_{2\alpha}$ treatment [35]. Interestingly, we have shown that KN-62, a CaMKII inhibitor attenuated t10,c12 CLA-mediated ERK and JNK phosphorylation,

ROS production, induction of inflammatory genes, $PGF_{2\alpha}$ secretion, and suppression of PPARγ protein levels and insulin-stimulated glucose uptake [12]. We found that R59022 also decreased CaMKIIβ gene expression (data not shown), thus providing another mechanism by which DGKs mediate 10,12 CLA-induced inflammation. Collectively, these findings support mechanisms by which t10,c12 CLA may trigger intracellular calcium accumulation and subsequent activation of inflammatory pathways.

DGK-generated PA has also been shown to activate other kinases such as mTOR and S6K. Both of these proteins have been implicated in regulating insulin resistance [22-23]. For example, it was shown in 3T3-L1 adipocytes that expression of a dominant negative mutant of S6K blunted the suppression of insulin-stimulated glucose uptake mediated by tumor necrosis factor (TNF)α. The insulin-resistant effects of S6K were found to be due to phosphorylation of IRS-1 on Ser-265/270 [22]. It was also found that insulin-resistant ob/ob mice [22] or mice fed a high-fat high-sucrose diet [23] had increased levels of mTOR and S6K activation compared to lean controls [22-23]. Interestingly, we have previously shown that t10,c12 CLA robustly increases the phosphorylation of mTOR and S6K in primary human adipocytes [21]. Furthermore, it was shown that inhibitors of GPCRs (PTX), mTOR (rapamycin), phosphatidylinositol 3-kinase (LY-294002), and protein kinase C (calphostin C) blocked the phosphorylation of mTOR and S6K [21]. The activation of these proteins could provide a mechanism by which DGKs mediate t10,c12 CLA-induced insulin resistance in human adipocytes. However, future studies are needed to test this hypothesis.

Treatment times in this study differed based on the experimental outcome measured. Historically, we have shown in our primary cultures of newly differentiated primary human adipocytes that t10,c12 CLA increases calcium accumulation from 1-7 min, phosphorylation of MAPKs between 6-24 h, induces inflammatory gene expression and protein secretion between 12-48 h, decreases adipogenic gene expression from 18-72 h, PPARγ protein and TG levels from 24-48 h, and FA and insulin-stimulated glucose uptake after 48 h [10, 11, 12, 36, 37, 38]. Therefore, we chose a 12 h time point to examine MAPK phosphorylation, 18 h for inflammatory gene expression, 24 h for cytokine/chemokine secretion, and 24 h for adipogenic, lipogenic, and insulin-sensitizing gene expression, and 24-48 h for measuring levels of TG and glucose and fatty acid uptake. The timing of these events fits our hypothesis that DGK activation occurs prior to t10,c12 CLA-mediated induction and secretion of inflammatory proteins, leading to the suppression of adipogenic/lipogenic gene and protein levels.

Based on the data reported herein and reports from the literature, we propose the working model presented in **Fig 5.7**, whereby t10,c12 CLA activates a GPCR linked to PLC, that generates IP3 and DAG. DGKs convert DAG into PA, and together with IP3, PA stimulates calcium secretion from the ER. Elevated calcium levels activate calcium-sensitive kinases such as CAMKII, which promotes ROS production and MAPK activation, leading to the induction of inflammatory genes by NFκB and AP-1. Secreted inflammatory proteins, such as IL-6 and TNFα, exacerbate inflammatory signaling in adipocytes in an autocrine and paracrine fashion. Together with direct inhibition by NFκB or AP-1, these inflammatory signals antagonize PPARγ, leading to decreased

glucose and FA uptake, resulting in delipidation and insulin resistance in adipocytes (**Fig 5.7**). In summary, this study suggests that DGKs may be an important target for preventing t10,c12 CLA-mediated inflammation and insulin resistance.

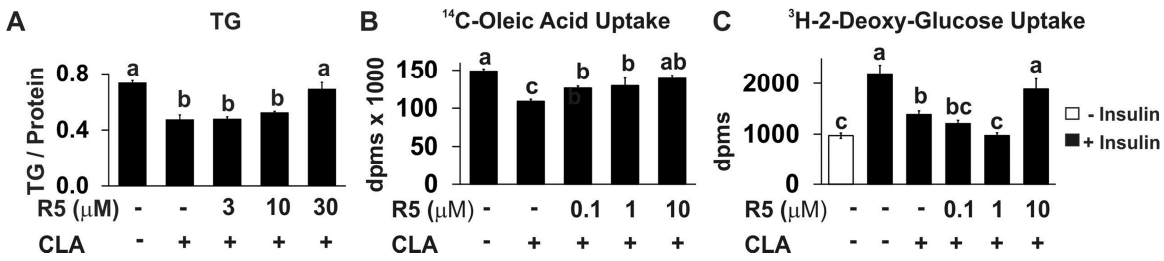

FIGURE 5.1. R59022 (R5) attenuates *trans*-10, *cis*-12 (t10,c12) CLA-mediated suppression of triglyceride (TG) levels, ^{14}C-oleic acid uptake, and insulin-stimulated ^{3}H-2-deoxy-glucose uptake. A) Cultures of newly-differentiated human adipocytes were pretreated with 3, 10, or 30 μM R5 and subsequently treated with BSA vehicle or 50 μM t10,c12 CLA (CLA) for 24 h on d 12. Cells were harvested and TG content was determined using a colorimetric assay. **B-C)** Cultures of newly-differentiated human adipocytes were supplemented with low-glucose DMEM on day 10. The following day, cultures were pretreated with 0.1, 1, or 10 μM R5 for 30 minutes and subsequently treated with BSA vehicle or 50 μM t10,c12 CLA for 48 h. Cultures were stimulated for 10 min with 100 nM insulin and treated with ^{3}H-2-deoxyglucose and ^{14}C-oleic acid for 90 min or 120 min, respectively. Data are representative of two (**B**) or three (**A,C**) independent experiments. Means ± SEM (n = 4) not sharing a common superscript differ significantly ($p < 0.05$).

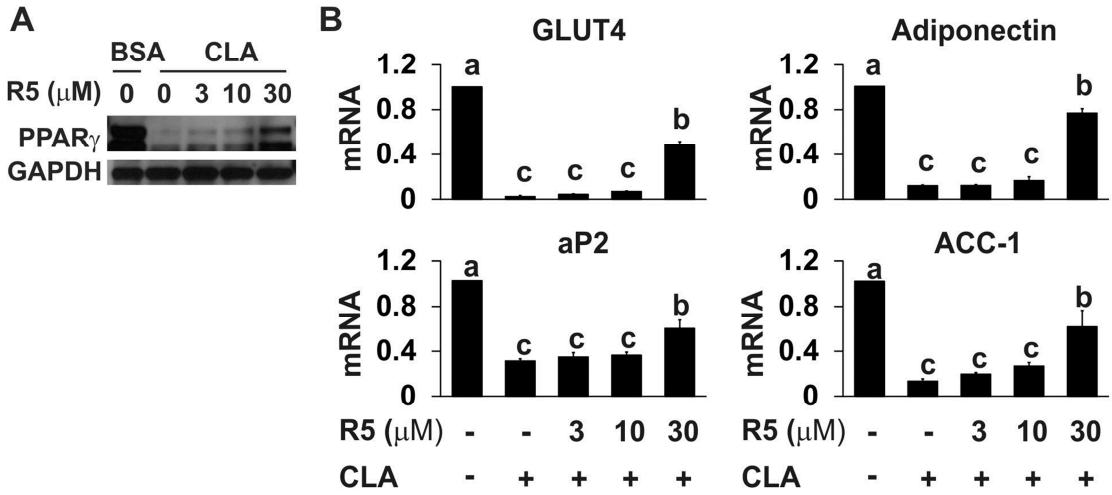

FIGURE 5.2. R59022 (R5) attenuates *trans*-10, *cis*-12 (t10,c12) CLA-mediated suppression of PPARγ protein levels and adipogenic/lipogenic gene expression. A-B) Cultures of newly-differentiated human adipocytes were pretreated with 3, 10, or 30 μM R5 and subsequently treated with BSA vehicle or 50 μM t10,c12 CLA (CLA) for 24 h (**A**) or 18 h (**B**) on d 8-10. **A)** Cells were harvested for protein and immunoblotted for PPARγ and GAPDH control. **B)** Cells were harvested for RNA and mRNA levels were measured via RT-qPCR. Data are representative of two (**B**) or three (**A,C**) independent experiments. Means ± SEM (n = 3-4) not sharing a common superscript differ significantly ($p < 0.05$).

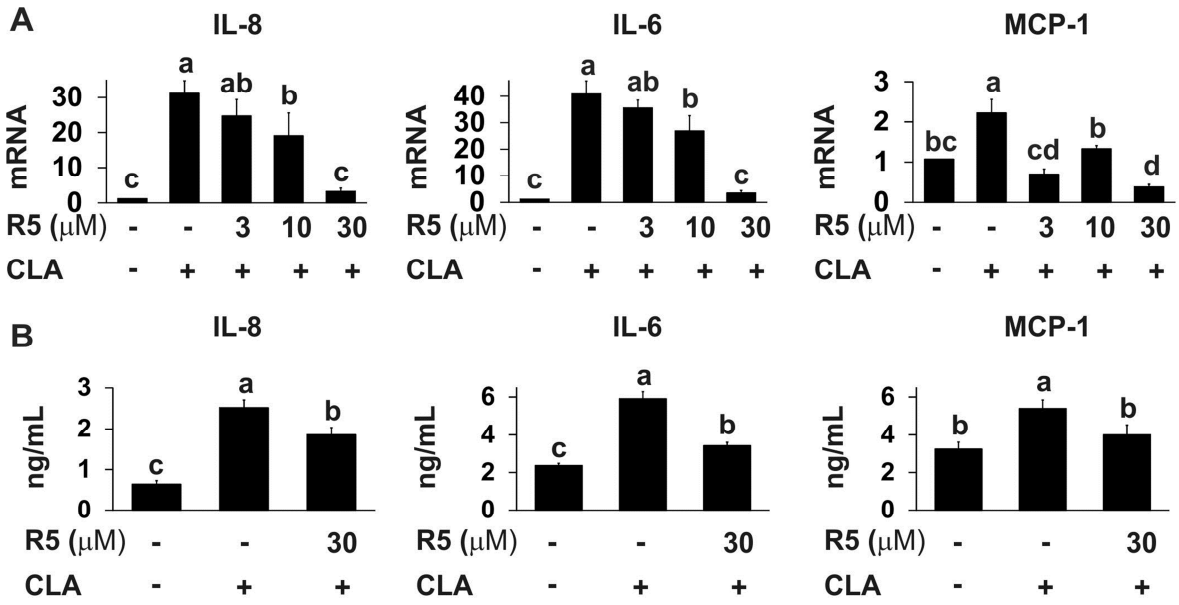

FIGURE 5.3. R59022 (R5) attenuates *trans*-10, *cis*-12 (t10,c12) CLA-induced inflammatory gene expression and protein secretion. A-B) Cultures of newly-differentiated human adipocytes were pretreated with 3, 10, or 30 μM R5 and subsequently treated with BSA vehicle or 50 μM t10,c12 CLA (CLA) for 18 h (**A**) or 24 h (**B**) on d 7-10. **A)** Cells were harvested for RNA and mRNA levels were measured via RT-qPCR. **B)** Media was collected and IL-8, IL-6, and MCP1 were measured via BioPlex® Suspension Array System from Bio-Rad. Data are representative of three independent experiments. Means ± SEM (n = 3-4) not sharing a common superscript differ significantly ($p < 0.05$).

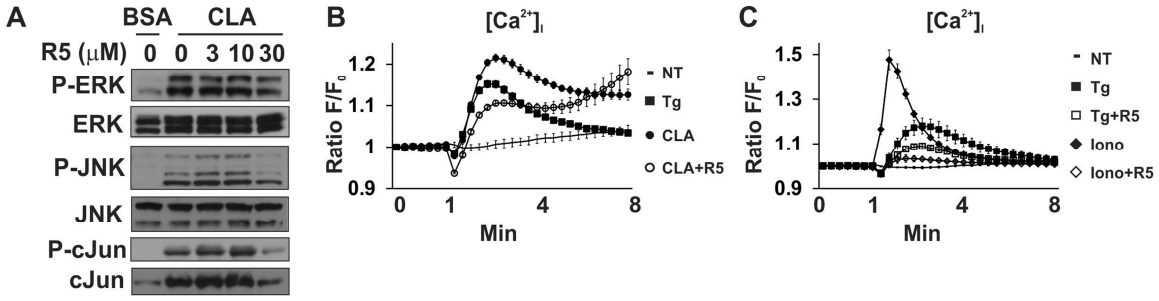

FIGURE 5.4. R59022 (R5) attenuates *trans*-10, *cis*-12 (t10,c12) CLA-mediated MAPK and cJun phosphorylation and intracellular $[Ca^{2+}]_i$ accumulation. A) Cultures of newly-differentiated human adipocytes were pretreated with 3, 10, or 30 μM R5 and subsequently treated with BSA vehicle or 50 μM t10,c12 CLA (CLA) for 24 h on d 8-10. **B-C**) Cultures of newly differentiated human adipocytes were preloaded with 5 μM Fluo-3 AM. Cultures were injected with vehicle (-), 5 μM thapsigargin (Tg; filled square), ionomycin (iono; filled diamond) 150 μM CLA (filled circle), or pretreated with R5 for 10 min and injected with 150 uM CLA (open circle), 5 μM Tg (open square), or ionomycin (open diamond) for 7 min at 20 s intervals. Baseline fluorescence was measured prior to treatment injection for 1 min at 10 s intervals. Data are representative of three independent experiments. **B-C)** Emitted fluorescence intensities were collected over time using a multidetection microplate reader. Excitation wavelength was 485 nm, and fluorescence was collected at 528 nm. Data are expressed as a ratio to baseline fluorescence (F_0). Means ± SEM (n = 3-4) not sharing a common superscript differ significantly ($p < 0.05$).

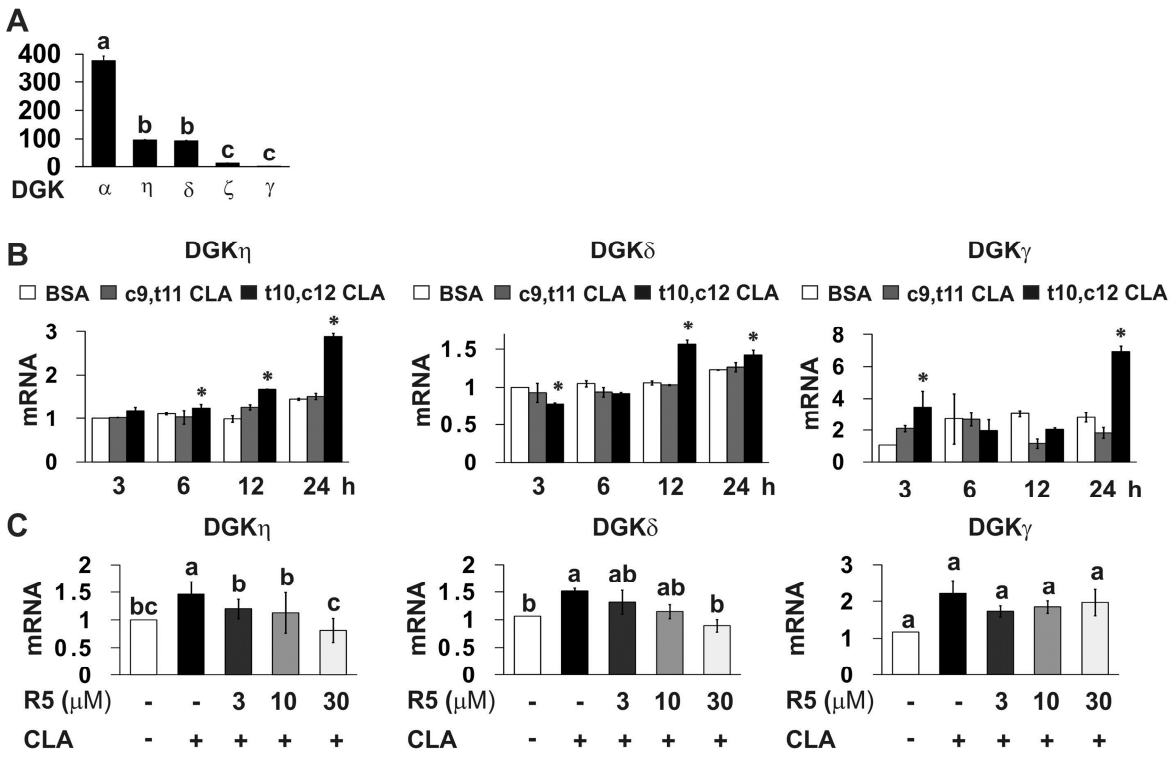

FIGURE 5.5. R59022 (R5) attenuates *trans*-10, *cis*-12 (t10,c12) CLA-induced DGK gene expression. A) Basal expression of DGK α, η, δ, ζ, and γ was measured in cultures of newly-differentiated human adipocytes on d8. **B)** Cultures were treated with BSA vehicle or 50 μM t10,c12 CLA (CLA) for 3-24 h, or **C)** pretreated with 3, 10, or 30 μM R5 and subsequently treated with BSA vehicle or 50 μM t10,c12 CLA (CLA) for 18 h and mRNA levels were measured via RT-qPCR. Data are representative of two **(A-B)** to three **(C)** independent experiments. Means ± SEM (n = 2-3) not sharing a common superscript differ significantly ($p < 0.05$).

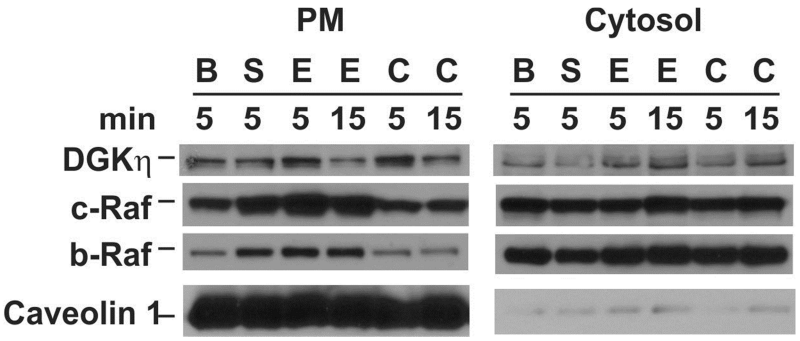

FIGURE 5.6. Epidermal growth factor (EGF) and *trans*-10, *cis*-12 (t10,c12) CLA treatment triggered DGKη translocation to the plasma membrane (PM). Cultures of newly-differentiated human adipocytes were treated with BSA (B) vehicle control, 20% serum (S), 100 ng/ml EGF (E), or 50 μM t10,c12 CLA (C) for 5 or 15 min. Total cell extracts were harvested and cytosolic and plasma membrane fractions were isolated by differential centrifugation. Each fraction was immunoblotted for DGKη, c-Raf, b-Raf, and caveolin-1. Data are representative of two of seven independent experiments.

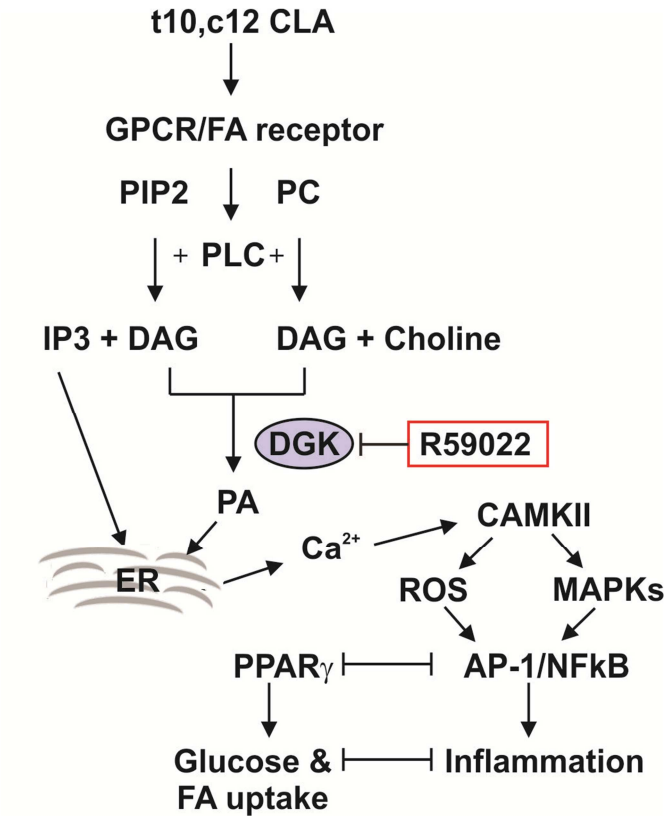

FIGURE 5.7. Working Model. t10,c12 CLA activates a g-protein coupled receptor linked to PLC. PLC cleaves phosphatidylinositol-bisphosphate (PIP2) or phosphatidylcholine (PC) into DAG and IP3 or choline, respectively. IP3 activates the IP3 receptor on the ER, and PA produced by DGK, stimulates Ca^{2+} release from the ER. Intracellular Ca^{2+} activates calcium-sensitive kinases like calmodulin kinase (CAMK) II leading to ROS production and MAPK activation. Activated AP-1 or NFkB suppress PPARγ activity leading to reduced glucose and FA uptake, thus promoting delipidation and insulin resistance in adipocytes.

References

1. World Health Organization. (Accessed March 7, 2011). Obesity and Overweight. www.who.int/mediacentre/factsheets/fs311/en/index.html.

2. Behan DF. (2010) Obesity and its Relation to Mortality and Morbidity Costs. Society of Actuaries.

3. Kennedy A, Martinez K, Schmidt S, Mandrup S, Lapoint K, McIntosh M (2010) Anti-obesity Mechanisms of Action of Conjugated Linoleic Acid. J Nutr Biochem 21:171-179.

4. Risérus U, Arner P, Brismar K, Vessby B (2002) Treatment with dietary trans10cis12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. Diabetes Care 25:1516-1521.

5. Moloney F, Yeow TP, Mullen A, Nolan JJ, Roche HM (2004) Conjugated linoleic acid supplementation, insulin sensitivity, and lipoprotein metabolism in patients with type 2 diabetes mellitus. Am J. Clin Nutr 80:887-895.

6. Steck SE, Chalecki AM, Miller P, Conway J, Austin GL, Hardin JW, Albright CD, Thuillier P (2007) Conjugated linoleic acid supplementation for twelve weeks increases lean body mass in obese humans. J Nutr 137:1188–1193.

7. Thrush AB, Chabowski A, Heigenhauser GJ, McBride BW, Or-Rashid M, Dyck DJ (2007) Conjugated linoleic acid increases skeletal muscle ceramide content and decreases insulin sensitivity in overweight, non-diabetic humans. Appl Physiol Nutr Metab 32:372-382.

8. Tholstrup T, Raff M, Straarup EM, Lund P, Basu S, Bruun JM (2008) An oil mixture with trans-10, cis-12 conjugated linoleic acid increases markers of inflammation and in vivo lipid peroxidation compared with cis-9, trans-11 conjugated linoleic acid in postmenopausal women. J Nutr 138:1445-51.

9. Moloney F, Toomey S, Noone E, Nugent A, Allan B, Loscher C, Roche H (2007) Antidiabetic effects of cis-9, trans, 11 conjugated linoleic acid may be mediated via anti-inflammatory effects in white adipose tissue. Diabetes 56:574-582.

10. Brown JM, Boysen M, Chung S, Fabiyi O, Morrision R, Mandrup S, McIntosh M (2004) Conjugated linoleic acid (CLA) induces human adipocyte delipidation: autocrine/ paracrine regulation of MEK/ERK signaling by adipocytokines. J Biol

Chem 279:26735-47.

11. Chung S, Brown JM, Provo JN, Hopkins R, McIntosh M (2005) Conjugated linoleic acid promotes human adipocyte insulin resistance through NFkappaB-dependent cytokine production, J Biol Chem 280:38445-56.

12. Kennedy A, Martinez K, Chung S, LaPoint K, West T, Hopkins R, Schmidt S, Andersen K, Mandrup S, McIntosh M (2010) Inflammation and insulin resistance induced by trans-10, cis-12 conjugated linoleic acid are dependent on intracellular calcium levels in primary cultures of human adipocytes. J Lipid Res 51:1906-1917.

13. Poirier H, Shapiro H, Kim R, Lazar M (2006) Nutritional supplementation with trans-10, cis-12 conjugated linoleic acid induces inflammation of white adipose tissue. Diabetes 55:1634-1641.

14. Ruan H, Hacohen N, Golub T, Van Parijs L, Lodish H (2002) Profiling gene transcription in vivo reveals adipose tissue as an intermediate target for tumor necrosis alpha: implications for insulin resistance. Diabetes 51:1319-1336.

15. Ruan H, Pownall H, Lodish H (2003) Troglitazone antagonizes tumor necrosis factor-a induced reprogramming of adipocyte gene expression by inhibiting the transcriptional regulatory functions of NFkB. J Biol Chem 278:28181-28192.

16. Suzawa M, Takada I, Yanagisawa J, Ohtake F, Ogawa S, Yamauchi T, Kadowaki T, Takeuchi Y, Shibuya H, Gotoh Y, Matsumoto K, Kato S (2003) Cytokines suppress adipogenesis and PPAR-gamma function through the TAK1/TAB1/NIK cascade. Nat Cell Biol 5:224-230.

17. Adams M, Reginato M, Shao D, Lazar M, Chatterjee V (1997) Transcriptional activation by peroxisome proliferator activated receptor gamma is inhibited by phosphoryaltion at a consensus mitogen-activated protein kinase site. J Biol Chem 272:5128-5132.

18. Camp H, Tafuri S (1997) Regulation of peroxisome proliferators-activated receptor γ activity by mitogen activated protein kinase. J Biol Chem 272:10811-10816.

19. Sakane F, Imai SI, Kai M, Yasuda S, and Kanoh H. (2007) Diacylglycerol Kinases: Why So Many Of Them? *Biochim Biophys Acta - Mol Cell Biol Lipids* 1771:793-80.

20. Avila-Flores A, Santos T, Rincon E, Merida I. (2005) Modulation of the mammalian target of rapamycin pathway by diacylglycerol kinase-produced phosphatidic acid. J Biol Chem.

21. Chung S, Brown JM, Sandberg MB, McIntosh M. (2005) Trans-10, cis-12 CLA

increases adipocyte lipolysis and alters lipid droplet-associated proteins: role of mTOR and ERK signaling. J Lipid Res. 46:885-895.

22. Zhang J, Gao Z, Yin J, Quon MJ, Ye J. (2008) S6K directly phophorylates IRS-1 on Ser-270 to promote insulin resistnace in reponse to TNF-α signaling through IKK2. J Biol Chem. 283:35375-35382.

23. Khamzina L, Veilleux A, Bergeron S, Marette A. (2005) Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. Endocrinology. 146:1473-1481.

24. Camina J, Casabiell X, Casanueva FF. (1999) Inositol 1,4,5-trisphophate-independent Ca2-mobilization triggered by a lipid factor isolated from vitreous body. J Biol Chem. 274:28134-28141.

25. Yasuda S, Kai M, Imai S, Takeishi K, Taketomi A, Toyota M, Kanoh H, Sakane F. (2009) Diacylglycerol kinase η augments c-raf acgtivity and b-raf/c-raf heterodimerization. J Biol Chem. 284:29559-29570.

26. Kai M, Yasuda S, Imai S, Toyota M, Kanoh H, Sakane F. (2009) Diacylglycerol kinase α enhances protein kinase cζ-dependent phosphorylation at Ser311 of p65/relA subunit of nuclear factor-κB. FEBS Letters. 583:3265-3268.

27. Alibin CP, Kopilas MA, Anderson HDI. (2008) Suppression of cardiac myocyte hypertrophy by conjugated linoleic acid: role of peroxisome proliferator-activated receptors α and γ. J Biol Chem. 283:10707-10715.

28. Jiang Y, Sakane F, Kanoh H, Walsh J. (2000) Selectivity of the diacylglycerol kinase inhibitor 3-{2-(4-[bis-(4-Fluorophenyl)methylene]-1-piperidinyl)ethyl}-2,3-dihydro-2-thioxo-4(1*H*)quinazolinone (R59949) among diacylglycerol kinase subtypes. Biochemical Pharmacology. 59:763-772.

29. Brown JM, Sandberg-Boysen M, Skov S, Morrison R, Storkson J, Lea-Currie R, Pariza M, Mandrup S, and McIntosh M. 2003. Isomer-specific regulation of metabolism and PPARγ by conjugated linoleic acid (CLA) in human preadipocytes. J Lipid Res. 44:1287-300.

30. Stoddart LA, Smith NJ, Milligan G. (2008) International Union of Pharmacology. LXXI. Free fatty acid receptors FFA1, -2, and -3: Pharmacology and pathophysiological functions. Pharmacological Reviews. 60:405-417.

31. Schmidt J, Liebscher K, Merten N, Grundmann M, Mielenz M, Sauerwein H,

Christiansen E, Due-Hansen ME, Ulven T, Ullrich S, Gomeza J, Drewke C, Kostenis E. (2011) Conjugated linoleic acids mediate insulin release through islet g protein coupled receptor FFA1/GPR40. J Biol Chem. In press.

32. Marley PD, and Thomson KA. (1996) The Ca++/calmodulindependent protein kinase II inhibitors KN62 and KN93, and their inactive analogues KN04 and KN92, inhibit nicotinic activation of tyrosine hydroxylase in bovine chromaffin cells. Biochem Biophys Res Commun. 221:15 – 18.

33. Suh HN, Huong HT, Song CH, Lee JH and Han HJ. (2008) Linoleic acid stimulates gluconeogenesis via Ca2+/PLC, cPLA2, and PPARs pathways through GPR40 in primary cultured chicken hepatocytes. Am J Physiol Cell Physiol. 295:C1518– C1527.

34. Andrei CP, Margiocco A, Poggi LV, Lotti MR, Torrisi, and Rubartelli A. (2004) Phospholipases C and A2 control lysosomemediated IL-1 beta secretion: implications for inflammatory processes. Proc Natl Acad Sci USA. 101:9745 – 9750.

35. Miller CW, Casimir DA, and Ntambi JM. (1996) The mechanism of inhibition of 3T3-L1 preadipocyte differentiation by prostaglandin F2alpha. Endocrinology. 137:5641- 5650.

36. Martinez K, Kennedy A, West T, Milatovic D, Aschner M, (2010) McIntosh M Trans-10, cis-12 Conjugated Linoleic Acid Promotes Inflammation to a Greater Extent in Human Adipocytes Compared to Preadipocytes. J Biol Chem 285:17701-17712.

37. Kennedy A, Chung S, LaPoint K, Fabiyi O, McIntosh MK (2008) Trans-10, cis-12 conjugated linoleic acid antagonizes ligand-dependent PPARgamma activity in primary cultures of human adipocytes. J Nutr 138:455-61.

38. Kennedy A, Overman A, Lapoint K, Hopkins R, West T, Chuang CC, Martinez K, Bell D, McIntosh M (2009) Conjugated linoleic acid-mediated inflammation and insulin resistance in human adipocytes are attenuated by resveratrol. J Lipid Res 50:225-32.

CHAPTER VI

EPILOGUE

The prevalence of obesity has risen dramatically over the past 30 years in the U.S. and is becoming a global health issue. Although combating obesity would be best achieved by diet and exercise, supplemental agents have become increasingly popular. One such agent is conjugated linoleic acid (CLA). Supplementation of CLA has been shown to reduce body weight and fat mass in several animal models and in some humans. However, the safety and efficacy of CLA in reducing body weight and fat mass is controversial, as it has been reported to promote inflammation and insulin resistance in animals and humans. Therefore, it is imperative to better understand CLA's mechanism of action and adverse side effects in white adipose tissue (WAT). Furthermore, the cell type in WAT that is responsible for mediating inflammatory signaling in response CLA is unclear. Thus, it is equally important to identify the cell type initiating CLA's adverse side effects.

Results from our lab suggest that t10,c12 CLA-mediated delipidation is linked to inflammatory signaling and insulin resistance. For example, we have demonstrated that t10,c12 CLA induces inflammation and insulin resistance via elevation of intracellular calcium levels, activation of extracellular signal-regulated kinase (ERK), nuclear factor kappa B (NFκB), and activator protein (AP)-1, which upregulate inflammatory proteins such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. Secretion of these

cytokines and direct inhibition by NFκB attenuates the activation of transcription factors, such as peroxisome proliferator-activated receptor (PPAR)γ and sterol regulated enhancer binding protein (SREBP)-1c, that promote glucose and fatty acid uptake, fatty acid synthesis, and TG storage in adipocytes. However, the cell type in our cultures of newly differentiated primary human adipocytes responsible for initiating inflammatory signaling is unknown. Furthermore, the upstream signaling mechanisms activated by 10,12 CLA that trigger intracellular calcium accumulation, inflammatory protein activation (i.e., ERK and NFκB), and result in insulin resistance and delipidation are unknown. Therefore, I investigated 1) the cell type that is responsible for initiating the inflammatory response to CLA and 2) the upstream mechanisms involved, where I specifically examined the role of cJun N-terminal kinase (JNK) and diacylglycerol kinase (DGK) in CLA-mediated inflammation, insulin resistance, and delipidation.

Based on the results from these studies, I propose that adipocytes are essential for the inflammatory response to CLA. Additionally, treating primary cultures of newly differentiated human adipocytes with 10,12 CLA increases the phosphorylation of mitogen-activated protein kinases (MAPK)s including ERK 1/2, JNK, and p38, and the activation of the transcription factor AP-1 [i.e., c-Jun and activating transcription factor (ATF)3], which leads to the production of inflammatory adipocytokines and prostaglandins (PG)s through upregulating inflammatory genes [i.e., cyclooxygenase (COX)-2, IL-6, IL-1β, IL-8, monocyte chemoattractant protein (MCP)-1, and ATF3]. These inflammatory signals subsequently activate preadipocytes, leading to inflammatory cytokine secretion from preadipocytes, thus continuing the inflammatory cycle. It was

also found using chemical inhibitors targeting JNK and DGK, that these kinases are involved in t10,c12 CLA-mediated inflammatory signaling and/or insulin resistance. Based on these findings, I have developed the following research questions: 1) Which DGK isoform is responsible for mediating t10,c12 CLA's effects and will knocking down this isoform prevent t10,c12 CLA-mediated inflammatory signaling and insulin resistance?, 2) What role does phospholipase C (PLC) play in t10,c12 CLA-mediated inflammation?, and 3) Is t10,c12 CLA-mediated inflammatory signaling dependent on G-protein coupled receptor (GPCR) activation?

Q1. Which DGK isoform is responsible for mediating t10,c12 CLA's effects and will knocking down the gene prevent t10,c12 CLA-mediated inflammatory signaling?

In order to fully address the role of DGKs in t10,c12 CLA-mediated inflammation and insulin resistance, several experiments are needed. First, it is still unclear which isoform is activated by t10,c12 CLA. Although we have preliminary data showing that t10,c12 CLA activates DGKη translocation to the plasma membrane (PM) (n=2), this effect should be confirmed with other activity assays, such as immunostaining DGKs to detect translocation. Alternatively, phosphatidic acid (PA) production could be measured, which is an indirect but common method of determining DGK activity. In addition, it is also important to identify the specific DGK isoforms involved in t10,c12 CLA-mediated signaling. It was attempted to evaluate the activity of five DGK isoforms (i.e., DGKη, δ, γ, α, ζ) by investigating their translocation to the PM. However, either no effect was evident with these DGK isoforms or the antibody was poor. Therefore, it will be

important in the future to obtain better antibodies for a more accurate evaluation of DGK activity or use different methods. Lastly, data generated thus far implicating a role for DGKs in t10, c12 CLA signaling is based on the use of a chemical inhibitor, which may not be specific to the target of interest. Therefore, it will be important to knockdown candidate DGK isoforms to confirm their role in t10,c12 CLA-mediated inflammatory signaling and insulin resistance.

Q2. What role does PLC play in t10,c12 CLA-mediated inflammation?

We have previously published data showing that D609, a PLC inhibitor, attenuated intracellular calcium levels, reactive oxygen species (ROS) production, and inflammatory gene expression induced by t10,c12 CLA. This inhibitor has been reported to be specific for phosphatidylcholine (PC)-PLC. However, other PLCs like phosphatidylinositol 4,5-bisphosphate (PIP2)-PLC may also be involved in regulating intracellular calcium levels via inositol 3-phosphate (IP3)-mediated endoplasmic reticulum (ER) calcium mobilization. Therefore, future studies will be conducted using the chemical inhibitor U73122, which targets PIP2-PLC. Activation of candidate PLC isoforms will be measured by determining their phosphorylation status with t10,c12 CLA treatment in primary human adipocytes. Once a candidate PLC isoform is identified, siRNA will be used to knockdown the gene to confirm its role in regulating t10,c12 CLA-mediated inflammation and insulin resistance.

Q3. Is t10,c12 CLA-mediated inflammatory signaling dependent on GPCR activation?

We have previously shown that pertussis toxin (PTX), a general GPCR inhibitor, attenuates t10,c12 CLA-mediated MEK/ERK phosphorylation and suppression of insulin-stimulated glucose uptake. Furthermore, data generated from our lab in the past 5 years strongly suggest that t10,c12 CLA activates a GPCR, as calcium levels are elevated and chemical inhibition of proteins involved in calcium signaling, like PLC, DGK, and calcium/calmodulin-dependent protein kinase (CaMK)II attenuates t10,c12 CLA signaling. Therefore, future studies will be conducted to determine the GPCRs potentially activated by t10,c12 CLA. Microarray data generated in our lab by Dr. Arion Kennedy will be analyzed to determine the GPCRs expressed in our cultures of primary human adipocytes, and subsequently confirmed using TaqMan assays from Applied Biosystems. In addition, a GPR40 inhibitor will be obtained from Dr. C. Jayawickreme at GlaxoSmithCline, RTP, NC to determine its role in t10,c12 CLA-mediated inflammatory signaling and insulin resistance. Interestingly, we have preliminary data showing that a GPR120 agonist, which has been shown to antagonize GPR40 signaling, dose-dependently attenuates t10,c12 CLA-induced inflammatory gene expression (data not shown). Lastly, candidate GPCRs will be knocked down using siRNA to determine their involvement in elevated intracellular calcium levels, inflammatory gene expression, insulin resistance, and delipidation.

In summary, further research is needed to better understand the upstream mechanisms responsible for t10,c12 CLA-mediated inflammation, insulin resistance, and delipidation. The proposed studies will provide further insight into the t10,c12 CLA's

mechanism of action, which will be important for evaluating the efficacy, specificity, and potential side effects of CLA consumption for use as a weight loss agent.